

Optimization of growth determinants of a potent cellulolytic bacterium isolated from lignocellulosic biomass for enhancing biogas production

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Abstract Anaerobic digestion of lignocellulosic biomass is limited by inefficient hydrolysis of recalcitrant substrates, leading to low biogas yields where bacteria can be utilized successfully. In this study, we have chosen ten cellulose-degrading bacteria from active anaerobic slurry identified as *Enterobacter ludwigii*, *Klebsiella pneumoniae*, *Pantoea agglomerans*, *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus anthracis*, *Pseudomonas* sp., *Enterobacteriaceae* bacterium *Staphylococcus warneri*, and *Bacillus safensis*; among them, *E. ludwigii* was found to be the most potent having an endoglucanase gene in the genome. The growth conditions of *E. ludwigii* were further optimized using Response Surface Methodology that designated 35 °C temperature, 6.5 pH, 5 % carboxymethylcellulose, 5 % yeast extract, 1 % ammonium nitrate as optimum growth conditions. The optimized growth module found to accelerate cellulase production at both transcription and translation level that in turn enhanced biogas production inside anaerobic digester as well. Finally, the growth-cellulase production relationship could be helpful in efficient industrial applications.

Keywords Cellulolytic bacteria · *Enterobacter ludwigii* · Biomass · Response surface methodology · Biogas

Introduction

Cellulose is a linear homo-polysaccharide of β -glucose, abundantly distributed in nature. Unfortunately, much of the cellulosic residuals are often disposed of by biomass burning, which is now considered as a global phenomenon. Conversion of cellulose to glucose (an industrially important product) through cellulolytic system has been proven as a much cheaper and an environment favorable process. Being the most common natural renewable biopolymer, cellulose is commonly degraded by the hydrolytic action of a multicomponent enzyme system—the cellulase and represents the key step for biomass conversion (Sukumaran et al. 2005). Recently, metagenomic analyses of cellulose-degrading microorganisms allowed us to understand cellulose metabolism in the environment (Duan and Feng 2010). On the other hand, many investigators have focused on cellulases principally for cellulose degradation which have been improved biotechnologically by site-specific mutagenesis or over-expression (Ni et al. 2010). However, since the application of genetically engineered microorganisms is restricted, naturally isolated cellulose-degrading microorganisms are still considered as useful tool to serve the aforementioned purposes (Liang et al. 2014).

Numerous cellulose-degrading microorganisms have been isolated and identified from natural sources. Among those, most of the studies are emphasized on fungi due to their capacity of producing higher quantity of extractable cellulases with market value (Liang et al. 2014; Sukumaran et al. 2005). Although fungi have been widely studied for cellulase production in recent years (Liang et al. 2014; Sukumaran et al. 2005), the isolation and characterization of novel cellulose-

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hydrolyzing bacteria are still a thrust research area, due to their (bacterial) higher growth rate, expression of multi-enzyme complexes, and resistance to extreme environments make them successful candidate for industrial applications (Duan and Feng 2010; Sadhu et al. 2013; Maki et al. 2009). Bacteria belonging to the genera *Clostridium*, *Cellulomonas*, *Cellulosimicrobium*, *Thermomonospora*, *Bacillus*, *Ruminococcus*, *Erwinia*, *Bacteroides*, *Acetivibrio*, *Fibrobacter*, *Streptomyces*, *Microbispora*, and *Paenibacillus* have been observed to produce different kinds of cellulases under anaerobic or aerobic conditions (Liang et al. 2014; Wilson 2011; Sukumaran et al. 2005). Knowledge of such bacteria is of significant importance with respect to nutrition, biodegradation, biotechnology, and the carbon-cycle, providing insights into the metabolism, physiology, and functional enzyme systems of the cellulolytic bacteria responsible for the largest flow of carbon in the biosphere (Wilson 2011; Maki et al. 2009; Mulka et al. 2015). Interestingly, microbial cellulose utilization is growth associated and is influenced by various factors and their interactions are believed to drive the biogeochemical carbon flow (Sukumaran et al. 2005).

Present study aims to isolate cellulose-degrading bacteria from active anaerobic digester (AD) slurry and to optimize the growth of most potent bacterium for enhancing biogas production. Here, we are reporting *Enterobacter ludwigii* (first reported by Hoffmann et al. (2005) from clinical specimens) as a potent cellulose-degrading bacterial strain (for the first time) which was found to promote biogas production significantly ($p < 0.05$). The future industrial applicability of the said bacterium requires an accurate optimization of its growth. Thus, major emphasis was given to optimize the influential determinants which could influence the growth of the cellulolytic bacterium under laboratory conditions. Response Surface Methodology (RSM) with a Box–Behnken Design was adopted to obtain a regression equation that could predict the optimum growth conditions alongside a cost effective approach. To date, RSM has been implicated in the optimization of process variables for various purposes including growth conditions optimization of microbe (Mukherjee et al. 2015). Herein, RSM with a Box–Behnken design was used to optimize the growth of a potent cellulolytic bacterium *E. ludwigii* for feasible biotechnological applications of the bacterium (Mukherjee et al. 2015).

Materials and methods

Isolation of cellulose-degrading bacteria

Cellulolytic bacteria were isolated from the slurry of active, anaerobic digester (AD) which was utilized to investigate the yield of biogas, and methane production through batch scale anaerobic digester (AD) using water

hyacinth (*Eichhornia crassipes*) as the feedstock collected from the water bodies of Santiniketan, West Bengal, India (Mathew et al. 2015). Briefly, slurry was collected from digester on tenth day, suspended in 1 mL sterile saline (0.85 %; w/v) and serially diluted. Finally, the aliquot of 100 μ L from each dilution was spread onto Bushnell Haas medium (BHM) agar plates supplemented with low viscous carboxymethyl cellulose (CMC; Sigma) containing (g/L): CMC (10.0), K_2HPO_4 (1.0), KH_2PO_4 (1.0), $MgSO_4 \cdot 7H_2O$ (0.2), NH_4NO_3 (1.0), $FeCl_3 \cdot 6H_2O$ (0.05), $CaCl_2$ (0.02), and agar (20.0). The plates were incubated aseptically at 35 °C for 96 h and growth was observed at regular intervals.

Qualitative screening of cellulolytic bacteria by plate staining method

Morphologically dissimilar and discrete colonies were picked from different dilution plates and streaked on separate BHM–CMC plate and incubated at 35 °C for 48 h. The replica plates were also prepared separately for staining. Briefly, the replica plates were flooded with 0.1 % congo red for 20 min, stain was poured off, and the plates were washed with 1 M NaCl. Congo red served as an indicator for cellulose degradation in an agar medium to screen cellulolytic bacteria (Sadhu et al. 2013). Isolates showing clear zones around the colonies were picked from master plate and their hydrolysis capacity (ratio of diameter of clearing zone and colony) was measured. Finally, cultures showing cellulolytic activity were maintained on nutrient agar slants, sub-cultured every week, and used for further studies (identification, CMCase production).

Quantitative determination of extracellular CMCase

Selected isolates were grown in 50 mL enzyme production medium (at pH 6.5) composed of (g/L): CMC (10.0), K_2HPO_4 (1.0), KH_2PO_4 (1.0), $MgSO_4 \cdot 7H_2O$ (0.2), NH_4NO_3 (1.0), $FeCl_3 \cdot 6H_2O$ (0.05), $CaCl_2$ (0.02) as used previously during isolation but supplemented with 0.5 % yeast extract to provide additional nitrogen source and enhance the growth rate (Sadhu et al. 2013). After every 8 h (up to 48 h), the culture was retrieved and centrifuged at 12,000 g for 20 min at 4 °C. The cell-free culture supernatant (CFS) containing the crude enzyme was used for estimating CMCase activity by means of hydrolyzed glucose concentration. The isolate showing highest CMCase activity (as described later) was selected for characterization and further studies.

CMCase activity assay

CMCase activity (U/mL) in terms of reducing sugars liberated from CMC was assayed by incubating the crude enzyme (CFS) with CMC for 15 min at 35 °C employing

D-glucose as standard (Nelson 1944). See Supplementary materials for more

PCR amplification and sequencing of bacterial 16S rDNA

Genomic and plasmid DNA were isolated using Qiagen DNA extraction kit and Qiagen plasmid isolation kit, respectively. The bacterial 16S rDNA gene was amplified with universal primers (forward 5'-ccgaattcgctgacaaacAGAGTTTGATCCTGGCTCAG-3' and reverse 5'-cccggatc-caagcttACGGCTACCTTGTTACGACTT-3') employing the amplification conditions depicted else before (Goswami et al. 2015). The PCR amplicons were purified (QIA quick PCR purification kit) and sequenced commercially (Xcelris

Labs Ltd., Ahmedabad, India). Bacteria were identified using 16S rDNA sequence homology analyses by BLASTn program (www.ncbi.nlm.nih.gov) and sequences were submitted to Genbank (www.ncbi.nlm.nih.gov). In order to study the phylogenetic relationship, 16S rDNA sequence of the isolate alongside 9 of its closest neighbors (www.ncbi.nlm.nih.gov) was subjected to both pairwise and multiple sequence alignment employing ClustalW program provided by the software MEGA 6.0 (Tamura et al. 2011; Thompson et al. 1994). An un-rooted phylogenetic tree was constructed using maximum parsimony method employing Subtree-Pruning-Regrafting (SPR) algorithm using MEGA 6.0 and reliability of the tree was tested by the bootstrap method (500 replicates) (Tamura et al. 2011; Nei and Kumar 2000). Interpretation of the phylogenetic tree was further tested by pairwise sequence alignment separately

