



Research Article

Enhancement of Substrate Decomposition through Potential Hydrolytic Bacteria for Cumulative Biogas Production

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Abstract

Scenarios focus on the practical behavior of anaerobic decomposition systems to enhance biogas production, in addition to assure economic progression and ecological sustainability. The present study has framed to identify the potential hydrolytic bacteria from five different sources since principally the efficacy of hydrolytic bacteria determines the rate of hydrolysis of anaerobic decomposition and thereby biogas production. Among the 40 dominant bacteria isolated from diverse bases, 10 isolates were selected as efficient through preliminary screening. Consequently, the premier enzyme activity obtained from the isolate G5 obtained from goat rumen fluid for cellulase (44.16 ± 1.00 U/ml), protease (260.63 ± 1.35 U/ml) and lipase (33.20 ± 0.81 U/ml). Morphological, biochemical and molecular characterization revealed that G5 is *Bacillus sp.* DDG5 (KM093856.1). A range of pH (7.0-7.5) and temperature (40°C) was sufficient for the highest activity of hydrolytic enzymes experienced. Biogas production using cow dung showed an improved efficiency of 9.54 % in *Bacillus sp.* DDG5 treated tank (70.16 ± 1.54 %) in contrast to control (58.13 ± 1.02 %) at 30th day. However, this study established that *Bacillus sp.* DDG5 obtained from goat rumen fluid is the promising hydrolytic bacteria, since it can be applied for proficient hydrolysis of various organic materials to enhance methane production in outlook.

Keywords: Anaerobic decomposition; Hydrolysis; Microorganisms; Enzymes; Screening; Biogas.

Introduction

The reliance of fossil fuels has been restricted due to its non-renewable nature, which surrogate biomass being the major contributor of energy generation in the upcoming scenarios by the rationale of increasing energy consumption. Biogas production from biomass through anaerobic decomposition has great interest at the moment due to its renewable energy generation potential in addition to eco-friendly applications

for greenhouse gas reduction, waste recycling and biofertilizer production. During anaerobic decomposition, different microbial groups are employed in respective stages and subsequently release biogas under anaerobic circumstances, where methane is accomplished as the flammable fraction (Divya et al., 2014). Among the four stages such as hydrolysis, acidogenesis, acetogenesis and methanogenesis were recognized in anaerobic

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decomposition, hydrolysis is established as the substantial rate limiting step in which complex organic polymers are being converted into monomers by the action of hydrolytic bacteria. Generally, hydrolysis destined by the conversion of polysaccharides, proteins and lipids of organic materials in to sugars, amino acids and fatty acids. Throughout the decomposition process, the final products of one stage would be the key substance for the subsequent stage. Whereas, the lack of sufficient hydrolytic enzymes would drastically affect the entire process. Therefore, the principle coupled with significance of hydrolysis is the intact decomposition is depending upon the rate of hydrolysis since the subsequent stages of anaerobic decomposition would be frequently brought on the exact manner only once absolute hydrolysis occurs. Usually, hydrolysis of complex organic material is difficult by the majority of the hydrolytic bacteria or a group of hydrolytic bacteria is needed to attain proper hydrolysis during digestion. However, the adeptness of biomass conversion during decomposition can be enhanced by mounting the rate of hydrolysis (Romano *et al.*, 2009).

Diverse microorganisms play significant role in anaerobic decomposition; correspondingly the magnitude of biogas production is largely influenced by the potentiality of microbes involved in the process. The probable hydrolytic bacteria are adequate for the production of hydrolytic enzymes with highest activity to assure the fluidity of organic substrates (Hendriks and Zeeman, 2009). However, few records demonstrated that the substrate fluidity can be enhanced by ensuring hydrolytic enzymes (Plochl *et al.*, 2009). The major enzymes involved in hydrolysis are cellulase, protease and lipase; which convert polysaccharides, proteins and lipids into sugars, amino acids and fatty acids, respectively. A range of microorganisms are capable of hydrolysis whereas sufficient quantity of entire enzymes for absolute hydrolysis can be accomplished by few microbes (Saraswati *et al.*, 2012). In general, simplest organic sources were recognized as wealthy habitat for the microbes those persuade fermentative degradation. The criterion of the carbohydrate potential of various sources such as cow dung slurry, sugar cane effluent, sewage water, municipal solid waste and goat rumen fluid crafts them to an assumption to hamper diverse hydrolytic bacteria (Eze and Agbo, 2010; Budiyono *et al.*, 2010; Malik and Bharati, 2009; Zhu *et al.*, 2009). Therefore, this study preferred the mentioned sources for the recognition of potential hydrolytic bacteria. Moreover, the deployment of potential hydrolytic bacterium nullifies the lag phase required for the production of each enzyme while using a group of hydrolytic bacteria or consortia for the purpose. With these concepts, the present study has been focused on the identification of potential hydrolytic bacteria among five different sources, to enhance hydrolysis of substrates to achieve flourishing biogas production in meanwhile.

A wide variety of substrates have been used in earlier, whereas cow dung is established as the feasible substrate for biogas production. Cow dung possesses a moisture content of 88% and carbon/nitrogen ratio of 15-35 in the range (Divya *et al.*, 2015). These characteristics are often favorable for biogas production that bring into being an increased attraction of cow dung as substrate for the purpose from the last few decades. Even though the absolute substrate decomposition along with desired quantity and quality of biogas has not yet attained from the simple feedstock experienced formerly, including cow dung. Another concern behind decomposition process is that long duration of 30-45 days are adequate for ceiling biogas production from most of the substrates due to prolonged hydrolysis. Hence this study pays an attention towards the significance of potential hydrolytic bacteria on cow dung for enhancing methane production with the intention of complete digestion of both simpler and complex substrates to realize commercialization of biogas production technology in future. However, these kinds of approaches are necessary at this energy demanding style to accomplish economic and ecological welfare.

Materials and Methods

Collection of Microbial Sources

Among the diverse prosperous sources chosen, cow dung slurry was collected from 24 years old biogas plant and sugar cane effluent collected from sugar factory, positioned under COR- foundation, Pandalam, Kerala. Sewage water and municipal solid waste was collected in and around Tiruchengode, Tamilnadu. Though, goat rumen fluid was collected from the Veterinary Hospital, Namakkal, Tamilnadu with the assistance of senior veterinary surgeon. All the five sources were collected aseptically and instantly brought to the laboratory of Vivekanandha College, Tamilnadu.

