DIRECT DEGRADATION OF CELLULOSIC BIOMASS TO FERMENTABLE SUGARS BY THE CELLULOLYTIC ISOLATES FROM BIOGAS PLANT

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Abstract:

The increased concern regarding the depletion of oil supply and emission of greenhouse gases urges measures to find renewable fuel alternatives. Ethanol is one among such fuel, which could be produced from lignocellulosic biomass especially agricultural wastes. This study provides the first evidence of the lignocellulolytic and saccharifying ability of a biogas plant derived *Stenotrophomonas* sp. JSR-001, a Gram-negative proteobacterium. The isolate has exhibited the highest filter paper unit effect, endoglucanase, exoglucanase, cellobiohydrolase, β-glucosidase, xylanase and ligninase production with hydrolytic potential and efficient saccharification competence in ammonia pretreated biomass. It was also found to degrade the paddy and sorghum into simple, reducing sugars through its lignocelluloses enzyme complex with the limited utilization of sugars.

Key words: Biogas slurry, Lignocellulosic substrates, Cellulolytic microorganisms, Saccharification, Pretreatment, Hydrolysis.

Introduction:

The world's demand for energy is ever increasing. Every year, roughly 3.7 billion tons of carbon are naturally fixed by the plants as its structural and non-structural carbohydrate components. Lignocellulosic biomass represents the world's most abundant carbohydrate reserve material and has the perspective to become a major resource of fermentable sugars for the production of ethanol. Being not included in the feed or food material list and availability in immense quantity is the added advantages. Because of its remarkable characters like cheaper cost, easy accessibility, easy processability, harmless, non-abrasive, recyclable and environmentally friendly nature, this Lignocellulosic substrate (crop residues) were used as a biofuel feedstock for ethanol production. However, due to its heterogeneous complexity and recalcitrance nature, pretreatment is required to break the lignin seal and/or disrupt the structure of crystalline cellulose to increase the accessibility of cellulase enzymes (Saha, 2004). For the successful conversion of lignocellulosic material into ethanol, there are three major steps involved firstly, pretreatment– a preprocessing step that improves enzyme access to the cellulose; secondly, enzymatic hydrolysis/saccharification – use of acid or enzymes (cellulases and hemicellulases); and to conclude, fermentation of released sugars by specialized organism (Singh and Bishnoi, 2012).

Cellulose has a microcrystalline structure and it is more difficult to hydrolyze into simple sugar compared than starch. Cellulose degrading enzymes are most commonly produced by microbial communities like bacterial and fungal species. Most of the cellulolytic microorganism extracellularly secretes three types of cellulolytic enzymes: endoglucanases (EG), cellobiohydrolases (CBH), and β-glucosidases (BGL). EG acts randomly against the amorphous region of the cellulose chain to produce reducing and nonreducing ends containing

sugars. CBH acts against crystalline cellulose and produces sugars containing reducing or non-reducing ends. This type of enzymatic degradation is an eco-friendly and low energy consuming process.

A promising strategy for efficient utilization of this renewable resource is the microbial hydrolysis of lignocellulosic biomass resulting in the reducing sugars for the production of desired metabolites or biofuels (Lynd *et al.,* 2002). Cellulase also has immense biotechnological potential in various other fields like food processing, oil extraction, agricultural industries, carotenoid extraction, waste management, brewery, animal feed, textile and various other fields like bio stoning, laundry, pulp, paper, deinking, detergent industry and color clarification (Kuhad *et al.,* 2011). Most of the microorganisms have the capacity to degrade cellulosic biomass into glucose molecules. A large group of fungi has been identified to degrade cellulose and hemicelluloses, but only some of the microorganisms exhibit both depolymerase and fermentative capacity (Lezinou *et al.,* 1995). For instance, *Neurospora crassa is* able to synthesize and secrete three types of enzymes, which are involved in cellulose degradation (Yazdi et al., 1990), in addition to endoxylanase and xylosidase activities (Mishra et al., 1984; Deshpande *et al.,* 1986) and it is known as well ethanol producer, capable of utilizing agricultural residues (Rao *et al.,* 1985). A number of microorganisms are associated with rumen of sheep, cattle, buffalo, and cattle, helping in the digestion process of these animals (Ware *et al.,* 1988). The common microflora of the cattle gut includes *Bacillus, Bifidobacterium, Lactobacillus,* and Yeast (Wallace and Newbold, 1993). Most of the *Bacillus sp* (*Bacillus substillus, Bacillus cereus, Bacillus lichiniformis*), *Enterococcus faecium* and *Saccharomyces cerevisiae* were identified as feed additives. These microbes have beneficial aspects on cows, pigs, rabbits, and cattle (Breul,1998). *B. substilus* strains were one of the most abundant groups of bacteria present in cow dung microflora (Swain *et al.,* 2006). These microbes play a vital role in the biocontrol of pathogenic fungi, plant growth promotion, sulfur oxidation, solubilization of rock phosphorous and production of industrially important enzymes like amylase and cellulase. Comparatively, microbial diversity in biogas plant digesters is greater in number (Ramasamy *et al.,* 1991) which is dominated by more than seventeen fermentative bacterial species (Wolin MJ, 1979).
A potential technology for generating simple fermentable sugars from the abundantly available lignocellulosic biomass is still in its juvenile stage though it is need of the hour. In this paper, we have examined the potential utility of biogas plant microbes for its saccharification and fermentation of cellulosic substances to simple sugars by cellulase activities.