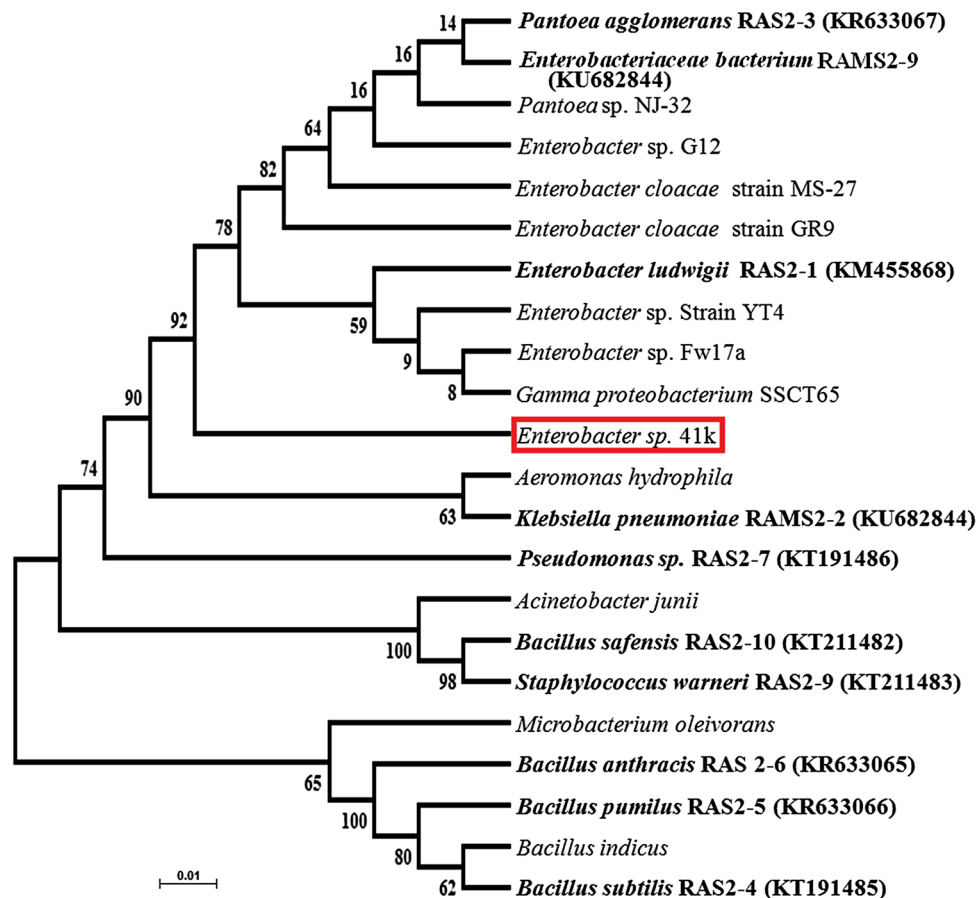


Fig. 1 Molecular phylogeny of cellulose-degrading bacteria based on 16S rDNA analysis. The evolutionary history was inferred using the Maximum Parsimony method. Tree #1 out of 5 most parsimonious trees (length = 7) is shown. The consistency index is (0.600000), the retention index is (0.800000), and the composite index is 0.571429 (0.480000) for all sites and parsimony-informative sites (in parentheses). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial

trees were obtained by the random addition of sequences (10 replicates). The analysis involved 10 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1394 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. Scale bar represents 0.01 nucleotide substitutions per site. Species indicated in red box represents the outgroup

among *E. ludwigii* and other isolates using EMBOSS Stretcher program provided by EMBL-EBI (www.ebi.ac.uk).

Characterizations of the most potent cellulolytic bacterial isolate

Morphological and biochemical characterizations

The cell morphology of the selected isolate was observed under scanning electron microscope (Zeiss). Nitrate reductase and urea hydrolysis property were determined according to Garcia de Salamone et al. and Christensen (Garcia de Salamone et al. 1996; Christensen 1946), whereas amino acid decarboxylation was performed in following a previous report (Asis and Adachi 2003). Other tests including experimental details have been given in Supplementary materials.

Functional characterization

Antimicrobial sensitivity The antimicrobial sensitivity of the bacterial isolate was determined by conventional disc diffusion method on Mueller-Hinton agar plates (HiMedia) using freshly prepared inocula from exponential phase of growth and tested against different antimicrobials. The inhibition zones appearing around each antimicrobial disc were measured and the sensitivity was determined from the zone diameters appearing on the plates following manufacturer's instructions (CLSI 2006). Experimental detail is given in Supplementary materials.

Antibacterial and antifungal activity Antimicrobial activity of the bacterial isolate was determined against two different bacteria *Escherichia coli* (MTCC- 443) and *Bacillus subtilis* (MTCC- 441) and one fungus *Pichia guilliermondii* (GenBank accession no. # KC771883; reported by Mukherjee et al. (2015)) on nutrient agar (NA) plates at 35 °C. Experimental detail is given in Supplementary materials.

PCR-based localization study of cellulase (endoglucanase) gene

In order to amplify cellulase gene of the bacterial isolate, primers (forward 5'-TTCGCGTGTATCTCTGGACG-3'; reverse 5'-GGTTTACCGCTTGCACATC-3') were designed from the available sequence of *Enterobacter cloacae* strain JV (Vasan et al. 2011) cellulase gene (endoglucanase; GenBank accession no.: GQ368735.1) using Primer 3 software. Conditions applied for the PCR amplification were as follows: initial denaturation at 94 °C for 4 min followed by 30 cycles of 30 s at 94 °C, 30 s at 48 °C, 1 min at 72 °C, and a final elongation step

at 72 °C for 5 min executed in a Gradient Thermocycler (Bio-Rad, USA). PCR product was resolved in 1.8 % agarose gel electrophoresis and observed under UV illumination in a Gel Doc™ (Bio-Rad, USA). All the primers were commercially obtained from Xcelris Genomics (Xcelris Labs Ltd., Ahmedabad, India) and validated accordingly (Supplementary Fig. 1).

Optimization of growth of *E. ludwigii*

Effect of different parameters on bacterial growth

To determine the effect of initial pH and temperature on growth, actively growing bacterial isolates were inoculated in BHM broth (amended with 2 % yeast extract) at various pH values (ranging from pH 4.5–pH 8.5) and temperatures (15–45 °C) and growth monitored at regular intervals at 600 nm at 35 °C. In an another study, to determine the effect of carbon concentrations on growth, actively growing bacterial isolates were inoculated in BHM broth with increase in concentration of CMC ranging from 0 to 5 % (0.25 % yeast extract amended as additional nutrient source) and growth monitored at regular intervals at 600 nm at 35 °C. In a separate set, BHM broth was prepared separately with increase in concentration of yeast extract (0–5 %) and NH₄NO₃ ranging from 0 to 10 % (no yeast extract added), respectively, and sterilized. To determine the effect of various nitrogen sources on growth, actively growing bacterial isolates were inoculated and changes in growth were monitored at regular intervals at 600 nm at 35 °C. All the experiments were repeated thrice.

Development of suitable design matrix, mathematical modeling, and optimization of bacterial growth

In this study, two-step RSM was employed to determine the optimum growth conditions of the isolated cellulolytic bacterium. In the first step, optimum levels of six influential growth determinants viz., CMC (%), yeast extract (%), ammonium nitrate (%), pH, temperature (°C), and time (h) were determined statistically using RSM. The experimental design and statistical analyses were performed using design expert software 8.0.4.1 (Stat-Ease Inc., Minneapolis, USA). All the experiments were designed using a Box–Behnken design with a quadratic model in order to study the combined and individual effects of the aforementioned influential experimental parameters on the growth of the most potent cellulolytic isolate. In the RSM studies, each parameter had two levels (–1 and +1) which were A: CMC (0–5 %), B: yeast extract (0–5 %), C: ammonium nitrate (1–10 %), D: pH (4.5–8.5), E: temperature (20–50 °C), and F: time (4–48 h). A total of 54 sets of experiments were performed to determine the optimum growth of the isolate (Table 1). Relationship between the six

Table 1 Box–Behnken design matrix showing combination of experiments

Run	A: CMC (%)	B: Y. extract (%)	C: A. nitrate (%)	D: pH	E: Temp. (°C)	F: Time (h)	Bact. Growth: OD _{600 nm}
1	2.5	5	10	6.5	50	26	0.6
2	2.5	2.5	1	8.5	35	48	1.188
3	2.5	5	5.5	6.5	50	4	0.002
4	5	2.5	5.5	4.5	20	26	0.008
5	5	5	5.5	4.5	35	26	0.009
6	2.5	0	5.5	6.5	50	48	0.012
7	2.5	0	5.5	6.5	20	48	0.294
8	0	2.5	5.5	4.5	50	26	0.019
9	2.5	2.5	5.5	6.5	35	26	0.294
10	2.5	2.5	1	4.5	35	48	0.288
11	0	2.5	1	6.5	35	48	1.027
12	2.5	2.5	10	8.5	35	48	0.067
13	2.5	2.5	10	4.5	35	4	0.015
14	0	2.5	5.5	8.5	50	26	0.005
15	5	0	5.5	8.5	35	26	0.07
16	2.5	5	5.5	6.5	50	48	0.019
17	5	2.5	5.5	8.5	20	26	0.067
18	5	2.5	10	6.5	35	48	0.217
19	5	2.5	5.5	8.5	50	26	0.03
20	2.5	0	10	6.5	20	26	0.077
21	2.5	2.5	5.5	6.5	35	26	0.168
22	2.5	2.5	5.5	6.5	35	26	0.168
23	5	5	5.5	8.5	35	26	0.063
24	2.5	0	5.5	6.5	50	4	0.025
25	0	5	5.5	4.5	35	26	0.035
26	2.5	2.5	1	4.5	35	4	0.032
27	2.5	2.5	5.5	6.5	35	26	0.168
28	0	0	5.5	8.5	35	26	0.019
29	0	2.5	5.5	4.5	20	26	0.014
30	2.5	0	1	6.5	50	26	0.021
31	2.5	5	1	6.5	50	26	0.02
32	0	2.5	5.5	8.5	20	26	0.071
33	2.5	0	5.5	6.5	20	4	0.028
34	0	5	5.5	8.5	35	26	0.011
35	5	2.5	1	6.5	35	48	1.108
36	2.5	5	5.5	6.5	20	4	0.007
37	2.5	5	1	6.5	20	26	0.714
38	5	2.5	10	6.5	35	4	0.028
39	2.5	2.5	10	4.5	35	48	0.03
40	2.5	0	1	6.5	20	26	0.217
41	2.5	0	10	6.5	50	26	0.41
42	0	2.5	10	6.5	35	48	0.142
43	2.5	5	5.5	6.5	20	48	0.314
44	5	0	5.5	4.5	35	26	0.014
45	5	2.5	1	6.5	35	4	0.086
46	0	2.5	10	6.5	35	4	0.035
47	5	2.5	5.5	4.5	50	26	0.021
48	2.5	2.5	1	8.5	35	4	0.112

Table 1 continued

Run	A: CMC (%)	B: Y. extract (%)	C: A. nitrate (%)	D: pH	E: Temp. (°C)	F: Time (h)	Bact. Growth: OD _{600 nm}
49	2.5	2.5	5.5	6.5	35	26	0.294
50	0	2.5	1	6.5	35	4	0.147
51	0	0	5.5	4.5	35	26	0.021
52	2.5	2.5	5.5	6.5	35	26	0.19
53	2.5	2.5	10	8.5	35	4	0.014
54	2.5	5	10	6.5	20	26	0.044

parameters and the response i.e., bacterial growth was analyzed using RSM. All the experimental combinations and values of the corresponding response studied are depicted in Table 1. Analysis of variance (ANOVA) was performed for the analyses of regression coefficient, prediction of equations, and case statistics. Both independent and dependent values were subjected to ANOVA analyses to obtain regression equations that could predict the responses within a given range. The generalized second-order regression equation used for the response surface study was as follows:

$$Y = \beta_0 + \sum_{i=1}^7 \beta_i X_i + \sum_{i,j=1}^7 \beta_{ij} X_i X_j + \sum_{i=1}^7 \beta_{ii} X_i^2 \quad (1)$$

where Y is the predicted response; β_0 , β_i , β_{ii} , and β_{ij} are the regression coefficients for intercept, linear, quadratic terms.