Bacterial Isolation and Screening

The isolation was carried out through spread plate technique. Bacterial colonies obtained were differentiated on the basis of colony characteristics and prominent colonies showed different morphology were cultured for pure cultures. The isolates were preliminary screened for the evaluation of major hydrolytic enzymes cellulase, protease and lipase on carboxyl methyl cellulose (CMC) agar, protease specific gelatin agar and tributyrin agar, respectively. Subsequently, cellulase producers were selected based on halo zone obtained due to substrate hydrolysis after the addition of 0.1% congo-red solution and followed by 1M sodium chloride solution (Saraswati *et al.*, 2012). For protease producing isolates, mercuric chloride solution flooded to respective plates to obtain clear zones (Alnahdi, 2012). A clear zone around the isolates on tributyrin agar indicated that the isolates capable of lipase production (Bonala and Mangamoori, 2012).

Enzyme Production and Assay

The isolates which possesses large zone of clearance from each sample were selected for enzyme assay in order to prefer the most potential isolate possess highest enzyme activity. The efficient isolates were subjected for assay of the enzymes cellulase, protease and lipase. Basal medium containing 0.1% carboxyl methyl cellulose substrate was used as production media for cellulase. For assay 1% carboxyl methyl cellulose was used, where one unit of enzyme activity is determined as the amount of enzyme that released 1 μ M of glucose (Saraswati *et al.*, 2012). For protease assay, efficient isolates were incubated in protease specific broth and assay was carried out with casein as substrate and tyrosine as standard. One unit of enzyme activity refers to the amount of enzyme that released 1 μ g of tyrosine per ml per minute under the standard conditions of supernatant solution (Alnahdi, 2012; Lowry *et al.*, 1951). Mineral media supplemented with olive oil was used as the production media for lipase. For lipase assay, 10% v/v olive oil emulsion in 2% v/v gum acacia was used and the reaction mixture was then titrated against 0.05N sodium hydroxide using phenolphthalein indicator to find out liberated fatty acids. One unit of lipase activity is determined as the amount of enzyme needed to liberate 1 μ M of fatty acids under assay conditions (Sirisha *et al.*, 2010).

Characterization of Potential Bacteria

Potential isolate showed highest enzyme activity was identified through morphological, biochemical and molecular characterization. Gram's staining, spore staining, IMViC tests, catalase, oxidase, starch hydrolysis, lipid hydrolysis, gelatin hydrolysis, triple sugar iron, hydrogen sulfide production, nitrate reduction, and sugar fermentation tests were carried out as indicated by Bergy's manual of discriminative bacteriology. Although, 16S rRNA sequencing was performed for molecular identification, where universal primers such as 8-27f (5'-AGAGTTGATCCTGGCTCAG-3') and 1500r (5'-AGAAAGGAGGTGATCCAGGC-3') are employed (Kowsalya and Gurusamy, 2013). The amplified fragments were purified using PCR clean up kit. DNA sequencing was carried out through automated DNA sequencing using automated ABI3100 genetic analyser. Accordingly, sequence similarity search was performed with the reference sequence by using the tool BLAST and the sequence was then deposited in Genbank. Multiple sequence alignment of the respective bacterial sequence along with ten closely related strains were carried out through CLUSTAL W and subsequently phylogenetic analysis was performed by the software Mega- 4.0 where Neighbor- Joining method was used for the erection of the phylogenetic tree (Saitou and Nei, 1987; Tamura *et al.*, 2007).

Optimization of Growth Parameters

The imperative control measures such as pH and temperature were optimized to accomplish better enzyme yield. The potential bacteria designated was grown in relevant production media for cellulase, protease and lipase at a selective range of pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5) and temperature (25, 30, 35, 40, 45, 50, 55, 60°C). Furthermore, enzyme assay has been performed to analyze the significance of each parameter to realize the optimum conditions for the highest production of hydrolytic enzymes.

Reactor Design, Biogas Production and Analysis

A set of batch reactors with each possess an intact volume of 20 liters were used in this investigation. The reactor offers an inlet, outlet, sampling unit, thermometer, pressure gauge and gas collection unit to analyze the rate of biogas production. Fresh cow dung was collected in cellophane bags from a dairy farm located at Namakkal, Tamilnadu. Two kilograms of cow dung was mixed with 15 liters of distilled water to achieve feasible decomposition of substrate. A final volume of 15 liters cow dung slurry was mixed with 10% of inoculums cultured under optimum pH and temperature. Followed by the substrate-inoculums mixture was transferred to the anaerobic digester for biogas production. Simultaneously, reactor contains feedstock without inoculum was kept as control for the comparative analysis of process efficiency. The reactors were then sealed tightly to ensure anaerobic environment and biogas production carried out for 30 days and subjected samples during respective time intervals (1st, 10th, 20th & 30th day) were collected and analyzed through gas chromatography (GC3800) in order to characterize various components of biogas. The gas analyzer possesses a flame ionization detector (FID) and a glass column (1.6m long, 3mm i.d.) packed with 80/100 porapak Q; with a nitrogen flow rate of 25ml/min as well as a column and the detector temperature of 32°C and 50°C, respectively (Merlin Christy *et al.*, 2014a).

Results

Sample Collection and Bacterial Isolation

Plentiful colonies obtained on the nutrient agar from the five sources demonstrated that the collected sources are wealthy with different types of bacteria. Based on the colony characteristics, a total of 40 bacterial colonies chosen of which 8 distinct dominant colonies (C1, C2, C3, C4, C5, C6, C7, C8) from cow dung slurry, 7 colonies (S1, S2, S3, S4, S5, S6, S7) from sugar cane effluent, 8 colonies (E1, E2, E3, E4, E5, E6, E7, E8) from sewage water, 9 bacterial colonies (M1, M2, M3, M4, M5, M6, M7, M8, M9) from municipal solid waste and 8 colonies (G1, G2, G3, G4, G5, G6, G7, G8) were from goat rumen fluid.