Materials and Methods:

2.1. Sample collection and isolation of bacterial isolates:

A 10g of biogas slurry samples were randomly collected from the outlet chamber and the overflow tank of the household fixed dome type biogas plant which is continuously in operation successfully for the past 25 years located in Kanyakumari District, Tamil Nadu, India. All the samples were collected from 10 to 20 cm depth, stored in a sterile container (coring) and transferred to the laboratory under sterile conditions to avoid the external contamination and processed immediately for the isolation of lignocellulolytic microorganisms. The slurry samples were pooled together, mixed well and 1.0 g sample was serially diluted up to 10^{-4} dilutions and spread plated on agar-cellulose medium (2.0 g NaNO3, 1.0g K2HPO4, 0.5g MgSO4, 0.5g KCl, 20µM FeSO4, 15g agar, 1.0 L Distilled water, pH 7.6) containing 10.0 g of microcrystalline cellulose (Sigmacell cellulose, Sigma Aldrich) as the carbon source,. The colony forming units (CFU) were counted after 4 to 7 days of incubation at 28°C. The selected isolates were purely cultured after a few consecutive transfers on the cellulosic medium and evaluated for their ability to utilize cellulose by subjecting it to different lignocellulolytic assays.

2.2. Screening of lignocellulolytic microorganisms:

2.2.1. Plate assay for β-glucan hydrolysis using congo red staining:

A preliminary analysis of a cellulolytic activity was conducted by spread plate method using Carboxymethylcellulose (CMC) agar medium (10.0 g CMC, 2.0 g $NaNO_3$, 0.5 g KCl, 1.0 g K_2HPO_4, 0.5 g $MgSO_4$, 20 M $FeSO_4.5H_2O$, 15.0 g agar, 1.0 L water, pH 7.6) and incubated at 28°C for 3 days. Then the plates were observed for the clearance zone, demonstrating the cellulase activity was detected by flooding the plate with a 0.1% congo red solution for 15 mins followed by destaining with 1M NaCl for 20 mins (Teather and Wood, 1982). The quotient of the clear zone diameter directly proportional to the highest cellulase activity was measured in order to select the chief cellulase producer (Ariffin *et al.,* 2006).

2.2.2. Plate assay for cellulose hydrolysis by Gram's iodine test:

The cellulase activity of the isolates was analyzed by growing the isolates on cellulose agar (10.0 g Cellulose, 2.0 g $NaNO_3$, 0.5 g KCl,1.0 g K_2HPO_4, 0.5 g $MgSO_4$, 20 M $FeSO_4 \cdot 5H_2O$, 15 g agar per L, pH7.6) at 30 ˚C for 48 h. After the incubation period, the plates were flooded with 3.0 ml of Gram's iodine solution (KI-2.0g & iodine -1.0g in 3.0ml distilled water) for 5 mins (Kasana *et al.,* 2008) and the appearance of the clear zone was measured. All these experiments were done in triplicates and the values are expressed as mean with standard deviation.

2.3. Pretreatment of lignocellulosic substrates:

To enhance the accessibility of microorganism on the substrate for cellulase and ethanol production, a pretreatment was carried out by the Ammonia fiber explosion method (Holtzapple *et al.,* 1991). AFEX is one of the alkaline physicochemical pretreatment processes. Here the biomass were exposed to liquid ammonia at a relatively high temperature of 100 °C for a period of one hour. The biowaste substrates such as paddy straw and sorghum stubbles were sun-dried individually to reduce the moisture content and to make a powder form. Then the 10.0g ground material was mixed with 250ml ammonia solution in an airtight container and incubated in a hot air oven at 100°C for 1 h (Santhi *et al.,* 2014). After fumigation, the residual ammonia was removed by drying in a fumigating hood. The pretreated biomass substrates obtained by this process were denoted as RP for paddy straw and SP for sorghum straw. Non-pretreated ground biomass from paddy straw and sorghum stubbles were denoted as RN and SN respectively. The control AFEX pretreated paddy straw substrate (RPM) used in this study was provided by the Institute of Chemical Technology (ICT), Mumbai, India. The pretreated biomass substrates were immediately utilized in the enzymatic saccharification experiment.