The adequacy and significance of the regression model was tested using ANOVA method. Test for significance on individual model coefficients and test for lack-of-fit were also estimated. Optimum conditions for the possible maximum growth of the bacterial isolate involving all six parameters were obtained using the predictive equation of RSM.

The validity and adequacy of the predictive extraction model was verified by considering all the six variables within or outside the design range was performed by constructing a model by taking random set of five experimental combinations to study bacterial growth. All three verification experimental runs have not been used in the RSM study but are within the range of the levels defined previously. The experimental values were obtained by performing experiment while predictive values were obtained by putting the values of the parameters in the RSM-generated regression equation. Experimental and predictive values were compared to validate the model.

Cellulase activity under optimized growth conditions

Bacterial isolate was grown under RSM-optimized conditions in separate Erlenmeyer flasks each containing cellulosic substrates either CMC (conventional substrate for

endoglucanase) or any of the three nonconventional ones such as straw, filter paper, and *Eichhornia* as the sole carbon source. The culture was retrieved and centrifuged at $12,000 \times g$ for 20 min at 4 °C. The cell-free culture supernatant was used for estimating the cellulase activity in terms of reducing sugars (glucose) liberated from CMC as well as other complex cellulosic substrates that have been mentioned elsewhere.

Expression of cellulase gene under optimized growth conditions

Bacterium was grown under optimum growth conditions in BHM containing CMC as carbon source (RSM-optimized bacteria). Cells were isolated by centrifugation, washed with sterile saline, and mRNA was isolated and subjected to real-time qPCR (Bio-Rad, USA) using cellulase (CMCase or endoglucanase)-specific primers (*cel48*; Xcelris Genomics, India) to study the expression of respective gene following Pereyra et al. (2010). Similarly, expression of cellulase enzyme was detected from bacterial cell lysate as well. Cell lysate was quantified for protein content, separated by SDS-PAGE, and immunoblotted with bacteria-specific anti-cellulase (rabbit) antibody (Biorbyt; Cat# orb20808) followed by secondary anti-rabbit IgG (Sigma).

Biogas production from lignocellulose biomass after addition of *E. ludwigii* grown under optimized conditions

Biomass collection, preparation, and physico-chemical analysis of biomass

Laboratory batch experiments like biomass collection, preparation, and physico-chemical analyses were carried out following our previous report by Mathew et al. (2015).

Batch-culture and biogas fermentation experiment

The wet anaerobic digestion of *Eichhornia* sp. (water hyacinth) was carried out using 2 L heavy duty vacuum

bottle (Tarsons, India) with cow dung as an inoculum. The biogas fermentation tank contained well-mixed slurry of *Eichhornia* sp. (water hyacinth) and cow dung at an inoculum to biomass ratio of 2:1 on volatile solids (VS) basis (Mathew et al. 2015) and 100 mL cultures of mesophilic cellulolytic bacterial isolate. However, the RSM-optimized (bacteria grown under RSM-optimized conditions) and unoptimized (bacteria grown in BHM amended with 2 % yeast extract for 28 h at 35 °C) *E. ludwigii* were applied independently in different fermentation tanks. The total solids (TS) content of *Eichhornia* sp. and cow dung mixture was approximately 5 % (w/w), respectively. Biogas fermentation was performed at a constant mesophilic temperature (35 °C) for 75 days in a thermostat-coupled shaking water bath under strict anaerobic conditions and biogas production was monitored following water displacement (Mathew et al. 2015; Navarro et al. 2012). An illustration of the experimental setup is shown in Supplementary Fig. 2.

Gas phase analysis

The compositions of biogas were measured on a GC-2010 gas chromatograph (Shimadzu, Japan) equipped with a thermal conductivity detector and Porapak-N column (Waters, USA). The detector, injector, and column temperatures were maintained at 200, 250, and 250 °C, respectively (Mathew et al. 2015). Purified helium was used as the carrier gas at a flow rate of 30 mL/min.

Statistical analysis

All the experiments were performed in triplicate and results have been expressed as mean \pm SE calculated for each parameter and pairwise comparisons were performed between the variables. Difference among data of $p < 0.05$ was considered statistically significant.

Results and discussion

Identification and phylogeny of cellulolytic bacteria

Degradation of cellulosic biomass by bacteria can be efficiently utilized in enhancing biogas production (Sadhu et al. 2013; Maki et al. 2009). In the present study, we have isolated potential cellulolytic bacteria from the active slurry of the AD. So far, the 16S rDNA-based identification revealed ten distinct bacteria namely *Enterobacter ludwigii* (RAS2-1, GenBank accession # KM455868), *Klebsiella pneumoniae* (RAMS2-2, GenBank accession # KU682843), *Pantoea agglomerans* (RAS2-3, GenBank accession # KR633067), *Bacillus subtilis* (RAS2-4, GenBank accession # KT191485),

Bacillus pumilus (RAS2-5, GenBank accession # KR633066), *Bacillus anthracis* (RAS2-6, GenBank accession # KR633065), *Pseudomonas* sp. (RAS2-7, GenBank accession # KT191486), *Enterobacteriaceae* bacterium (RAMS2-9, GenBank accession # KU682844), *Staphylococcus warneri* (RAS2-9, GenBank accession # KT211483), and *Bacillus safensis* (RAS2-10, GenBank accession # KT211482) (Fig. 1, Supplementary Fig. 3).

Since all the ten isolates were found to possess cellulolytic property, phylogeny was required to be studied (Fig. 1). We have studied the phylogenetic relationship involving the ten isolates depicted in this study alongside another twelve bacterial strains those have been reported for cellulose-degrading property. The un-rooted maximum parsimony tree shown in Fig. 1 demonstrates that the positions made in the tree were well supported with high BT values. The species pairs comprising *B. safensis*—*S. warneri* and *K. pneumoniae*—*A. hydrophila* were interpreted as monophyletic groups supported with passable BT values of 98 and 63 %, respectively (Fig. 1). However, other species pairs viz. *B. pumilus* RAS2-5—*B. subtilis* RAS2-4 and *Enterobacter cloacae*-*Enterobacteriaceae* bacterium. RAMS2-9 were also predicted as monophyletic groups but supported with low bootstrap score of <50 % (Fig. 1). Among the ten isolated strains, *P. agglomerans* and *K. pneumoniae* were phylogenetically closer to *E. ludwigii* with 94.4 and 84.6 % similarity, respectively. In addition, *Enterobacteriaceae* bacterium and *B. subtilis* were the most distantly related species having 64.3 and 54.1 % similarity, respectively from *E. ludwigii*. Interestingly, most of the reported cellulose-degrading strains belong to the family of enterobacteriaceae and bacillaceae (Pourramezan et al. 2012); however, our study appears to be the first report on cellulolytic *E. ludwigii* and *B. anthracis* from AD.

Screening and characterization of potential cellulolytic isolate

Selection of the most potent strain among the ten isolates was required for successful industrial utilization of the same. So far, all the isolates showed clear halo zone around colonies after staining with congo red indicative of cellulose degradation (as shown in Supplementary Fig. 4; Supplementary Table 1). However, plate-screening method is not a quantitative measure because of poor correlation between enzyme activity and colony to clear zone ratio (Liang et al. 2014). In the present study, although some strains presented large and clear hydrolyzing zones, the CMCase activities produced by them might be lesser in liquid media containing CMC, suggesting that either the concentration of the enzyme produced by these strains was (very) low after cultivation in liquid medium or the ability of the strains to secrete CMCase was weak.

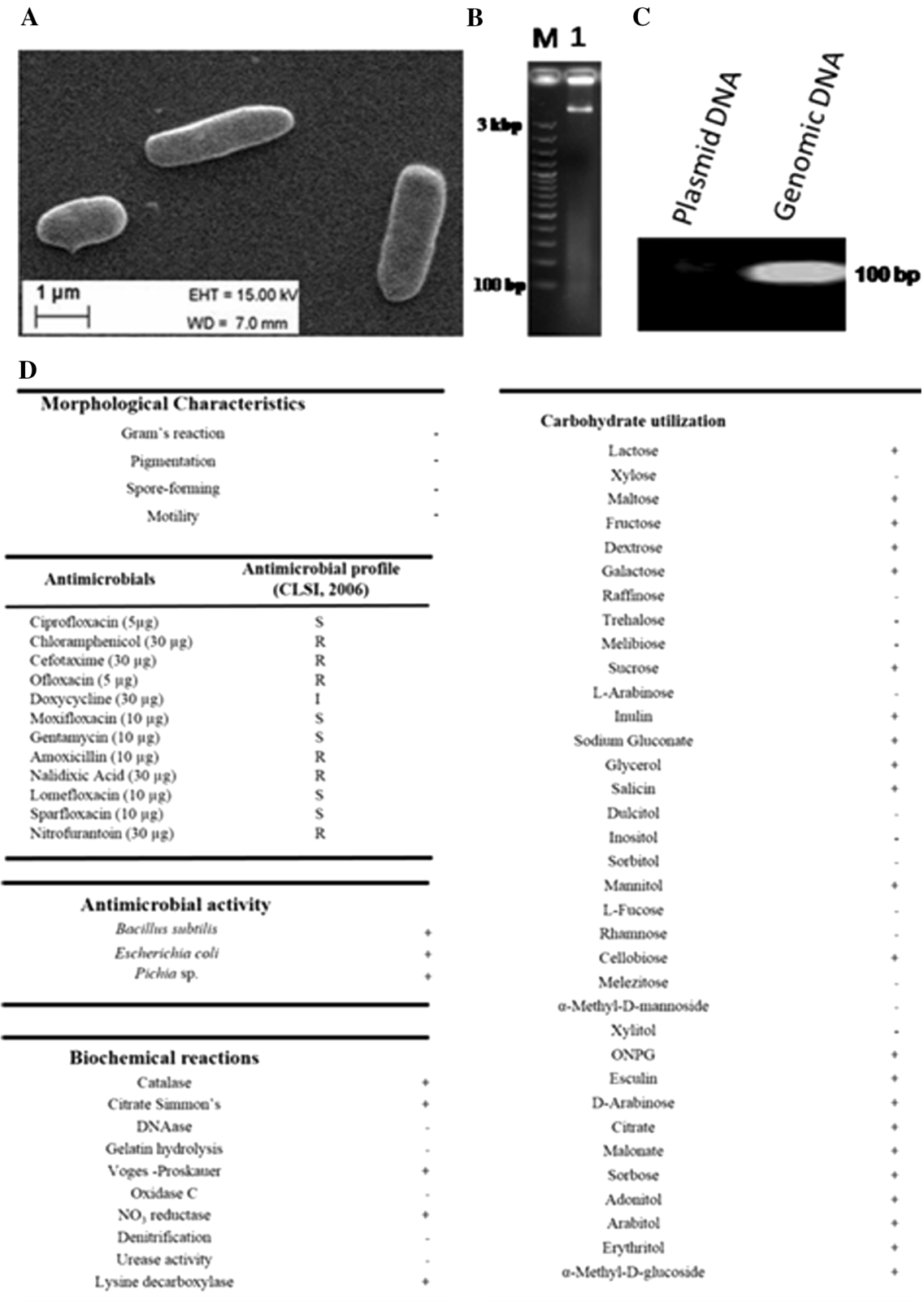


Fig. 2 Differential characteristics of *E. ludwigii*. **a** Scanning electron micrograph. **b** Agarose gel showing the presence of plasmid DNA (lane 1). **c** PCR amplification of cellulase gene. **d** Physiological and

biochemical properties. *M*, marker; *S*, sensitive; *R*, resistant; *I*, intermediate sensitivity; +, positive; -, negative