Table 1: Preliminary screening of bacteria isolated from different sources

Screening of cellulase, protease and lipase on different media- zone of clearance (cm)																			
Cow dung				Sugar cane waste				Sewage water				Municipal solid waste				Goat gut fluid			
Isolates	CMC	PSM	TA	Isolates	CMC	PSM	TA	Isolates	CMC	PSM	TA	Isolates	CMC	PSM	TA	Isolates	CMC	PSM	TA
C1	0.3	1.2	0.7	S1	0.7	1.5	1.0	E1	0.3	0.8	0.7	M1	0.3	0.9	0.4	G1	0.2	1.0	0.9
C2	0.7	1.5	1.0	S2	1.2	1.9	1.5	E2	0.6	1.4	0.8	M2	0.2	1.8	0.6	G2	0.5	1.1	0.2
C3	-	1.2	0.8	S3	0.4	1.2	1.2	E3	0.4	1.3	0.8	M3	0.5	1.1	0.6	G3	1.5	1.4	0.4
C4	1.2	2.0	0.9	S4	1.4	1.5	1.2	E4	0.2	1.5	0.8	M4	0.6	1.2	0.8	G4	-	0.5	1.5
C5	-	-	0.8	S5	-	-	0.7	E5	0.5	1.4	0.5	M5	1.1	2.0	1.2	G5	2.5	2.2	2.0
C6	0.2	1.3	0.9	S6	0.3	0.9	0.9	E6	0.6	1.6	0.9	M6	-	-	0.9	G6	0.3	1.9	1.0
C7	0.4	0.7	0.8	S7	0.5	1.3	0.9	E7	0.4	1.5	0.6	M7	-	-	-	G7	0.2	0.4	-
C8	0.4	1.1	0.6					E8	1.0	1.8	1.1	M8	0.5	1.2	0.6	G8	1.8	1.9	1.4
												M9		1.0	1.9	1.0			

CMC: carboxyl methyl cellulose agar; PSM: protease specific media; TA: tributyrin agar.

Enzyme Screening and Assay

Preliminary screening of 40 bacterial isolates revealed that 33 isolates were positive for the three enzymes experienced. Amongst 40 bacteria isolated throughout the study, ten isolates such as C2, C4, S2, S4, E6, E8, M5, M9, G5 and G8 were selected as efficient hydrolytic bacteria based on its largest zone of clearance for all the three hydrolytic enzymes, as demonstrated in the Table 1. Interestingly, all the isolates from sewage water showed respective zone of clearance. Results of enzyme assay of efficient strains render that G5 obtained from goat rumen fluid was promising for the production of cellulase, protease and lipase. The highest enzyme activity of 44.16 ± 1.0 U/ml for cellulase, 260.63 ± 1.35 U/ml for protease and 33.2 ± 0.81 U/ml for lipase was observed for G5 (Table 2)

Table 2: Enzyme assay of efficient isolates

Isolates	Enzyme activity (U/ml)		
	Cellulase	Protease	Lipase
C2	26.5 ± 0.6	224.7 ± 0.8	25.3 ± 0.6
C4	22.7 ± 0.4	243.2 ± 0.9	24.2 ± 0.6
S2	32.4 ± 0.5	237.2 ± 0.5	31.5 ± 0.7
S4	37.3 ± 0.8	255.1 ± 1.1	28.7 ± 1.0
E6	29.2 ± 0.4	242.3 ± 1.0	22.0 ± 0.2
E8	23.6 ± 0.4	218.5 ± 0.6	28.1 ± 0.4
M5	31.5 ± 0.6	256.5 ± 1.3	29.4 ± 0.6
M9	30.1 ± 0.4	249.2 ± 0.5	26.8 ± 0.7
G5	44.1 ± 1.0	260.6 ± 1.2	33.2 ± 0.8
G8	38.5 ± 0.6	251.4 ± 1.0	27.3 ± 0.4

Potential enzyme activity- G5

Bacterial Identification and Phylogenetic Analysis

Morphological analysis reveals that the preferred bacterium is a rod shaped Gram positive, spore former (Fig. 1). The bacteria showed positive results for Voges Proskaur, citrate, catalase, starch hydrolysis, gelatin hydrolysis, lipid hydrolysis, triple sugar iron, carbohydrate fermentation tests and nitrate reduction test and negative results for indole, methyl-red, oxidase, and hydrogen sulfide production test. Moreover, PCR analysis of bacterial DNA explored that the amplified DNA possesses base pairs about to 1500bp on agarose gel in contrast to the isolated genomic DNA (Fig. 2). Similarity study of the sequence obtained by

16S rRNA sequencing through BLAST search revealed that the bacterium possesses close relationship with *Bacillus* sp. and *Bacillus subtilis*, simultaneously the sequence was productively deposited in the GenBank as *Bacillus* sp. DDG5 and obtained the accession number KM093856.1. Phylogenetic analysis of *Bacillus* sp. DDG5 (KM093856) with ten closely related strains revealed that the bacterium shows least divergence to all the *Bacillus* strains analyzed. However, the rate of divergence was too low which was supported by the low bootstrap values depicted in the phylogenetic tree. Even though *Bacillus* sp. DDG5 was found as closer to *Bacillus* sp. SNC1 (JX495609) and *Bacillus subtilis* NB-01 (HM214542) (Fig. 3).