2.4. Enzymatic hydrolysis:

Biogas plant-associated bacterial isolates were grown on cell-free extracellular enzyme production medium (0.5 g $(NH_4)_2SO_4$, 0.2 g NaCl, 5.0 g K_2HPO_4, 1.5 g KH_2PO_4, 0.1 g $MgSO_4.7H_2O$, 0.1 g yeast extract and 0.01% cellulose) at 30° C for 48 h. After incubation, the cultures were centrifuged and supernatants were used as a source of crude enzyme. The crude enzyme solution was utilized for determination of enzyme activity. It was further concentrated by acetone precipitation method. The concentrated protein samples were dissolved in 50 mM citrate buffer, pH 4.8 and used for the determination of enzymatic activities. The cellulolytic activities such as filter paper unit (FPU) effect, exoglucanase, endoglucanase, cellobiohydrolase, Xylanase and b-glucosidase assays were performed using the 50 mg substrates (Whatman No.1 filter paper, Sigmacell cellulose, carboxymethyl cellulose, cellobiose, xylan, p-nitrophenyl-b-D-glucopyranoside respectively). The enzymatic activities were calculated by using the standard methods as mentioned previously (Ghose *et al.,* 1987; Teather and Wood, 1982; Sternberg, 1977; Adney and Baker, 2008; Mandels *et al.,* 1976; Kubicek, 1982). Lignin peroxidase (Lip) assay was performed using the substrate veratryl alcohol. (Tien and Kirk, 1983). Reducing sugar estimation was done by DNS method (Miller *et al.,* 1959) using glucose as a standard. One unit of an endoglucanase, exoglucanase and filter paper activity is defined as the amount of enzyme liberating 1 mM of glucose per min under the assay conditions and one unit of b-glucosidase & xylanase is defined as the amount of enzyme liberating 1 mM of p-nitrophenyl and D-Xylose per min under the

assay conditions. The protein concentration of the enzyme extract was determined using Quick StartTM Bradford protein assay kit (Bio-Rad, CA, USA).

2.5. Saccharification of pretreated and non-pretreated lignocellulosic substrates:

For Saccharification studies chemically treated and non-treated lignocellulosic biomass substrates were autoclaved and inoculated with each cellulolytic bacterial isolates. This culture was harvested after 72 hours by centrifugation at 10,000 rpm for 10 mins at 4°C. The supernatant was used as the crude extracellular enzyme source. The amount of reducing sugar released was quantified by DNS (Miller *et al.,* 1959) method and the saccharification efficiency was calculated (Selig *et al.,* 2008) using the following formula

$$Saccarification\ (\%) = \frac{Concentration\ of\ reducing\ the\ sugar * 0.9 * 100}{Carbohydrate\ content\ of\ the\ biomass}$$

2.6. Phylogenetic analysis of efficient cellulase producer by 16S rRNA gene sequence analysis

Based on the result of qualitative, quantitative and saccharification efficiency the efficient cellulase producer was selected and the Genomic DNA was isolated by the modified method of Sharma and Singh (Sharma and Singh, 2005). Genomic DNA was used as a template for 16S rRNA gene amplification using a method by Solomon et al (Solomon *et al.,* 2013) using gene-specific 27F and 1492R universal primers. The amplified product was purified with the HiPurA^{TM} PCR purification kit (HiMedia, Mumbai, India) and sequenced (Eurofins, Bangalore). The phylogenetic analysis was performed by BLAST and Ribosomal Project Database (RPD). The phylogenetic tree was constructed based on a neighbor-joining method using MEGA software (Kumar *et al.,* 2016).

2.7. Statistical analysis:

All the experiments and enzymatic assays were performed in triplicates, statistically evaluated by Microsoft Excel and the results have been presented as mean ± SD (Standard Deviation).

3. Results and Discussion

3.1. Isolation of lignocellulolytic microorganisms from biogas plant:

The biogas slurry samples were selected for searching the best lignocellulolytic bacteria with the ability to completely degrade the fresh leaf litters into compost within three days (personal observation). Among the microbial colonies grown on agar-cellulose medium, a total of 24 isolates were observed with clearing zone. They were again grouped based on their cellulolytic and saccharification efficiency.

3.2 a) Plate assay for β-glucan hydrolysis using congo red staining:

After incubation, CMC-agar plates which were flooded with Congo red and destained with 1M NaCl were observed for the cellulolytic zone of clearance around the colony. Congo red exhibits a strong interaction with complex polysaccharides composed of contiguous-(1→4) linked D-glucopyranosyl entities. It also shows a significant interaction with β-(1→3), (1→3)-D-glucan units, thus identify the bacterial strains possessing -(1→4),(1→3)-d-glucanohydrolase,-(1→4)- d-glucanohydrolase, and -(1→3)-d-glucanohydrolase activities (Teather and Wood, 1982). Among 24 isolates JSR-001 showed higher cellulolytic activity (4.0±0.02) by congo red staining method. This revealed that the isolate JSR-001 displayed the β-d-glucanase activity (Table 1).