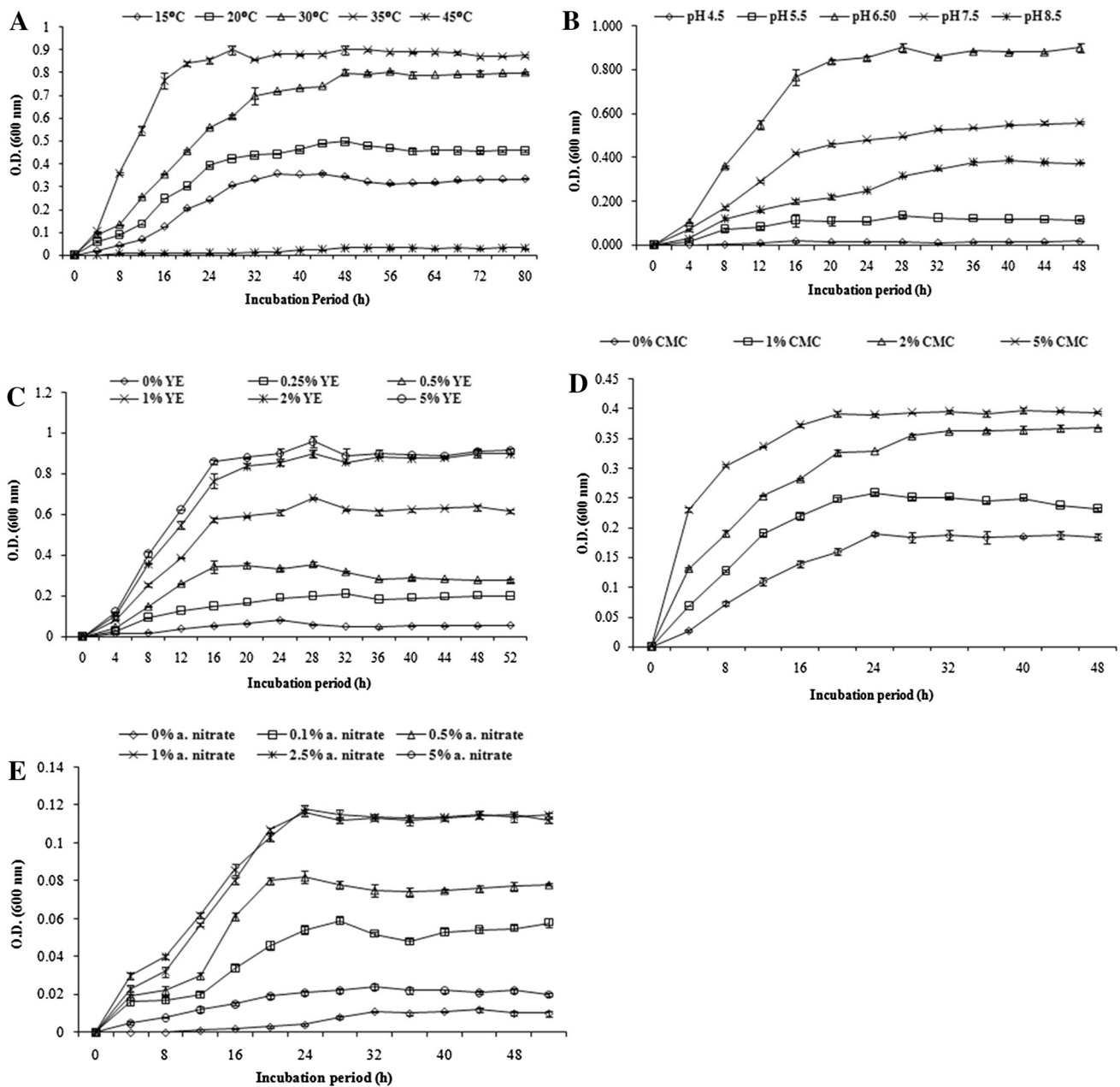


Fig. 3 Effects of growth determinants on *E. ludwigii*. Effect of temperature (a), pH (b), yeast extract (c; % YE), carboxymethyl cellulose (d; % CMC), and ammonium nitrate (e; % a. nitrate) on growth, respectively. The figure is a representative of three consecutive observations

For further studies, the most potent cellulolytic isolate was screened on the basis of CMCase production (after 48 h) in liquid medium. Among the isolates, *E. ludwigii* (RAS 2-1) exhibited maximum CMCase activity of approximately 1.20 U/mL followed by *B. safensis*—(0.92 U/mL), *B. anthracis* (0.80 U/mL), *B. subtilis* (0.79 U/mL), *K. pneumonia* (0.70 U/mL), *B. pumilus* (0.69 U/mL), *S. warneri* (0.67 U/mL), *Enterobacteriaceae bacterium* (0.60 U/mL), *Pseudomonas sp.* (0.55 U/mL), *P. agglomerans* (0.47 U/mL), respectively (see Supplementary Table 1 for details). To our knowledge, till date, no study has been reported about CMCase production by *E. ludwigii* (RAS

2-1) although other species of *Enterobacter* have been found to produce cellulose (Rezaei et al. 2009) and this value was higher than the activity of CMCase produced from some known natural isolates like *Cellulomonas sp.* (0.0336 U/mL), *Micrococcus sp.* (0.0152 U/mL), *Geobacillus sp.* (0.0113 U/mL) (Liang et al. 2014; Singh et al. 2013). Thus, the *E. ludwigii* RAS2-1 was revealed to be an efficient CMCase producer species and would be utilized in further studies. The morphological and biochemical studies further revealed that the cells of the strain RAS2-1 were rod-shaped with a length of 1.12–2.06 μm observed in scanning electron micrograph (Fig. 2a). The bacterium was found to

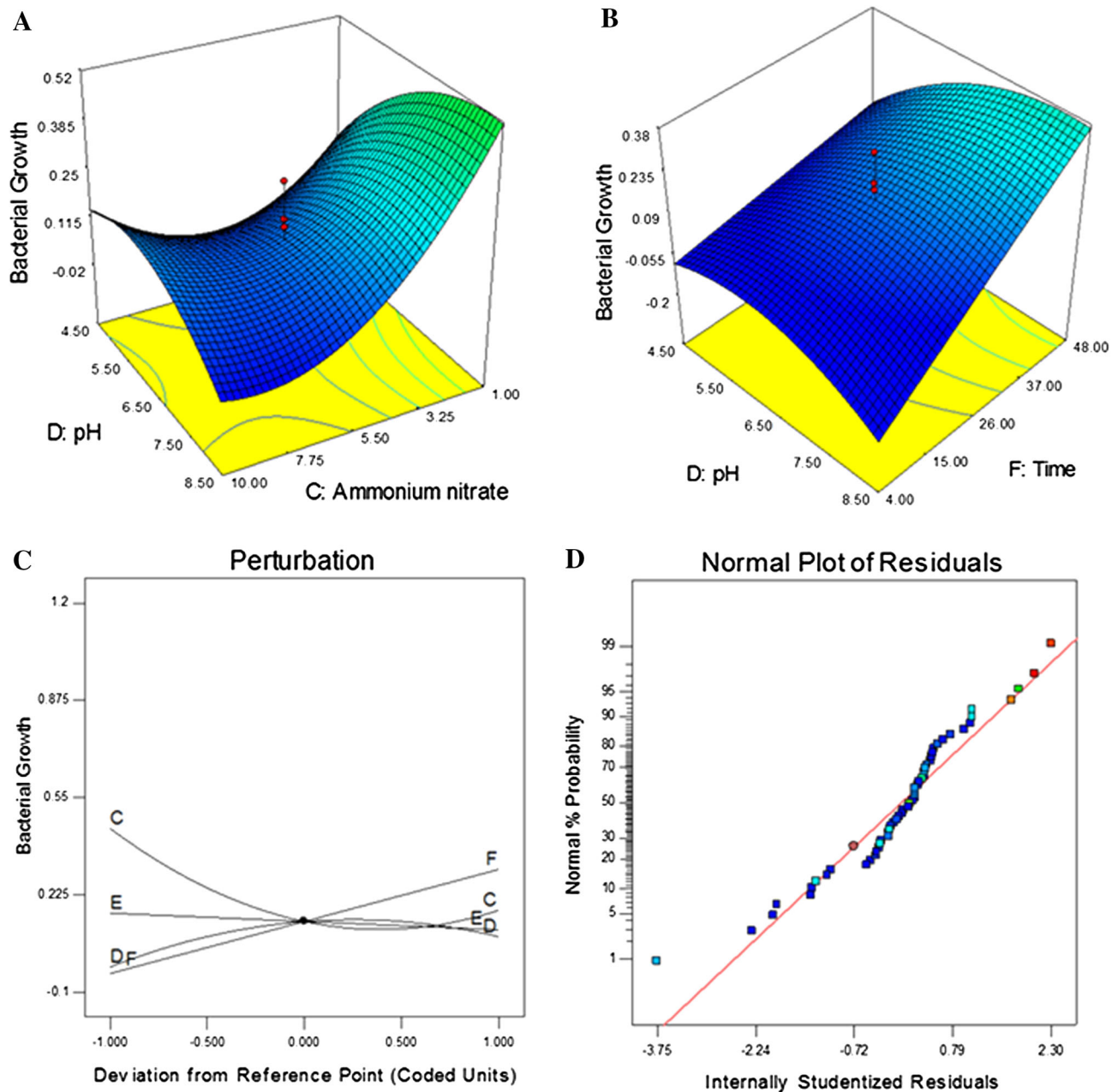


Fig. 4 Synergistic effects of growth determinants on *E. ludwigii*. **3D** response surface plots depicting interaction effects of pH and ammonium nitrate proportion (**a**) and pH and time (**b**) on bacterial

growth. **c** Perturbation plot showing relative influence of the parameters on bacterial growth. **d** Normal probability plot showing accuracy of ANOVA analysis

possess a plasmid (mol. size ~ 3 Kb) (Fig. 2b). The isolate was found to be a Gram-negative, nonsporulating bacterium, and the details of physiochemical parameters of the bacterium have been shown in Fig. 2d.