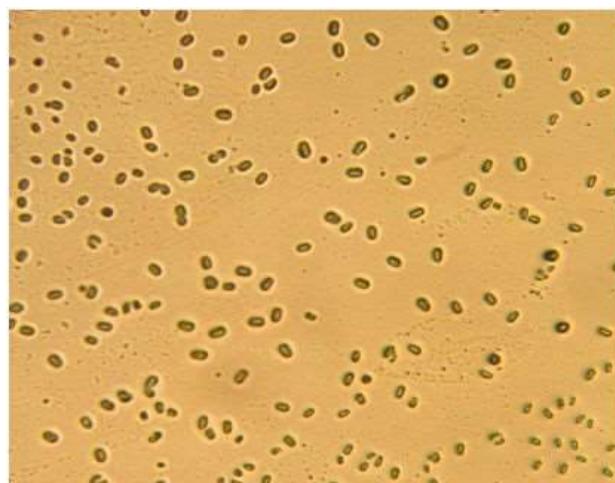


Fig. 1: Gram staining of G5

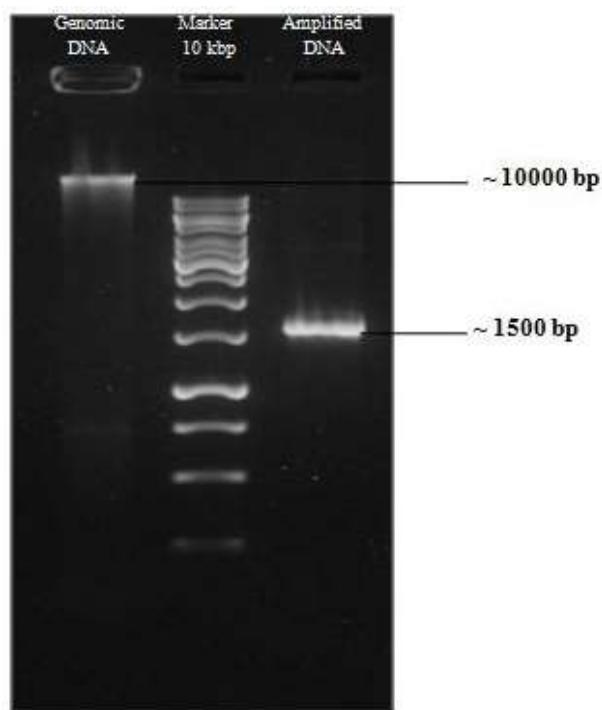
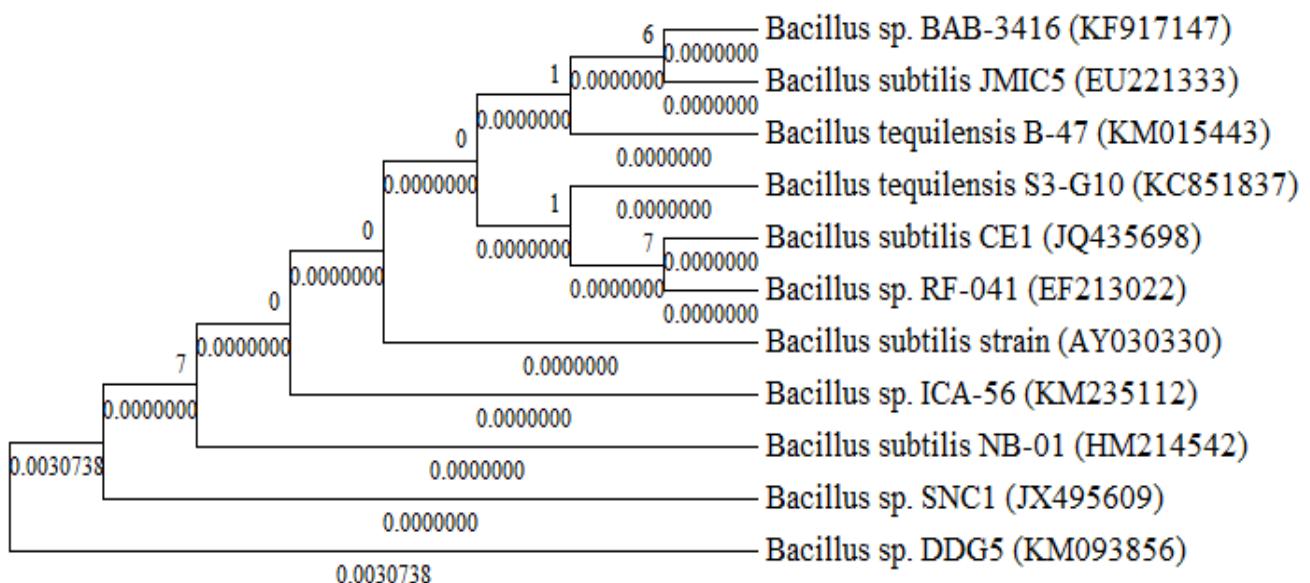


Fig. 2: Isolated and amplified DNA on agarose gel

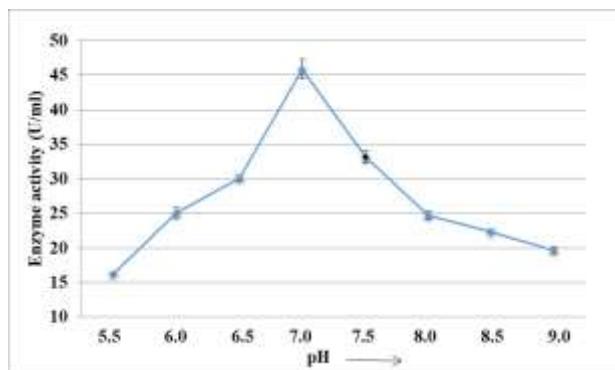
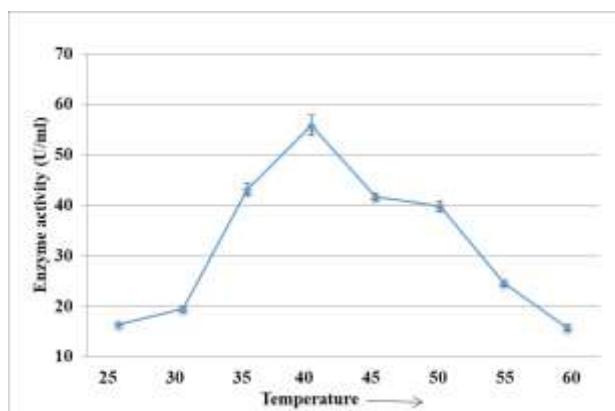
**Fig. 3:** Phylogenetic analysis of *Bacillus* sp. DDG5 (KM093856)

Parameter Optimization

The optimum pH for cellulase, protease and lipase production by *Bacillus* sp. DDG5 was recognized as 7.0 (45.90 ± 1.4 U/ml), 7.5 (486.36 ± 2.3 U/ml) and 7.5 (38.63 ± 0.8 U/ml) while optimum temperature was identified as 40°C (55.86 ± 1.9 U/ml), 40°C (513.13 ± 2.4 U/ml) and 40°C (41.63 ± 0.8 U/ml), respectively (Fig. 4, 5 & 6). Controversially, it was interesting to note that hydrolytic enzyme production from *Bacillus* sp. DDG5 was moderately constant at a range of pH 7.0-7.5 and temperature of 40°C .

Biogas Production

The foremost reflection of the present study is that biogas production from cow dung with the addition of hydrolytic bacteria *Bacillus* sp. DDG5 showed $70.16 \pm 1.54\%$ methane production at 30th day, which was comparatively higher than that of control where the methane content was $58.13 \pm 1.02\%$ at 30th day (Fig. 7). In the due course of 30 days digestion, the concentration of carbon dioxide and hydrogen sulphide was gradually decreased while methane concentration was increased from 1st day to 20th day in both control and test. However, an increased methane efficiency of 9.54% has been obtained in the treated tank when compared to control.