Table 1: Ranking of different isolates on the basis of the zone of hydrolysis by biogas slurry associated bacterial isolates on agar plate supplemented with cmc from 1 to 4 days of incubation. zone of hydrolysis was expressed as mean value of triplicates ± standard error mean and '-denotes no hydrolytic zone

S.No	Isolate name	Cellulolytic zone (cm)			
		Day 1	Day 2	Day 3	Day 4
1	**JSR-001**	**1.7 ± 0.03**	**2.8 ± 0.01**	**3.4 ± 0.03**	**4.0 ± 0.02**
2	JSR-002	--	0.5 ± 0.05	1.4 ± 0.01	2.1 ± 0.03
3	JSR-003	1.2 ± 0.01	1.7 ± 0.03	2.5 ± 0.02	3.1 ± 0.03
4	JSR-004	0.7 ± 0.02	1.2 ± 0.01	2.2 ± 0.02	2.7 ± 0.06
5	JSR-005	0.6 ± 0.02	1.2 ± 0.01	2.2 ± 0.04	2.4 ± 0.04
6	JSR-006	0.5 ± 0.05	1.8 ± 0.05	3.6 ± 0.02	3.0 ± 0.02
7	JSR-007	0.4 ± 0.02	1.2 ± 0.01	2.5 ± 0.03	3.7 ± 0.01
8	JSR-008	0.7 ± 0.02	1.7 ± 0.01	3.2 ± 0.03	3.6± 0.05
9	JSR-009	0.9 ± 0.03	1.3 ± 0.01	2.0 ± 0.02	2.5 ± 0.01
10	JSR-010	0.5 ± 0.01	1.5 ± 0.03	1.8 ± 0.04	2.1 ± 0.03
11	JSR-011	0.6 ± 0.01	1.5 ± 0.02	2.7 ± 0.03	3.5 ± 0.04
12	JSR-012	0.7 ± 0.03	1.5 ± 0.02	2.3 ± 0.01	2.9 ± 0.04
13	JSR-013	0.8 ± 0.03	1.2 ± 0.06	2.0 ± 0.01	2.6 ± 0.02
14	JSR-014	1.2 ± 0.01	1.3 ± 0.03	2.7 ± 0.04	3.0 ± 0.01
15	JSR-015	0.8 ± 0.02	1.2 ± 0.07	1.9 ± 0.02	2.2 ± 0.07
16	JSR-016	0.4 ± 0.01	0.8 ± 0.09	1.9 ± 0.03	2.4 ± 0.01
17	JSR-017	0.5 ± 0.03	0.9 ± 0.03	1.8 ± 0.07	2.1 ± 0.03
18	JSR-018	0.8 ± 0.01	1.3 ± 0.01	1.8 ± 0.04	2.2 ± 0.04
19	JSR-019	1.2 ± 0.04	1.7 ± 0.01	3.4 ± 0.07	3.1 ± 0.01
20	JSR-020	0.8±0.03	1.0±0.01	1.2±0.06	1.7±0.04
21	JSR-021	0.8 ± 0.01	1.5 ± 0.04	2.5 ± 0.06	2.9 ± 0.03
22	JSR-022	0.6 ± 0.01	1.9 ± 0.06	3.0 ± 0.04	3.6 ± 0.02
23	JSR-023	0.6 ± 0.02	0.9 ± 0.03	1.3 ± 0.02	1.5 ± 0.03
24	JSR-024	0.8 ± 0.01	1.6 ± 0.01	3.0 ± 0.04	3.2 ± 0.01

3.2 b) Plate assay for cellulose hydrolysis by Gram's iodine test:

Hydrolysis of cellulose was observed as a zone of clearance with the hydrolyzed region in the cellulosic agar medium flooded with Gram's iodine, which produces a bluish-black complex only with cellulose but not with simple sugars (Kasana *et al.,* 2008). Initial tests using different isolates of cellulolytic bacteria showed that hydrolysis of carboxymethyl cellulose (CMC) are very rapid compared with hydrolysis of microcrystalline cellulose. The efficient cellulose metabolizers with highest cellulolytic activities were screened with the microcrystalline substrate Sigmacell. Under these conditions, cellulolytic clearing zones were observed after 24 to 96 h of inoculation and maximal cellulolytic activities were observed during 4 days of incubation (Table 2). The bacterial isolate JSR-001 showed the high cellulolytic activity (3.8±0.01) on the 4th day of incubation.