Cellulase gene predominantly present in *E. ludwigii* genome

Cellulose-degrading ability of the isolate made us interested to understand the underlying mechanism. A PCR-based approach using the specific primers was used to

study the occurrence of cellulase gene in *E. ludwigii*. Experimental outcomes revealed that cellulose-degrading gene appeared to be present in genomic DNA (Fig. 2c). A clear amplicon size of ~ 100 bp was evident after electrophoretic separation of the PCR amplicon (cellulase gene) from genomic DNA but not from plasmid DNA (Supplementary Fig. 4c). Presence of cellulase in genome indicated toward the possibility of acquiring the gene from its primary ancestors but not through horizontal gene transfer since it has not been detected in plasmid DNA.

Table 2 ANOVA (partial sum of squares—type III) for response surface reduced quadratic model

Source	Sum of squares	df	Mean square	F value	p value Prob > F (significant)
Model	3.01	10	0.3	13.57	<0.0001
C-ammonium nitrate	0.45	1	0.45	20.25	<0.0001
D-pH	0.061	1	0.061	2.76	0.104
E-temperature	0.019	1	0.019	0.85	0.3625
F-Time	0.73	1	0.73	32.79	<0.0001
CD	0.11	1	0.11	5.03	0.0301
CE	0.4	1	0.4	17.86	0.0001
CF	0.51	1	0.51	23.25	<0.0001
DF	0.092	1	0.092	4.16	0.0477
C2	0.39	1	0.39	17.56	0.0001
D2	0.13	1	0.13	5.98	0.0186
Residual	0.95	43	0.022		
Cor total	3.96	53			

Optimization of growth conditions of *E. ludwigii*

Direct effects of the determinants on bacterial growth

Several physico-chemical and environmental factors such as inoculum size, pH and temperature, presence of inducers, medium constituents, growth time can alter bacterial growth that in turn influence cellulose production and/or activity (Liang et al. 2014; Sukumaran et al. 2005). Temperature affects bacterial growth and cellulase activity changing the physical properties of the cell membrane (Rastogi et al. 2010), while pH impinge on the enzyme transport system across the cell membrane (Liang et al. 2014). On other side, production of extracellular cellulase has been shown to be affected by the inhibition of growth by different carbohydrates and nitrogen sources (Liang et al. 2014). Here, the maximum growth was observed at 35 °C, pH 6.5, 5 % CMC, 2–5 % yeast extract, and 1–2.5 % ammonium nitrate for the strain RAS 2-1 (Fig. 3). Such findings were obtained from the observations based on the effect of a single parameter on bacterial growth when rests were fixed at a particular value. Therefore, accurate optimization was needed to be carried out by studying the interaction effects of parameters on bacterial growth too.

Synergistic effects of the growth determinants

The synergistic effects of the growth parameters on *E. ludwigii* have been demonstrated in Fig. 3a and b. The parameters have been found to exert different degrees of influence over bacterial growth. Bacterial growth was found to decrease significantly with the increased concentration of ammonium nitrate in the growth medium (Fig. 4a). Higher concentration of ammonium nitrate decreases the growth of *E. ludwigii* by increasing osmotic pressure resulting in

dehydration creating hypertonic micro-environment unfavorable for viability (Mager et al. 1965). However, increase in pH up to 6.5 favored bacterial growth, whereas negative effect was observed beyond 6.5 (Fig. 4a). Maximum growth at pH 6.5 is due to highest metabolic activity that resulted from increased enzyme activity (Jalasutram et al. 2013) and this also has indicated *E. ludwigii* as mesophilic bacterium. On the other side, bacterial growth was found to increase consistently with the rise in incubation time up to 48 h (Fig. 4b). Longer incubation does make the bacterium enable to take-up nutrient required for necessary cellular metabolic or physiological changes prior to each binary fission. The synergistic effect of ammonium nitrate and time suggested that these parameters should have optimum values at which maximum bacterial growth could be obtained theoretically as well as experimentally. In addition, perturbation plot depicted in Fig. 4c reveals relative influence of the six parameters over bacterial growth. A steep slope curvature for ammonium nitrate is demonstrated as the most influential factor, whereas incubation time was found as the second most crucial parameter. The other combinations like ammonium nitrate concentration versus incubation time or others were not found as significant effectors and thus were excluded from the study. However, proportion of yeast extract and CMC, incubation temperature, do have influence on bacterial growth, therefore these parameters were needed to be optimized, despite the fact that these do not have a combinatorial effect on the responses.

ANOVA analysis of bacterial growth model was performed involving all experimental set up designed by RSM (Table 2). ANOVA analysis tested the adequacy of the developed model and a regression equation was eventually obtained that constituted significant model terms. The result of ANOVA analysis of the predicted model for the response i.e., bacterial growth shows the Model *F* value of

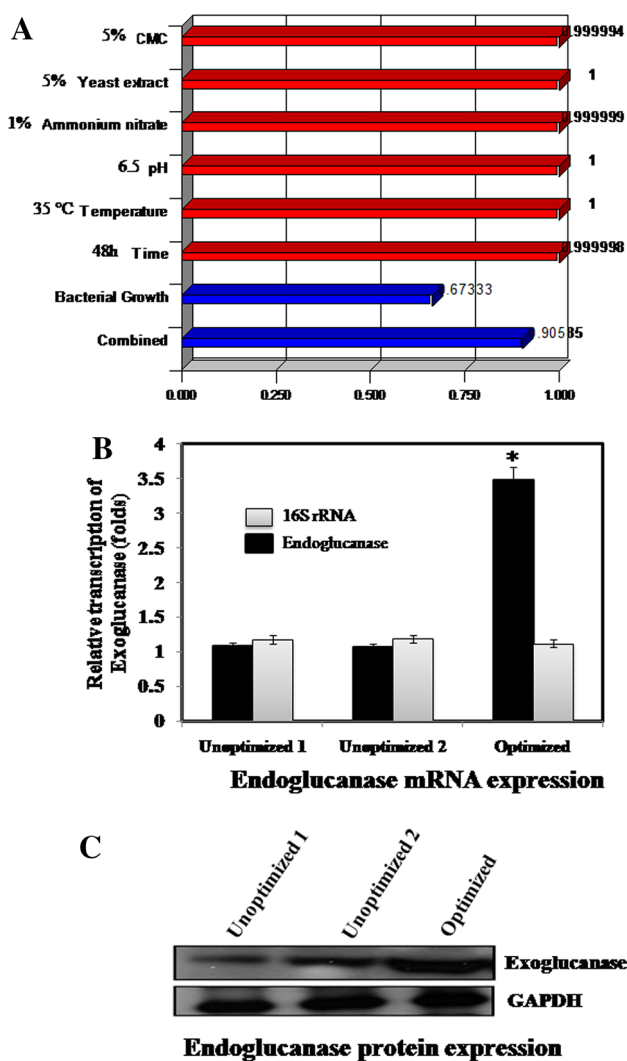


Fig. 5 RSM directed desirability function optimization (a) and expressions of endoglucanase mRNA (b) and protein (c) in *E. ludwigii* under optimum conditions. Values represented on bar diagram (a) are showing desirability of optimum levels of bacterial growth parameters. Real-time qPCR and Western blots are representative of experiments conducted in triplicate. Unoptimized 1 refers to: Run 2 from RSM design matrix; Unoptimized 2 refers to: Run 5 randomly chosen from the design. * $p < 0.05$

13.57 which implies that the model is significant (Table 2). There is only a chance of 0.01 % that the Model F-value could be large due to noise. Values of “Prob > F” less than 0.05 indicate model terms viz., C, F, CD, CE, CF, DF, C^2 , D^2 are significant. Furthermore, backward elimination procedure was adopted for the improvement of the model. The “Pred R-Squared” of 0.5188 is in reasonable agreement with the “Adj R-Squared” of 0.7035. “Adeq Precision” measures the signal-to-noise ratio. A ratio greater than 4 is desirable; our ratio of 15.958 indicated an adequate signal. Therefore, the model can be used to navigate the design space. The model shows standard deviation (SD), mean, and predicted residual sum of squares (PRESS) value of 0.15, 0.17, and 88.32. A correlation coefficient (R^2) of 0.7594 was obtained indicating high degree of correlation between the experimental parameters and bacterial growth. The experimental results of the Box–Behnken design were fitted by a second-order polynomial equation. The Eq. 2 depicts the empirical relationship between bacterial growth and the six independent variables in coded units obtained by applying RSM.

$$\begin{aligned}
 \text{Bacterial Growth (y)} = & -0.49676 - 0.10803 \\
 & \times \text{Ammonium nitrate} + 0.36455 \\
 & \times \text{pH} - 0.019983 \\
 & \times \text{Temperature} + 0.00202872 \\
 & \times \text{Time} - 0.013111 \\
 & \times \text{Ammonium nitrate} \times \text{pH} \\
 & + 0.00329444 \\
 & \times \text{Ammonium nitrate} \\
 & \times \text{Temperature} - 0.00181187 \\
 & \times \text{Ammonium nitrate} \times \text{Time} \\
 & + 0.00243750 \times \text{pH} \times \text{Time} \\
 & + 0.00860648 \\
 & \times \text{Ammonium nitrate}^2 \\
 & - 0.025430 \times \text{pH}^2
 \end{aligned}
 \tag{2}$$

Table 3 Average energy consumption of rural villagers in Santiniketan

Fuel	Quantity	Cost (INR)	CO ₂ emissions (kg CO ₂) ^a	Cost for fuel (INR)	Total CO ₂ emissions per household (kg)
Kerosene	35 L	40 per L	3.15	1400	110.25
Dung cake	140 kg	20 per kg	2.50	2800	350.0
Firewood	4200 kg	8 per kg	1.83	33,600	7686.0
Total				37,800	8146.25

^a Chakrabarty et al. (2013)

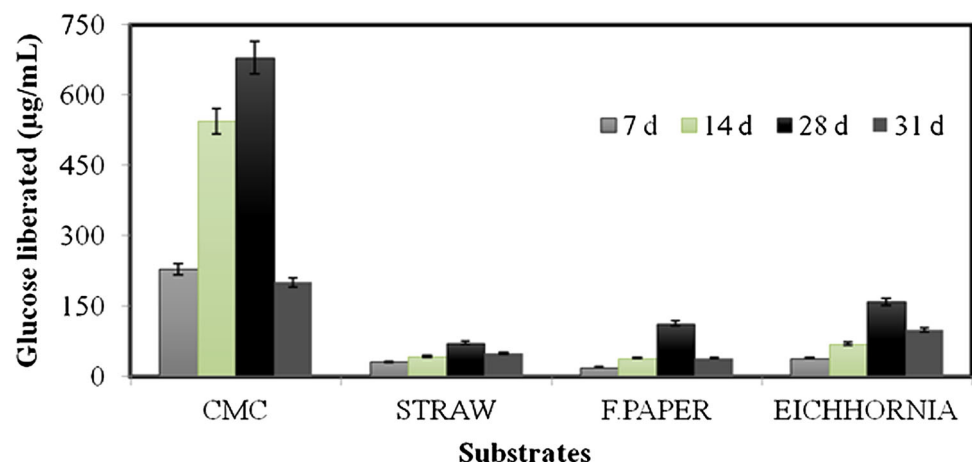
The aforementioned equation can be used to predict growth of *E. ludwigii* within the given range of parameters. Furthermore, the normal probability plot, shown in Fig. 4d, shows some scatters along the line which indicates that the residuals follow a normal distribution. This finding designates that the model satisfies the assumptions of the ANOVA which further demonstrate the accuracy and applicability of RSM in optimizing the six parameters to maximize growth of *E. ludwigii* under laboratory conditions.