**Fig. 4:** Cellulase activity at different pH**Fig. 5:** Cellulase activity at different temperature

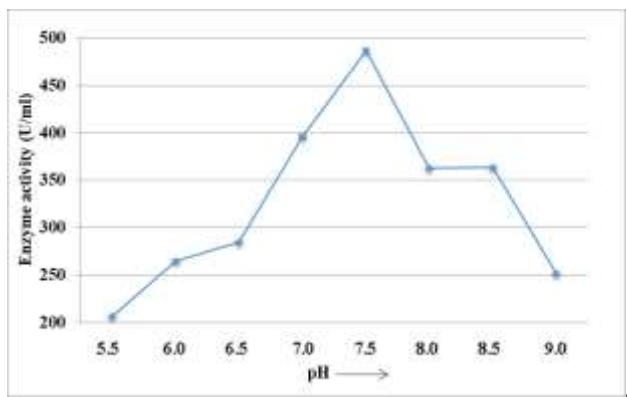


Fig. 6: Protease activity at different pH

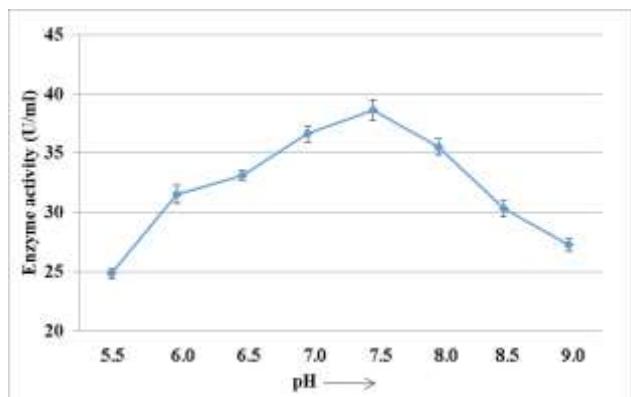


Fig. 8: Lipase activity at different pH

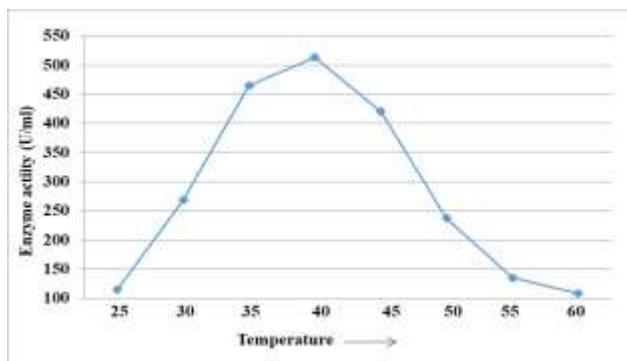


Fig. 7: Protease activity at different temperature

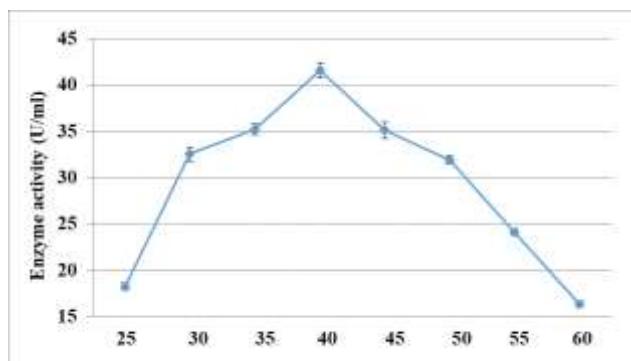


Fig. 9: Lipase activity at different temperature

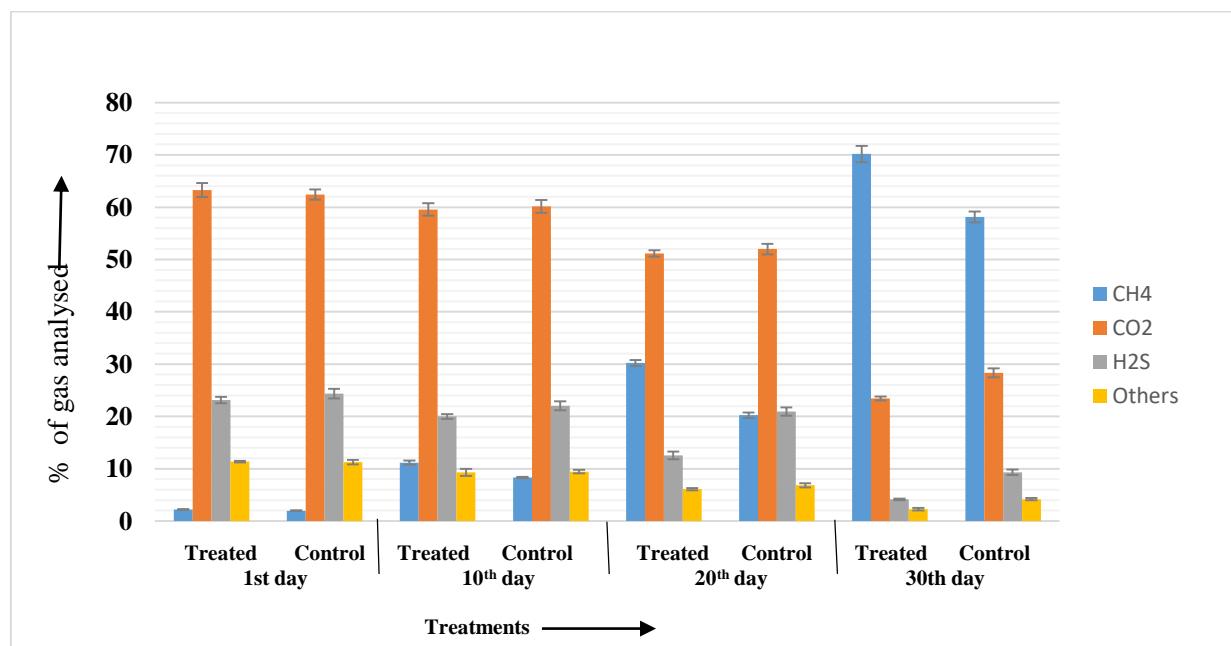


Fig. 10: Characterization of biogas through Gas chromatography

(CH₄: methane; CO₂: carbon dioxide; H₂S: hydrogen sulphide)