Table 2: Ranking of different isolates on the basis of the zone of hydrolysis by biogas slurry associated bacterial isolates on agar plate supplemented with sigma cell cellulose after 1 to 4 days of incubation. zone of hydrolysis was articulated as the mean value of triplicates ± standard error mean and '-denotes no hydrolytic zone.

3.3.

S.No	Isolate name	Cellulolytic zone (cm)			
		Day 1	Day 2	Day 3	Day 4
1	JSR-001	**0.6 ± 0.03**	**1.1 ± 0.01**	**1.7 ± 0.03**	**3.8 ± 0.01**
2	JSR-002	--	0.7 ± 0.01	1.5 ± 0.02	2.3 ± 0.01
3	JSR-003	1.0 ± 0.03	1.6 ± 0.02	2.3 ± 0.01	3.0 ± 0.02
4	JSR-004	0.6 ± 0.03	1.1 ± 0.02	2.1 ± 0.03	2.8 ± 0.04
5	JSR-005	0.7 ± 0.01	1.3 ± 0.02	2.3 ± 0.03	2.5 ± 0.02
6	JSR-006	0.5±0.01	1.1±0.02	1.4±0.02	1.6±0.03
7	JSR-007	1.0 ± 0.01	2.5 ± 0.02	3.3 ± 0.04	3.7 ± 0.03
8	JSR-008	0.9 ± 0.01	1.5 ± 0.02	2.0 ± 0.01	3.6 ± 0.04
9	JSR-009	0.8 ± 0.01	1.5 ± 0.03	2.1 ± 0.01	2.4 ± 0.02
10	JSR-010	0.6 ± 0.01	1.2 ± 0.01	1.9 ± 0.02	2.2 ± 0.01
11	JSR-011	0.7 ± 0.02	1.1 ± 0.01	2.9 ± 0.02	3.5 ± 0.03
12	JSR-012	0.8 ± 0.01	1.4 ± 0.02	2.5 ± 0.02	2.9 ± 0.02
13	JSR-013	0.7 ± 0.02	1.1 ± 0.02	1.9 ± 0.03	2.5 ± 0.03
14	JSR-014	1.1 ± 0.03	2.2 ± 0.01	2.6 ± 0.03	3.1 ± 0.02
15	JSR-015	0.9 ± 0.02	1.3 ± 0.04	2.1 ± 0.01	2.3 ± 0.05
16	JSR-016	0.5 ± 0.01	0.9 ± 0.02	1.8 ± 0.01	2.3 ± 0.02
17	JSR-017	0.6 ± 0.02	1.0 ± 0.01	1.9 ± 0.06	2.2 ± 0.01
18	JSR-018	0.9 ± 0.01	1.2 ± 0.04	1.7 ± 0.04	2.3 ± 0.02
19	JSR-019	0.5± 0.01	1.1± 0.02	1.5±0.03	1.9±0.02
20	JSR-020	1.6 ± 0.03	1.9 ± 0.01	2.1± 0.02	3.5 ± 0.01
21	JSR-021	0.7 ± 0.02	1.6 ± 0.03	2.6 ± 0.01	3.0 ± 0.01
22	JSR-022	0.9 ± 0.02	2.1 ± 0.02	2.9 ± 0.01	3.2± 0.04
23	JSR-023	0.7 ± 0.02	1.0 ± 0.03	1.4 ± 0.01	1.6 ± 0.02
24	JSR-024	1.0 ± 0.01	1.5 ± 0.04	2.9 ± 0.01	3.4 ± 0.02

Pretreatment of lignocellulosic substrates:

An ultimate goal of pretreatment is to increase the surface area of lignocellulosic material, making the complex polysaccharides more suitable for enzymatic hydrolysis. It has become a relentless fact that the different kind of pretreatment process of lignocellulosic biomass materials can enhance the conversion of recalcitrant polysaccharide components into fermentable sugar fractions (Alvira *et al.,* 2010 & Mussato *et al.,* 2016). The pretreatment of lignocellulosic materials results in changes in the different properties of these materials like compositional, imaging and crystallinity (Karimi and Taherzadeh, 2016). The AFEX pretreatment is a novel alkaline pretreatment process that effects a physicochemical alteration in the lignocellulosic ultra and macrostructure. In the present study, the substrates were treated chemically with Ammonia for a period of 1 hour. The results indicated that pretreatment can cause some structural changes in lignin and hemicellulose and also a decrease in the crystallinity of cellulose with increased porosity. Previous studies had reported that the AFEX pretreatment increases the biomass enzymatic digestibility several folds over the untreated lignocellulosic & result in the decrystallization of cellulose (Teymouri *et al.,* 2005 & Gollapalli *et al.,* 2002). Meanwhile, AFEX pretreatment process well preserved the cellulosic and hemicellulosic contents, with little or no degradation (Moniruzzaman *et al.,* 1997). A significant difference in enzyme activity was observed when the cultures were inoculated in the AFEX pretreated substrates. Morphological changes of AFEX treated and non-