Finally, desirability function optimization of the RSM has been employed for the optimization of the bacterial growth. The desired optimized growth module searches a combination of factor levels that could simultaneously satisfies the requirements placed on the response and factors in an attempt to establish the appropriate growth model. The aim of this optimization study was to find the optimum values of growth determinants in order to maximize the growth in a cost-effective way. RSM predicted that the optimum experimental conditions required for maximum growth of *E. ludwigii* are 5 % of both CMC and yeast extract, 1 % of ammonium nitrate, pH at 6.5, 35 °C temperature alongside 48 h incubation period. Moreover, desirability of this optimum combination is 90.58 % (Fig. 5a; Supplementary Table 2) which is acceptable. Our observation on optimum temperature as 35 °C is also corroborated with a previous finding by Hoffman et al. (2005).

Validation of the proposed model has been presented in supplementary Table 3. We have found reasonable degree of parity between predictive and experimental values confirming validity and adequacy of the proposed model. Furthermore, the validation experiments also revealed that the predicted values of bacterial growth could be satisfactorily achieved experimentally with more than 91 % of reproducibility.

Optimization of any physiological process like microbial growth or enzyme production majorly aims to improve the desired recital of that microbe (Bezerra et al. 2008; Mukherjee et al. 2015). Such optimization usually involves a number of parameters that usually exert an effect over the desired response like bacterial growth in our study. In traditional “one-variable-at-a-time” approach, optimum level of any parameter from a group of several parameters was determined by changing the level of that parameter while keeping others at a constant level (Bezerra et al. 2008; Mukherjee et al. 2014a). However, separate optimization of each parameter cannot provide the actual optimization conditions as several parameters might have interaction among them or there may be synergism or antagonism (Bezerra et al. 2008; Mukherjee et al. 2014a, b). Therefore, adequate optimization should include all the parameters at a time so that interaction effect, synergism, or antagonism among parameters can be taken in account to determine the actual optimum conditions. Thus, multi-variate statistical techniques are considered as most appropriate optimization of any analytical procedure like RSM, Taguchi method, factorial design. (Bezerra et al. 2008; Mukherjee et al. 2012; Basak et al. 2013). RSM possesses a number of recognized advantages in designing and executing optimization experiments (Bas and Boyaci 2007; Myers and Montgomery 2002; Bezerra et al. 2008; Mukherjee et al. 2012). A response like microbial growth that is influenced by several independent variables or parameters can be optimized accurately using RSM (Bas and Boyaci 2007; Myers and Montgomery 2002; Bezerra et al. 2008; Mukherjee et al. 2012). Moreover, it also provides all the information about the interaction between variables (Mukherjee et al. 2015, 2014a, b, 2012). Optimization study by RSM can be performed with a number of statistical design matrices like central composite design, Placket–Burman, Box–Behnken design, (Dong et al. 2008).

Fig. 6 Glucose liberation from different cellulosic substrates by optimally grown *E. ludwigii*



Box–Behnken is also extensively used as spherical and revolving design in optimization experiments studies owing to its reasoning design and admirable outcomes (Dong et al. 2008). In this study, we adopted Box–Behnken design coupled RSM since our aim was to optimize the growth of *E. ludwigii* in a time-consuming and cost-effective way. ANOVA further tested the accuracy of growth model depicted in the Box–Behnken design matrix constructed by RSM and the experimental values of bacterial growth were fitted in a polynomial equation (mentioned in earlier section) using the assumption made from ANOVA. The equation depicts the empirical relationship between the level of parameters that influences bacterial growth and the response i.e., bacterial growth. The said equation is also capable of predicting bacterial growth within the ranges of parameters given in the design matrix with high level of reproducibility as evident from the result of validation experiment.

Expression of cellulase in *E. ludwigii* in optimum growth conditions

Expression of cellulase gene in terms of transcription or cellulase enzyme production was evident in qPCR and immunoblot studies, respectively. It was found that optimum conditions (predicted by RSM) provided adequate expression of cellulase at mRNA and protein level (Fig. 5b, c). 3.23-fold increment in transcription was observed under optimum conditions as compared to the unoptimized conditions (Fig. 5b). Similarly, immunoblot data also corroborated with qPCR data and indicated enhancement of translation of cellulase mRNA in optimum growth conditions (Fig. 5c). Therefore, the optimum conditions for *E. ludwigii* depicted in earlier section not only increase cell division but also enhance their cellulolytic potential to acquire more nutrients from media required for growing bacterium.

In a further study, utilization of different cellulosic substrates by optimally grown *E. ludwigii* (RSM predicted) was tested separately in the presence of CMC (conventional substrate), straw, filter paper, and *Eichhornia* (non-conventional carbon source) and liberation of glucose ($\mu\text{g}/\text{mL}$) was measured. It was observed that glucose liberation ($\mu\text{g}/\text{mL}$) was increased with time with maximum glucose liberated after 28 days from CMC, and thereafter liberation was restricted. Glucose liberation by *E. ludwigii* from different cellulosic substrates has been shown in Fig. 6. Thus growth optimization not only increases the microbial biomass but also enhances their endoglucanase production potential to utilize glucose from a number of different cellulosic substrates.

Therefore, considering the aforementioned experiments on endoglucanase expression (mRNA and protein) and its

effect in utilizing cellulosic substrate, it is clear that optimization of growth does have a predominant role on endoglucanase production in *E. ludwigii*. However, it will be interesting to know about the possible mechanistic insight in growth optimization induced enhancement in endoglucanase production in near future. This also indicates that instead of treating a bacterium with endoglucanase inducer, accurate growth optimization can also upregulate endoglucanase production in a more cost-effective and feasible way.

Growth optimized *E. ludwigii* enhances biogas production

Microbial degradation of cellulosic materials has been the efficient approach for enhancing biodigestibility of cellulosic wastes under both aerobic and anaerobic conditions (Wongwilaiwalin et al. 2010). Symbiotic biomass-degrading microcosms are advantageous due to high cellulose-degrading capability and avoidance of the problem related to feedback inhibition and metabolite repression and offers an interesting candidate for enhancing efficiency in anaerobic digestion (Haruta et al. 2002; Gavrilescu 2002). But practically, construction of structurally stable biomass-degrading microcosms is a problem as it requires highly efficient screening, maintenance, and repeated subculturing of symbiotic microflora originated from different environmental sources active in lignocellulose degradation (Wongwilaiwalin et al. 2010). Besides that, it was also observed that individual bacterial species some time works in a better way rather than in consortium in the biogas system (Dhadse et al. 2012). As a result, application of stable biomass-degrading consortia in biotechnological application has been currently limited, for example to composting or silage (Wang et al. 2006). However, addition of single bacterial species for enhancing biogas production is gaining more and more attention now-a-days. Kume and Fujio (1990) reported that addition of aerobic bacteria, especially *Bacillus* sp., would produce extracellular enzymes in the digester which could act as a catalyst in improving the degradation efficiency (without doing any pretreatment) of the cellulosic matter in the sludge. So, we preferred to directly apply *E. ludwigii* in the anaerobic digester rather than using the isolate in/for pretreatment of biomass slurry which is not only helpful in enhancing biogas yield but also save valuable time.

In the present study, to investigate the effect of the addition of hemicellulolytic mesophilic microbial community on biogas formation, biogas (*Eichhornia*–cow dung) slurry and *E. ludwigii* RAS 2-1 were introduced in fermentation tank under mesophilic temperature conditions (i.e., 35 °C). Both the RSM optimized (5 % of both CMC and yeast extract, 1 % of ammonium nitrate, pH at 6.5,

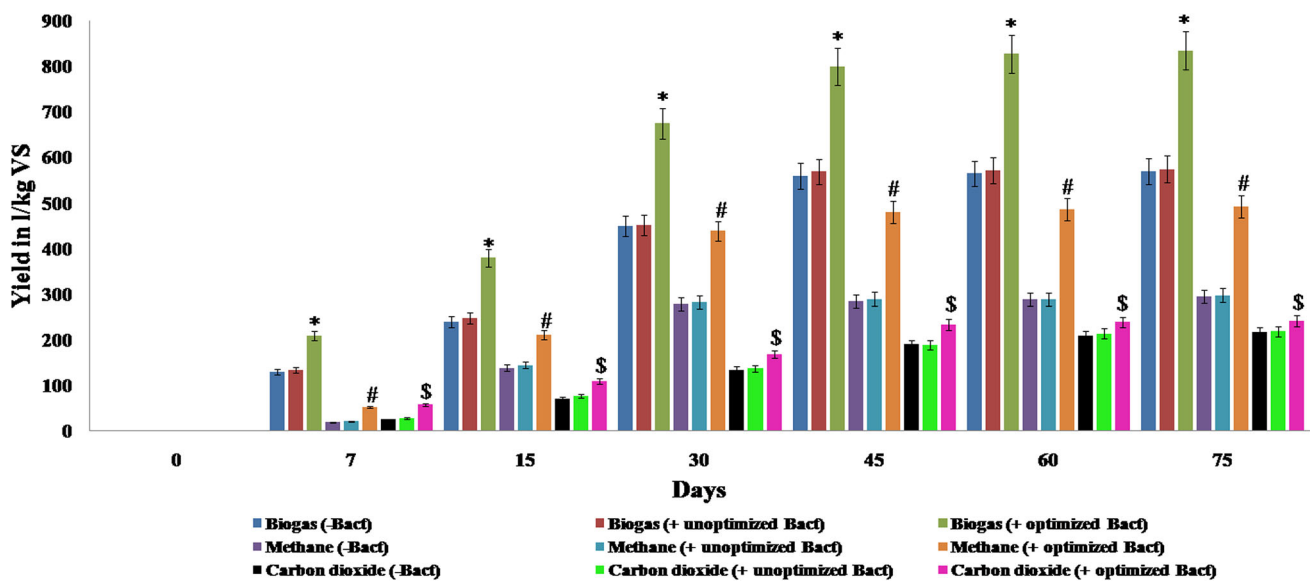


Fig. 7 Comparison of fermentation with and without addition of *E. ludwigii*. Addition of optimally grown *E. ludwigii* would significantly enhance biogas (* $p < 0.05$), methane (* $p < 0.05$), and carbon dioxide ($^{\$}p < 0.05$) production.—Bact: *E. ludwigii* not added inside anaerobic digester (AD); +unoptimized Bact: *E. ludwigii* grown in unoptimized conditions (BHM amended with 2 % yeast extract, 35 °C

temperature, and 28 h incubation period) added inside AD; +optimized Bact: *E. ludwigii* grown in RSM optimized conditions (5 % of both CMC and yeast extract, 1 % of ammonium nitrate, 6.5 pH, 35 °C temperature, and 48 h incubation period) added inside AD. The results represent mean \pm SE of three independent observations