Discussion

Usually, habitats with higher volatile organic compounds are the best sources in which to find a vast variety of degradative bacteria (Huang and Monk, 2004; Shaikh *et al.*, 2013). Generally, cow dung possesses a wide range of degradative microorganisms acquired from the rumen and gut of cow (Adegunloye *et al.*, 2007). As well it is expectant that cow dung slurry obtained after biodegradation from aged biodigester could possess promising fermentative microbes with genetic diversity. Conversely, hydrolytic bacteria are affluent in sugar cane effluent, sewage water and municipal solid waste that craft its effortless degradation of innate environment (Eleri *et al.*, 2014; Gautam *et al.*, 2012). However, the assurance of degradative microbes in sewage and municipal solid waste might be the scientific reason for applying this to large-scale biogas production alongside to ensure waste reduction. Rumen fluid is another source that could possess efficient fermentative microbes since bovine rumen fluid has been reported as promising inoculums for mounting biogas production in earlier (Sunarso *et al.*, 2010). Although, goat rumen fluid may possess potential hydrolytic bacteria those persuade efficient decomposition of organic matter as evidenced by fast digestion of feedstock occurred in ruminant animals. Whereas the microbial communities inhabited in the goat rumen fluid were not yet studied well. According to this concept cow dung slurry, sugar cane effluent, municipal solid waste, sewage water and goat rumen fluid were used for the present study to assure maximum possibility to obtain potential hydrolytic bacteria to accomplish successful biogas production.

Enzyme technology has received much more attention in biogas production at present since anaerobic digestion of organic matter is highly influenced by microbial enzymes (Plochl *et al.*, 2009). The entire fermentative microbes are gifted with relevant properties, however, the rate of hydrolysis is principally allied with the quantity and quality of hydrolytic enzymes produced. Fewer studies figured out that the most difficult task stumbles upon biodegradation is the identification of efficient hydrolytic bacteria (Sadhu and Maiti, 2013). Nevertheless, the current investigation pledges the recognition of potential hydrolytic bacteria among the 40 isolates obtained from five sources through preliminary screening and enzyme assay. A number of studies reported in earlier regarding with microbial screening of cellulase, protease and lipase individually, besides its optimization in course to identify potential producers for diverse industrial purposes extensively. As well as few microbes were tested for fermentative enzymes, especially cellulase production for biodegradation purposes (Gerhardt *et al.*, 2007; Hendriks and Zeeman, 2009). Commercial enzyme preparations were applicable for waste management at the moment whilst cost reduction turn over the major concern associated with the utilization of enzyme

mixtures, which driven the necessity of potential hydrolytic bacteria for biodegradation (Jordan and Mullen, 2007). Moreover, the present study is also accordance with the identification of potential hydrolytic bacteria for enhanced hydrolysis of organic material; exceptionally wealthy sources were used here to obtain the most effective producers of the major hydrolytic enzymes practiced.

The highest enzyme activity obtained during assay was satisfactory for the confirmation of G5 isolated from goat rumen fluid as a promising strain for hydrolysis. Generally, rumen of ruminant animals possesses microorganisms capable of degrading cellulosic materials (Aurora, 1983). The presence of efficient bacteria might be the underlying basis behind fast digestion of feedstock occurs within few hours inside of ruminant animals instead of requiring long time to digest the same at normal environment. For instance, the presence of degradative bacteria in sewage water owed by the effortless degradable nature of sewage water. However, the identification of such kind organism is an important criterion, for instance morphological, biochemical and molecular analysis is essential for the accurate identification of targeted microbe. Characterization studies showed G5 is *Bacillus subtilis* DDG5 and its cellulase activity was promising to contrast with the cellulase activity of 30.33 U/ml reported from *Bacillus subtilis* (Saraswati *et al.*, 2012). *Bacillus sp.* DDG5 was competent for protease production with *Bacillus sp* (243U/ml), *Bacillus subtilis* (240.45U/ml), *Bacillus amovivorus* (204U/ml) and *Bacillus licheniformis* (110U/ml) (Alnahdi, 2012; Sharmin *et al.*, 2005; Roja Rani *et al.*, 2012). Although the highest lipase activity obtained from fermentative bacteria like *Bacillus* (30U/ml, 18U/ml), *Pseudomonas* (10U/ml) and *Staphylococcus* (25U/ml) has signified the relevance of *Bacillus sp.* DDG5 for highest lipase production (Ertugrul *et al.*, 2007; Sirisha *et al.*, 2010; Padmapriya *et al.*, 2011). The current finding was quite reproducible and promisingly comparable with the earlier reports stated. Eventhough, *Bacillus Genera* is well known as the source of commercially available degradative enzymes (Horikoshi and Akiba, 1982). *Bacillus sp.*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis*, etc. were established as efficient for extracellular enzyme production in earlier (Alnahdi, 2012; Eftekhar *et al.*, 2003; Acharya and Chaudhary, 2012). Fermentative bacteria like *Bacillus sp.* and *Pseudomonas sp.* are of great importance in various biomass processing industries (Shaikh *et al.*, 2013; Pastor *et al.*, 2001). The better cellulolytic potential was also accomplished by fungi such as *Trichoderma* and *Aspergillus* (Vinod *et al.*, 2012; Lynd *et al.*, 2002). Expectantly, the reference strain *Bacillus sp.* DDG5 showed competent enzyme activity that renders the strain as an alternative to bacterial and fungal systems those reported high enzyme activity formerly.

The standardization of parameters is essential to fulfil the entire growth requirements of each microbe to attain maximum enzyme activity (Eftekhar et al., 2003). The most significant parameters optimized in this study were pH and temperature since fluctuations in pH and temperature during anaerobic digestion would drastically affect microbial growth and enzyme production that subsequently reduce biogas production (Divya et al., 2015). The finest pH and temperature range of 7.0-7.5 and 40°C recognized for highest enzyme production of *Bacillus subtilis* DDG5, which would be feasible for biogas production, since neutral pH and mesophilic temperature requirements offer effortless process maintenance. Concurrent study in 2011 (Sangkharak et al., 2011) reported that cellulase from *Cellulomonas sp.* exhibit stable enzyme activity at broad range of temperature. The optimization results were relatively higher than the outcome of earlier optimization studies accounted for cellulase production (32.4U/ml) and lipase production (39.6U/ml) at temperature 30°C, 40°C and pH 7.0, 9.0 (Saraswati et al., 2012; Padmapriya et al., 2011). The highest optimized protease activity reported was 524 U/ml at pH 8.5 and 549 U/ml at temperature 37°C (Sharmin et al., 2005). However, few studies reported moderately increased enzyme production in contrast to current results during standardization, where the highest enzyme activity has been acquired by alkaline pH, thermophilic/psychrophilic temperature or by extending incubation period, while those features cannot be adaptable for classic biogas production.