treated substrates were visualized by Scanning Electron Microscope (SEM) which is a powerful and widely used technique for analyzing surface modifications especially the lignocellulose surfaces (Amiri and Karimi, 2015). Figure.1 shows the SEM images of the surfaces of rice straw and sorghum stubbles before and after AFEX pretreatment. These images show significant disruption in AFEX treated samples. Smooth cell wall surfaces of control samples (non-treated) have become irregular in shape and deposition of broken cell wall matrix after AFEX treatment. It also exhibited a significant structural change in lignin re-localization along with increased porosity. Similar kind of structural changes was observed by several researchers using the same AFEX pretreatment with different lignocellulosic substrates such as corn stover (Li *et al.*, 2011) and switchgrass (Donohoe *et al.*, 2011). This AFEX process has been proved to be attractive economically compared with several other leading pretreatment technologies based on a recent study using an economic model (Eggeman and Elander, 2005) for evaluating bioethanol conversion of corn Stover.

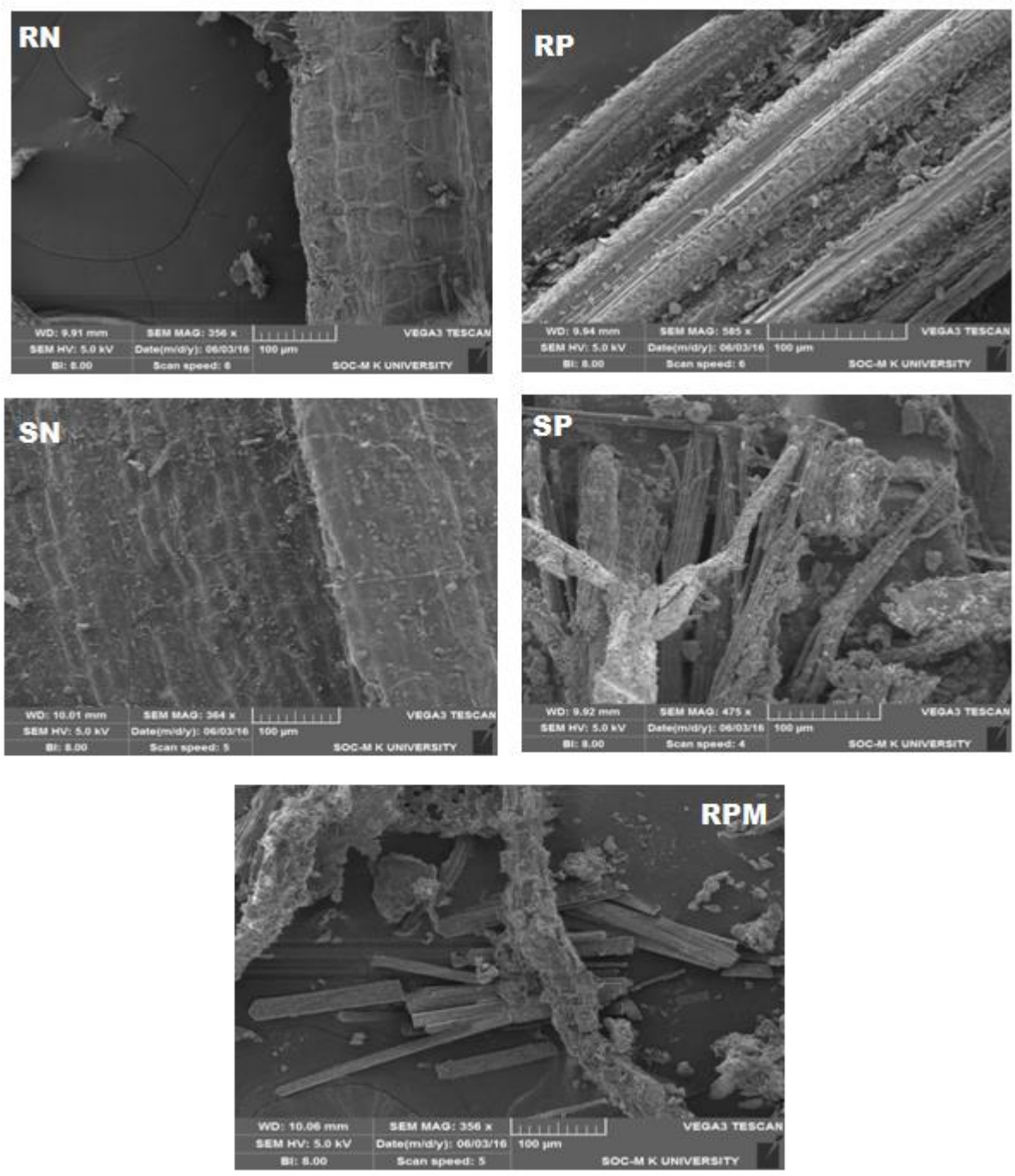

Fig : 1: SEM images of untreated and treated Rice straw & Sorghum stubbles; RN- Rice Non treated, RP- Rice Pretreated, SN- Sorghum Non treated, SP- Sorghum Pretreated & RPM- Rice Pretreated Mumbai (Control).