35 °C temperature, and 48 h incubation) and unoptimized bacteria (grown in BHM amended with 2 % yeast extract at 35 °C for 28 h) were used to study the improvement of biogas production but they were employed separately with biogas slurry in separate fermentation tanks. The physico-chemical properties of the biomass are mentioned in Supplementary Table 4. It was observed that addition of RSM optimized *E. ludwigii* (RAS 2-1) not only increased the biogas and corresponding methane and carbon dioxide volume significantly ($p < 0.05$) but also ensured faster gas generation and thus helped in rapid and cost-efficient biogas production (Fig. 7). The initial biogas production was found to be significantly increased until 45 days and then gas production was found to be stable till the end of digestion period ($p < 0.05$). Figure 7 shows a comparison of fermentation with and without addition of *E. ludwigii*, that is, their (RAS 2-1) effects on corresponding biogas yields cumulatively measured over a total fermentation period of 75 days. The total biogas yield with the addition of *E. ludwigii* inoculum (grown under optimized conditions) in the digester was 835 l/kg VS (approx. 59 % methane, 29 % carbon dioxide) which was approximately 47 % higher than the experimental set where no *E. ludwigii* was added externally (biogas volume: 570 l/kg VS, of them, approx. 52 and 38 % were methane and carbon dioxide, respectively). The details of production of biogas, methane, and carbon dioxide have been shown in Fig. 7 and Supplementary Fig. 5.

Earlier studies depicted that optimization of media compositions and different physical parameters (temperature, pH, agitation etc.) would be beneficial in yielding higher bacterial growth alongside cellulase production and activity of the bacterium inside the bioreactor (Deka et al. 2011, 2013; Immanuel et al. 2006). From our study, it can be predicted that optimization of different growth parameters would induce improved endoglucanase production and activity in optimally grown *E. ludwigii* which will be helpful in enhancing hydrolytic activity inside the anaerobic digester, resulting in higher biogas yield. Thus, microbial gas production could be maximized using optimally grown *E. ludwigii* cells as additional inoculum in the digester and therefore RSM-assisted growth optimization can be useful to provide maximum viable bacterial cells. Conversely, addition of *E. ludwigii* grown under unoptimized growth conditions (grown in BHM amended with 2 % yeast extract at 35 °C) would hardly generate any significant augmentation in biogas, methane, and carbon dioxide production when compared with the biogas yield where no *E. ludwigii* were added externally (Fig. 7).

Economic and environmental advantages of biogas production in Santiniketan area by applying *E. ludwigii*

Eichhornia sp. (water hyacinth) is one of the major aquatic weeds of Santiniketan (23.68°N, 87.68°E), West Bengal,

Table 4 Annual Energy benefit of biogas produced from *E. ludwigii* potentized biomass

Biomaterials	Biogas potential (m ³)/annum ^a	Gas requirement/household/day ^b	Gas requirement/annum ^b	No. of family (5 members) benefited
<i>Eichhornia</i> sp., and cow dung	5387	Cooking (0.2 m ³ per meal × 3) = 0.6 m ³	511 m ³ per household	11
<i>Eichhornia</i> sp., cow dung, and potentized <i>E. ludwigii</i>	7891	Lighting (0.1 m ³ × 4 h × 2 lamps) = 0.8 m ³ Total gas requirement = 1.4 m ³		15

^a Calculations for obtaining the biogas potential have been given in Supplementary data

^b Mathew et al. (2015)

India (major population comprises native tribal communities). Though it become noxious, it emerges to be a valuable resource for energy production through anaerobic digestion that could be helpful in solving serious problems in navigation and irrigation (Mathew et al. 2015).

In the present study, a comparative study of electricity generation potential of the biogas produced with or without addition of *E. ludwigii* was conducted. The electricity generation potential from the biogas produced was estimated using basic energy equivalents. The current study yielded 570 L biogas kg⁻¹ VS from the *Eichhornia* and cow dung sets, and 835 L biogas kg⁻¹ VS where optimally grown *E. ludwigii* was supplemented along with water hyacinth and cow dung sets during the 70 days of AD. From energy equivalents, 1 kWh is equal to 3.6 MJ. However, considering an electrical conversion efficiency of 35 %, the estimated electricity generation potential from 1 m³ biogas is estimated to be 2.14 kWh (Mathew et al. 2015). Hence, from the current study, approx. 1.22 and 1.78 kWh electricity could be generated (per kg VS) from *Eichhornia* + cow dung and *Eichhornia* + cow dung + optimally grown *E. ludwigii* inoculum sets, respectively, if the entire biogas is used for electricity generation.

Estimated amount of water hyacinth production is to be above 100 tons per annum in and around the villages of Santiniketan, within the area of 10 km in diameter. A preliminary survey has been carried out to understand about the livelihood and energy consumption of rural villagers on an annual basis per house hold and the data are summarized in Table 3. From Table 3, it is clear that, on an average, a tribal family (residing in and around Santiniketan) consisting of 5 people spends approximately INR 37,800 on a yearly basis to meet their daily energy demand and the CO₂ emissions were estimated to be approx. 8146 kg CO₂ per household on annual basis and this information highly corroborates with previous study by Mathew et al. 2015. Our survey also depicted that cattle rearing is a common practice of the indigenous villagers with an average of 3–4 cows per household and can be

utilized as a good source of cattle dung (inoculum) to feed into the anaerobic digester.

A preliminary study has been conducted to meet these energy demands by the biogas produced using RSM-optimized *E. ludwigii* keeping all the conditions unaltered as described earlier during describing the compositions and parameters of AD for biogas production (in Materials and Methods). The gas requirement per household of indigenous community in and around Santiniketan is estimated to be 1.4 m³ biogas per day (Mathew et al. 2015). A detail regarding the gas requirements per household is given in Table 4. From Table 4, it is clear that the annual biogas potential was estimated approx. 7891 and 5387 m³ with (grown under optimized conditions) or without addition of *E. ludwigii* in AD, respectively, from which 15 and 11 families (5 members in each) might be benefitted correspondingly (Table 4) and would be helpful in replacing the conventional fuel sources such as firewood, kerosene, and dung cake. Thus, application of *Eichhornia* derived biogas (after addition of optimally grown *E. ludwigii*) can be useful in improving the indoor environment of rural communities by providing clean energy. If the biogas could successfully replace the dependence on conventional fuel use of these families, significant reduction in CO₂ emissions along with reduction in indoor air pollution and considerable savings in conventional fuel cost can also be achieved (Mathew et al. 2015). Energy planning at village level through decentralized renewable-based power plants has huge potential to meet the increasing energy demand and rural development in India. Thus, new innovative initiatives in national energy policy are essential to accelerate social and economic development of rural areas. In addition, the digestate, by-product of AD can further be used to partially replace the inorganic fertilizer and improve the soil fertility. Moreover, enhanced biogas production depicted in the study using *E. ludwigii*-potentized *Eichhornia* biomass can be a useful tool to meet energy crises of the indigenous population.

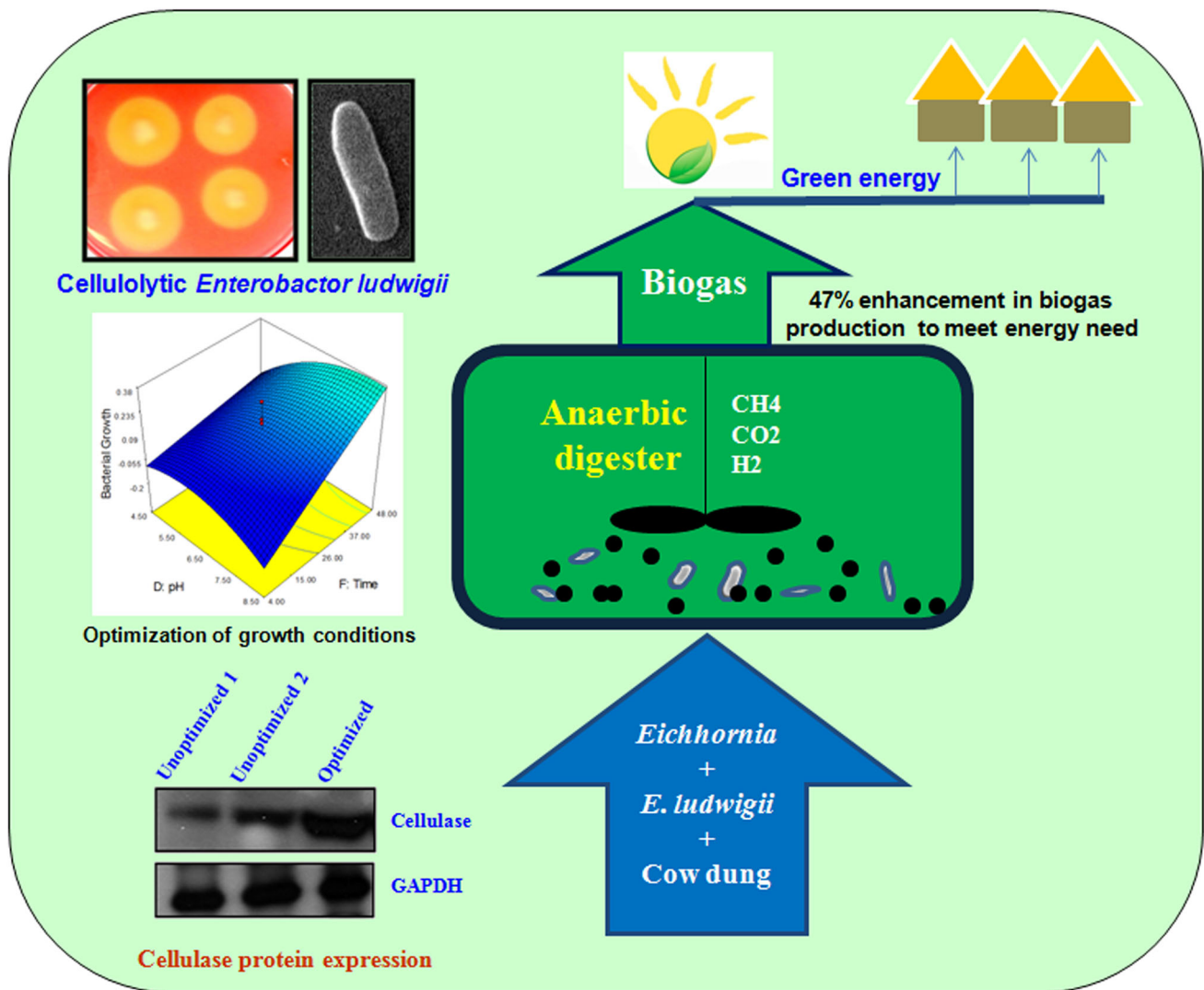


Fig. 8 Scheme depicting *E. ludwigii*-assisted enhanced biogas production in an environment friendly approach

Conclusion

Herein, we report *E. ludwigii* as a potent cellulolytic bacterium from active anaerobic slurry for the first time. The RSM-optimized growth conditions of *E. ludwigii* were found to contribute not only in increasing bacterial biomass but also in the expression of cellulase mRNA and protein to promote biogas production. Thus, the bacterium grown under most favorable conditions provides better performance in contrast to those grown under unoptimized conditions. The enhanced biogas production depicted in the study using *E. ludwigii*-potentized *Eichhornia* biomass can be a useful tool to solve the indoor pollutions and meet the energy crises of the indigenous tribes. Summary of our findings with *E. ludwigii*-assisted biogas production has been presented as schematic model (Fig. 8).