Nevertheless, the addition of potential fermentative bacteria in to anaerobic digester might enhance the degradation rate of feedstock materials and thereby augment biogas production. Since lower concentration of methane in biogas produced from various feedstock has been identified as the major constraint that hinder the production and utilization of biogas in earlier. Concurrently, highest methane production from cow dung was observed as 64% at 30th day, which was relatively lower than the highest production rate of $70.16 \pm 1.54\%$ observed at 30th day during the current study while using the additive *Bacillus sp.* DDG5 (Merlin Christy et al., 2014b). This study also supported the forecast that biological additives assist to improve biogas production (Divya et al., 2015; Merlin Christy et al., 2014a]. A similar prospective study explored that flourishing degradation of organic materials for ethanol production can be achieved by treating with cellulolytic bacteria *Bacillus sp.* (Acharya and Chaudhary, 2012). Moreover, through the present study it is evident that the addition of potential hydrolytic bacteria enhances methane production extensively alongside to reduce the duration of anaerobic digestion process for biogas production.

Conclusion

The present study was a realistic approach explored the viability of employing promising hydrolytic bacteria to

accomplish increased production of economically valuable renewable energy source biogas through enhancing hydrolysis. Accordingly, the potential isolate G5 obtained from goat rumen fluid was promising for biodegradation than the other characteristic strains in course of highest enzyme activity. As well the characterization studies revealed that G5 is *Bacillus sp.* DDG5 and optimization studies shown effortless enzyme production by preferred bacteria at ordinary conditions. However, 9.54% increased methane concentration at 30th day indicated that *Bacillus sp.* DDG5 is a promising strain for enhancing biogas production. Hence the significant effect of *Bacillus sp.* DDG5 for hydrolysis recommends further opportunities of using for fermentation as well as biodegradation purposes to attain sufficient methane production. Moreover, the current research offers a prospect for the utilization of probable hydrolytic bacteria in favor of desirable degradation of complex materials to increase substrate availability, alongside to ensure socio-economic advancement of developing countries in the meantime.

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References

- Acharya S and Chaudhary A (2012) Optimization of fermentation conditions for cellulases production by *Bacillus licheniformis* MVS1 and *Bacillus sp.* MVS3 isolated from Indian hot spring. *Braz Arch Biotechol* **55**: 497-503. DOI: [10.1590/S1516-89132012000400003](https://doi.org/10.1590/S1516-89132012000400003)
- Adegunloye DV, Adetuyi FC, Akinyosoye FA and Doyeni MO (2007) Microbial analysis of compost using cow dung as booster. *Pak J Nutr* **6**(5): 506-510. DOI: [10.3923/pjn.2007.506.510](https://doi.org/10.3923/pjn.2007.506.510)
- Alnahdi HS (2012) Isolation and screening of extracellular proteases produced by new isolated *Bacillus sp.* *J Appl Pharmaceut Sci* **2**(9): 71-74. DOI: [10.7324/JAPS.2012.2915](https://doi.org/10.7324/JAPS.2012.2915)
- Aurora SP (1983) Microbial digestion in ruminants. Indian Council of Agricultural Research, New Delhi.
- Bonala KC and Mangamoori LN (2012) Production and optimization of lipase from *Bacillus tequilensis* NRRL B-41771. *Inter J Biotechnol Appl* **4**(1): 134-136. DOI: [10.9735/0975-2943.4.1.134-136](https://doi.org/10.9735/0975-2943.4.1.134-136)
- Budiyono IN, Widiasa S and Johari S (2010) The kinetic of biogas production rate from cattle manure in batch mode. *Inter J Chem Biol Eng* **3**(1): 39-44.
- Divya D, Gopinath LR and Merlin Christy P (2014) A review on Trends, issues and prospects for biogas production in developing countries. *Inter Res J Environ. Sci* **3**(1): 62-69.