3.4 Enzymatic hydrolysis:

Cellulolytic enzyme assays were performed for all the 24 biogas plant derived microorganisms on its extracellular (supernatant) proteins concentrated from the early phase of culture medium by acetone precipitation method. All of these isolates greatly exhibited the profound activity in cellulase assays (Endoglucanase assay, Exoglucanase assay, Filter paper unit assay and cellobiase assay in Fig.2) hemicellulolytic assays (Xylanase and β-glucosidase assays in Fig.3&4) and lignin peroxidase assay (Fig.5). The endo-acting enzyme like endo-β-D-glucanase, β-(1-4), (1-3)-D-glucanohydrolase, β-(1-4)-D-glucanohydrolase and β-(1-3)-D-glucanohydrolase were active in the culture supernatant and they have exhibited the CMC degradation in the range of 17.83 U/ml/min to 39.17 U/ml/min. Similar to this result, the endoglucanase activity was reported in the culture supernatant of several cellulolytic microorganisms (Nascimento *et al.,* 2009; Lopez-Contreras *et al.,* 2001). Samira *et al.,* 2011 also reported that the *Stenotrophomonas maltophilia* sp., isolated from the Persian Gulf showed 0.084 U/ml/min of CMCase activity. The exo-acting cellulase enzyme like exoglucanase was also present in the precipitated culture supernatant. Exoglucanase activity was ranging from 3.01 to 19.52 U/ml/min. However, the cellobiohydrolase enzyme activity exhibited by all the isolates was found to be very less compared to Endoglucanase and Exoglucanase activities. Cellobiohydrolase activities were observed in the range of 2.81 to 8.97 U/ml/min. In all these biogas associated bacterial isolates the total saccharifying cellulase activity was in substantial level as indicated by the filter paper unit assay. FPU activities were observed in the level of 6.35 to 24.84 U/ml/min. This result was much higher than the report of Samira *et al.,* 2011, FPU activity of *S.maltophilia* was observed as 0.072U/ml/min. Cellulolytic microorganisms can secrete cellulolytic enzymes at extracellular regions (Saratale & Oh, 2011). Recent reports suggest that the minimal amount of 10 FPU is sufficient to convert 1.0g of the cellulosic substrate to glucose at 85% level of ethanol yield (Patel *et al.,* 2006; Shaw *et al.,* 2008). The bacterial isolate JSR-001 produced 24.84μmol min^{-1} ml^{-1} (IU ml^{-1}) of FPU activity and this level is above the minimum requirement of FPU for cost-effective cellulose biotransformation into glucose for the ethanol production. This study displayed the bacterial cellulolytic enzymes were secreted into the culture medium in an active form to exert the polymeric cellulose breakdown using their different cellulose modules. In addition, the highest endoglucanase activity of 39.17U/ml/min observed in this study appreciable as it is comparable to a purified endoglucanase activity (50.2U/mg) from *Penicillium notatum* NCIM NO-923 produced under mixed solid-state fermentation of waste cabbage and bagasse (Das *et al.,* 2012). Therefore *S.maltophilia* was drawn in as a source of these enzymes for industrial use.

The composite structure of the plant cell wall requires a multienzyme complex to deconstruct the cell wall polysaccharides into their individual sugar molecules for further conversion to useful products by the fermentation process. Next, to cellulose, hemicelluloses are the most abundant heteropolymer in renewable biomass, which accounts for 25-35% of lignocellulosic biomass (Saha, 2003). The hemicellulosic components are the likely barrier in inhibiting the cellulose-degrading enzymes which can be surmounted by the action of hemicellulases (Marx *et al.,* 2013). Xylan becomes an important major portion of hemicelluloses, to hydrolyze this portion of feedstock lignocellulosic matter into fermentable sugars, different groups of hemicellulases such as endoxylanases and exoxylanases would also be needed (Kumar *et al.,* 2008). Xylan degradation by microbial enzymes is an important key element, which increases the efficient conversion of biomass for biofuel industries. Recent reports of Giridhar & Chandra (2010) and Xin & He (2013) were has shown the potential application of xylanase in hydrolyzing the lignocellulosic biomass to simple sugars, which can be easily utilized by fermentation process for ethanol production. Xylanase activity determined from the extracellular supernatant fractions produced by the biogas plant associated isolates was shown in Fig. 3. In this study, the xylanase activity exhibited by the bacterial isolate JSR-001 was found to be 13.9U/ml/min which is the maximum when compared to other isolates. This result was slighter than the report of Bosire et al., 2013 showing 35.52 U/ml/min of xylanase activity of marine wood borer *Dicyathifer mannii*. The β-glucosidase activity exhibited by the isolate JSR-001 was found to be maximum (0.273μmol/ml/min) from the extracellular protein extracted from the culture broth. The bacterial isolates JSR-011 and JSR-020 displayed a slight decrease in their activities (0.223μmol/ml/min & 0.236μmol/ml/min). Rest of the bacterial isolates displayed the very low levels of β-glucosidase activities.