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interests regarding the publication of this paper.

References

- Asis CA, Adachi K (2003) Isolation of endophytic diazotroph *Pantoea agglomerans* and nondiazotroph *Enterobacter asburiae* from sweet potato stem in Japan. *Lett Appl Microbiol* 38:19–23
- Bas D, Boyaci IH (2007) Modeling and optimization I: usability of response surface methodology. *J Food Eng* 78:836–845

- Basak B, Bhunia B, Mukherjee S, Dey A (2013) Optimization of physicochemical parameters for phenol biodegradation by *Candida tropicalis* PHB5 using Taguchi methodology. *Desalin Water Treat* 51:6846–6862
- Bezerra MA, Santelli RE, Oliveira EP, Villar LS, Escalera LA (2008) Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta* 76:965–977
- Chakraborty S, Boksh FIMM, Chakraborty A (2013) Economic viability of biogas and green self-employment opportunities. *Renew Sustain Energy Rev* 28:757–766
- Christensen WB (1946) Urea decomposition as means of differentiating *Proteus* and paracolon cultures from each other and from *Salmonella* and *Shigella* types. *J Bacteriol* 52:461–466
- CLSI (2006) Performance standards for antimicrobial disk susceptibility tests. Approved standard, 9th edn., CLSI document M2-A9. CLSI, Wayne
- Deka D, Bhargavi P, Sharma A, Goyal D, Jawed M, Goyal A (2011) Enhancement of cellulase activity from a new strain of *Bacillus subtilis* by medium optimization and analysis with various cellulosic Substrates. *Enzyme Res*. doi:10.4061/2011/151656
- Deka D, Das SP, Sahoo N, Das D, Jawed M, Goyal D, Goyal A (2013) Enhanced Cellulase Production from *Bacillus subtilis* by Optimizing Physical Parameters for Bioethanol Production. *ISRN Biotechnol*. doi:10.5402/2013/965310
- Dhadse S, Kankal NC, Kumari B (2012) Study of diverse methanogenic and non methanogenic bacteria used for the enhancement of biogas production. *Int J LifeSci Biotechnol Pharm Res* 1:176–191
- Dong J, Mandenius CF, Lubberstedt M, Urbaniak T, Nussler AKN, Knobloch D, Gerlach JC, Zeilinger K (2008) Evaluation and optimization of hepatocyte culture media factors by design of experiments (DoE) methodology. *Cytotechnology* 57:251–261
- Duan C-J, Feng J-X (2010) Mining metagenomes for novel cellulase genes. *Biotechnol Lett* 32:1765–1775
- Garcia de Salamone IE, Dobereiner J, Urquiaga S, Boddey RM (1996) Biological nitrogen fixation in *Azospirillum* strain- maize genotype associations as evaluated by ^{15}N isotope dilution technique. *Biol Fert Soils* 23:249–256
- Gavrilescu M (2002) Engineering concerns and new developments in anaerobic waste-water treatment. *Clean Technol Environ Policy* 3:346–362
- Goswami R, Mukherjee S, Rana VS, Saha DR, Raman R, Padhy PK, Mazumder S (2015) Isolation and characterization of arsenic-resistant bacteria from the surface-waters of contaminated water-bodies in West Bengal, India. *Geomicrobiol J* 32:17–26
- Haruta S, Cui Z, Huang Z, Li M, Ishii M, Igarashi Y (2002) Construction of a stable microbial community with high cellulose-degradation ability. *Appl Microbiol Biotechnol* 59:529–534
- Hoffmann H, Stindl S, Stumpf A, Mehlen A, Monget D, Heesemann J, Schleifer KH, Roggenkamp A (2005) Description of *Enterobacter ludwigii* sp. nov., a novel *Enterobacter* species of clinical relevance. *Syst Appl Microbiol* 28:206–212
- Immanuel G, Dhanusha R, Prema P, Palavesam A (2006) Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment. *Int J Environ Sci Technol* 3:25–34
- Jalasurem V, Kataram S, Gandu B, Anupaju GR (2013) Single cell protein production from digested and undigested poultry litter by *Candida utilis*: optimization of process parameters using response surface methodology. *Clean Technol Environ Policy* 15:265–273
- Kume S, Fujio Y (1990) Digestion of municipal sewage sludge by a mixture of thermophilic *Bacilli* and their culture extract. *J Gen Appl Microbiol* 36:189–194
- Liang Y-L, Zhang Z, Wu M, Wu Y, Feng J-X (2014) Isolation, screening, and identification of cellulolytic bacteria from natural reserves in the subtropical region of China and optimization of cellulase production by *Paenibacillus terrae* ME27-1. *BioMed Res Int*. doi:10.1155/2014/512497
- Mager J, Kuczynski M, Schatzberg G, Avi-Dor Y (1965) Turbidity changes in bacterial suspensions in relation to osmotic pressure. *J Gen Microbiol* 14:69–75
- Maki M, Leung KT, Qin W (2009) The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. *Int J Biol Sci* 5:500–516
- Mathew AK, Bhui I, Banerjee SN, Goswami R, Shome A, Chakraborty AK, Balachandran S, Chaudhury S (2015) Biogas production from locally available aquatic weeds of Santiniketan through anaerobic digestion. *Clean Technol Environ Policy* 17:1681–1688
- Mukherjee S, Bandyopadhyay B, Basak B, Mandal N, Dey A, Mandal B (2012) An improved method of optimizing the extraction of polyphenol oxidase from potato (*Solanum tuberosum* L.) Peel. *Not Sci Biol* 4:98–107
- Mukherjee N, Mukherjee S, Saini P, Roy P, Sinha Babu SP (2014a) Antifilarial effects of polyphenol rich ethanolic extract from the leaves of *Azadirachta indica* through molecular and biochemical approaches describing reactive oxygen species (ROS) mediated apoptosis of *Setaria cervi*. *Exp Parasitol* 136:41–58
- Mukherjee S, Mandal N, Dey A, Mondal B (2014b) An approach towards optimization of the extraction of polyphenolic antioxidants from ginger (*Zingiber officinale*). *J Food Sci Technol* 51:3301–3308
- Mukherjee S, Mukherjee N, Roy P, Saini P, Sinha Babu SP (2015) An approach towards optimization of the influential growth determinants of opportunistic yeast isolate *Pichia guilliermondii*. *Prep Biochem Biotechnol*. doi:10.1080/10826068.2015.1045614
- Mulka R, Szulczewski W, Szlachta J, Prask H (2015) The influence of carbon content in the mixture of substrates on methane production. *Clean Technol Environ Policy*. doi:10.1007/s10098-015-1057-z
- Myers RH, Montgomery DC (2002) Response surface methodology: Process and product optimization using designed experiments, 2nd edn. Wiley, New York
- Navarro AR, Rubio MC, Maldonado MC (2012) A combined process to treat lemon industry wastewater and produce biogas. *Clean Technol Environ Policy* 14:41–45
- Nei M, Kumar S (2000) Molecular evolution and phylogenetics. Oxford University Press, New York
- Nelson N (1944) A photometric adaptation of the Somogyi method for the determination of glucose. *J Biol Chem* 153:375–380
- Ni J, Takehara M, Watanabe H (2010) Identification of activity related amino acid mutations of a GH9 termite cellulase. *Bioresour Technol* 101:6438–6443
- Pereyra LP, Hiibel SR, Riquelme MVP, Reardon KF, Pruden A (2010) Detection and quantification of functional genes of cellulose-degrading, fermentative, and sulfate-reducing bacteria and methanogenic archaea. *Appl Environ Microbiol* 76:2192–2202
- Pourramezan Z, Ghezlbash GR, Romani B, Ziaei S, Hedayatkah A (2012) Screening and identification of newly isolated cellulose-degrading bacteria from the gut of xylophagous termite *Microceroterme diversus* (Silvestri). *Mikrobiologiya* 81:796–802
- Rastogi G, Bhalla A, Adhikari A, Bischoff KM, Hughes SR, Christopher LP, Sani RK (2010) Characterization of thermostable cellulases produced by *Bacillus* and *Geobacillus* strains. *Bioresour Technol* 101:8798–8806
- Rezaei F, Xing D, Wagner R, Regan JM, Richard TL, Logan BE (2009) Simultaneous cellulose degradation and electricity

- production by *Enterobacter cloacae* in a microbial fuel cell. *Appl Environ Microbiol* 75:3673–3678
- Sadhu S, Saha P, Sen SK, Mayilraj S, Maiti TK (2013) Production, purification and characterization of a novel thermotolerant endoglucanase (CMCase) from *Bacillus* strain isolated from cow dung. *SpringerPlus*. doi:10.1186/2193-1801-2-10
- Singh S, Moholkar VS, Goyal A (2013) Isolation, identification, and characterization of a cellulolytic *Bacillus amyloliquefaciens* Strain SS35 from *Rhinoceros* dung. *ISRN Microbiol*. doi:10.1155/2013/728134
- Sukumaran RK, Singhanian RR, Pandey A (2005) Microbial cellulases-production, applications and challenges. *J Sci Ind Res* 64:832–844
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28:2731–2739
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673–4680
- Vasan PT, Piriya PS, Prabhu DIG, Vennison SJ (2011) Cellulosic ethanol production by *Zymomonas mobilis* harboring an endoglucanase gene from *Enterobacter cloacae*. *Bioresour Technol* 102:2585–2589
- Wang X, Haruta S, Wang P, Ishii M, Igarashi Y, Cui Z (2006) Diversity of a stable enrichment culture which is useful for silage inoculant and its succession in alfalfa silage. *FEMS Microbiol Ecol* 57:106–115
- Wilson DB (2011) Microbial diversity of cellulose hydrolysis. *Curr Opin Microbiol* 14:259–263
- Wongwilaiwalin S, Rattanachomsri U, Laothanachareon T, Eurwilai-chitr L, Igarashi Y, Champreda V (2010) Analysis of a thermophilic lignocellulose degrading microbial consortium and multi-species lignocellulolytic enzyme system. *Enzyme Microb Technol* 47:283–290