- Divya D, Gopinath LR and Merlin Christy P (2015) A review on current aspects and diverse prospects for enhancing biogas production in sustainable means. *Renew Sustain Ener Rev* **42**: 690-699. DOI: [10.1016/j.rser.2014.10.055](https://doi.org/10.1016/j.rser.2014.10.055)
- Eftekhar F, Fouladi J and Faghihi M (2003) Isolation and identification of an alkaline protease producing *Bacillus* from soil. *Iran J Biotechnol* **1**(3): 183-185.
- Eleri AA, Isu NR, Tijjani N (2014) Biogas production from organic kitchen waste using cow dung as inocula. *Inter J Innov Res Stud* **3**(3): 733-737.
- Ertugrul S, Domez G, Takac S (2007) Isolation of lipase producing *Bacillus* sp. from olive mill waste water and improving its enzyme activity. *J Hazard Mater* **149**: 720-724. DOI: [10.1016/j.jhazmat.2007.04.034](https://doi.org/10.1016/j.jhazmat.2007.04.034)
- Eze JJ and Agbo KE (2010) Studies on the microbial spectrum in anaerobic biomethanization of cow dung in 10 m³ fixed dome biogas digester. *Inter J Phy. Sci* **5**(8): 1331-1337.
- Gautam, SP, Bundela PS, Pandey AK, Jamaluddin, Awasthi MK and Sarsaiya S (2012) Diversity of cellulolytic microbes and the biodegradation of municipal solid waste by a potential strain. *Inter J Microbiol* : DOI: [10.1155/2012/325907](https://doi.org/10.1155/2012/325907)
- Gerhardt M, Pelec V and Bauml M (2007) The use of hydrolytic enzymes in agricultural biogas production: Results from practical applications in Germany. *Biotechnol J* **2**(12): 1481-1484. DOI: [10.1002/biot.200700220](https://doi.org/10.1002/biot.200700220)
- Hendriks ATWM and Zeeman G (2009) Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresource Technology* **100**: 10-18. DOI: [10.1016/j.biortech.2008.05.027](https://doi.org/10.1016/j.biortech.2008.05.027)
- Horikoshi K, Akiba T (1982). Alkalophilic microorganisms, a new microbial world. NewYork: Springer-Verlag.
- Huang XP and Monk C (2004) Purification and characterization of cellulase from a newly isolated thermophilic aerobic bacterium *Caldibacillus cellulovorans*. *World J Microbiol and Biotechnol* **20**(1): 85-92. DOI: [10.1023/B:WIBI.0000013316.12730.e7](https://doi.org/10.1023/B:WIBI.0000013316.12730.e7)
- Jordan SN and Mullen GJ (2007) Enzymatic hydrolysis of organic waste materials in a solid-liquid system. *Was Manag* **27**: 1820-1828. DOI: [10.1016/j.wasman.2006.12.020](https://doi.org/10.1016/j.wasman.2006.12.020)
- Kowsalya R and Gurusamy R (2013) Isolation, screening and characterization of cellulase producing *Bacillus subtilis* KG10 from virgin forest of Kovai Kutralam, Coimbatore, India. *Res J Biotechnol* **8**(6): 17-24.
- Lowry OH, Rosenrough NJ, Farr AL and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265-275.
- Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS (2002) Microbial cellulose utilization: Fundamentals and Biotechnology. *Microbiol Mol Biol Rev* **66** (3): 506-577. DOI: [10.1128/MMBR.66.3.506-577.2002](https://doi.org/10.1128/MMBR.66.3.506-577.2002)
- Malik D, Bharati U (2009) Biogas production from sludge of sewage treatment plant at Haridwar (Uttarkhand). *Asian J Exp Sci* **23**(1): 95-98.
- Merlin Christy P, Gopinath LR and Divya D (2014a) A review on anaerobic decomposition and enhancement of biogas production through enzymes and microorganisms. *Renew Sustain Ener Rev* **34**: 167-73. DOI: [10.1016/j.rser.2014.03.010](https://doi.org/10.1016/j.rser.2014.03.010)
- Merlin Christy P, Gopinath LR and Divya D (2014b) Microbial dynamics during anaerobic digestion of cow dung. *Inter J Plan Anim Environ Sci* **4**(4): 86-94.
- Padmapriya B, Rajeswari T, Noushida E, Sethupalan DG and Venil CK (2011) Production of lipase enzyme from *Lactobacillus* spp. and its application in the degradation of meat. *World Appl Sci J* **12**(10): 1798-1802.
- Pastor MD, Lorda GS and Batti A (2001) Protease obtention using *Bacillus subtilis* 3411 and amaranth seed meal medium at different aeration ratio. *Braz J Microbiol* **32**: 1-8. DOI: [10.1590/S1517-83822001000100002](https://doi.org/10.1590/S1517-83822001000100002)
- Plochl M, Hilse A, Heiermann M, Surez Quinones T, Budde J and Prochnow A (2009) Hydrolytic enzymes improve fluidity of biogas feedstock. *Agri Eng Internat: CIGR Ej* **2009**: 1529.
- Roja Rani M, Lalitha kumari B, Hanuma sri M (2012) Siva Prasad D. Isolation and screening of protease producing bacteria and induction of overproducing *Bacillus licheniformis* mutants through UV irradiation. *IOSR J Pharm* **1**(1): 001-004.
- Romano RT, Zhang R, Teter S and McGarvey JA (2009) The effect of enzyme addition on anaerobic digestion of Jose tall wheat grass. *Bioresource Technology* **100**: 4564-4571. DOI: [10.1016/j.biortech.2008.12.065](https://doi.org/10.1016/j.biortech.2008.12.065)
- Sadhu S and Maiti TK (2013) Cellulase production by bacteria: A review. *Brit Microbiol Res J* **3**(3): 235-258. DOI: [10.9734/BMRJ/2013/2367](https://doi.org/10.9734/BMRJ/2013/2367)
- Saitou N and Nei M (1987) The Neighbor-Joining Method: A New Method of Reconstructing Phylogenetic Trees. *Mol Biol Evol* **4**: 406-425.
- Sangkharak K, Vangsirikul P, Janathachat S (2012) Strain improvement and optimization for enhanced production of cellulase in *Cellulomonas* sp. TSU-03. *Afr J Microbiol Res* **6**(5): 1079- 1084.
- Saraswati B, Ravi kumar M, Mukesh kumar DJ, Balashanmugam P, Bala kumaran MD and Kalaichelvan PT (2012) Cellulase production by *Bacillus subtilis* isolated from cow dung. *Arch Appl Sci Res* **4**(1): 269-279.
- Shaikh NM, Patel AA, Mehta SA and Patel ND (2013) Isolation and screening of bacteria inhabiting different environment and optimization of cellulase production. *Uni J Environ Res* **3**(1): 39-49.
- Sharmin S, Towhid Hossain M and Anwar MN (2005) Isolation and characterization of a protease producing bacteria *Bacillus amovivorus* and optimization of some factors of culture conditions for protease production. *J Biol Sci* **5**(3): 358-362. DOI: [10.3923/jbs.2005.358.362](https://doi.org/10.3923/jbs.2005.358.362)
- Sirisha E, Rajasekar N and Lakshmi Narasu M (2010) Isolation and optimization of lipase producing bacteria from oil contaminated soils. *Adv Biol Res* **4**(5): 249-252.

Sunarso S, Johari IN, Widiasa and Budiyono (2010) The effect of feed to inoculums ratio on biogas production rate from cattle manure using rumen fluid as inoculums. *Inter J Sci Eng* **1**(2): 41-45.

Tamura K, Dudley J, Nei M and Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis Software Version 4. 0. *Mol Biol Evol* **24**: 1596-1599. DOI: [10.1093/molbev/msm092](https://doi.org/10.1093/molbev/msm092)

Vinod KN, Marry ER, Gunaseeli R, Kannan ND and Sridhar J (2012) Modeling and structural analysis of cellulases using *Clostridium thermocellum* as template. *Bioinfor* **8**(22): 1105-1110. DOI: [10.6026/97320630081105](https://doi.org/10.6026/97320630081105)

Zhu BN, Gikas P, Zhang RH, Lord J, Jenkins B and Li XJ (2009) Characteristics and biogas production potential of municipal solid waste pretreated with a rotary drum reactor. *Bioresource Technology* **100**(3): 1122-1129. DOI: [10.1016/j.biortech.2008.08.024](https://doi.org/10.1016/j.biortech.2008.08.024)