Lignin gives compressive strength and stiffness to the plant cell wall (Raven *et al.*, 1999). Lignins are phenolic compounds which are formed by the polymerization of three different monomers (p-coumaryl, coniferyl, and synapyl alcohols). Lignin is covalently linked to hemicelluloses by ferulic acid ester linkages (Gray *et al.*, 2006). So to detach and degrade the lignin content present in the plant cell wall (Lignocellulosic biomass) must be degraded efficiently. Lignin demeaning enzymes are in actual fact extracellular in nature due to the large and complex structure of lignin that cannot enter the cell for intracellular action. This implies that enzymes that digest lignin are either produced by microorganisms in the gut or are secreted by glands in the digestive tract and released into the gut for the action. Isolate JSR-001 exhibited the highest lignin peroxidase activity (85.35 U/ml/min). The bacterial isolates JSR-006 & JSR-007 showed relatively same level of activities (82.56 & 82.79 U/ml/min). The lowest level of lignin peroxidase activities was displayed by the isolates JSR-012 & JSR-022 (23.02 & 30.98 U/ml/min).

On the whole, the culture supernatant of the cellulolytic bacteria isolated from biogas slurry has shown the significant level of cellulase, hemicellulase and lignolytic activity and it could be clearly inferred that isolate JSR-001 is the potential source for the bacterial cellulase to aid in saccharification process for biofuel conversion.

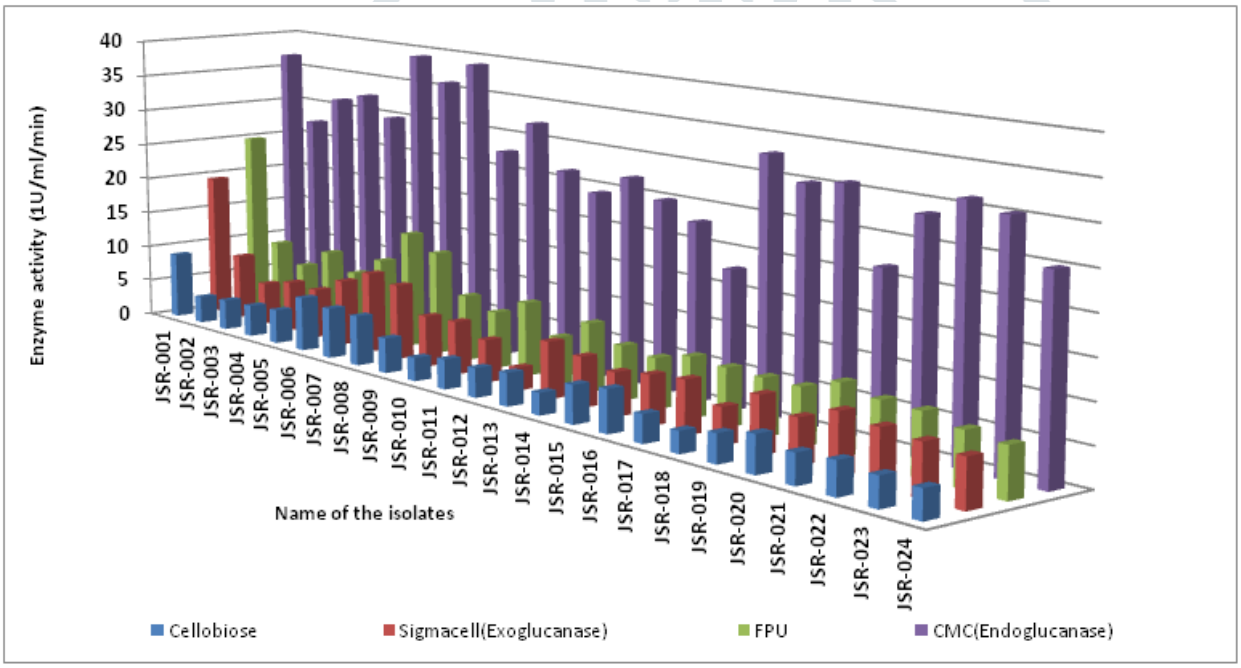

Fig.2. Cellulolytic enzyme activities exhibited by the extracellular protein fraction of the biogas associated bacteria.

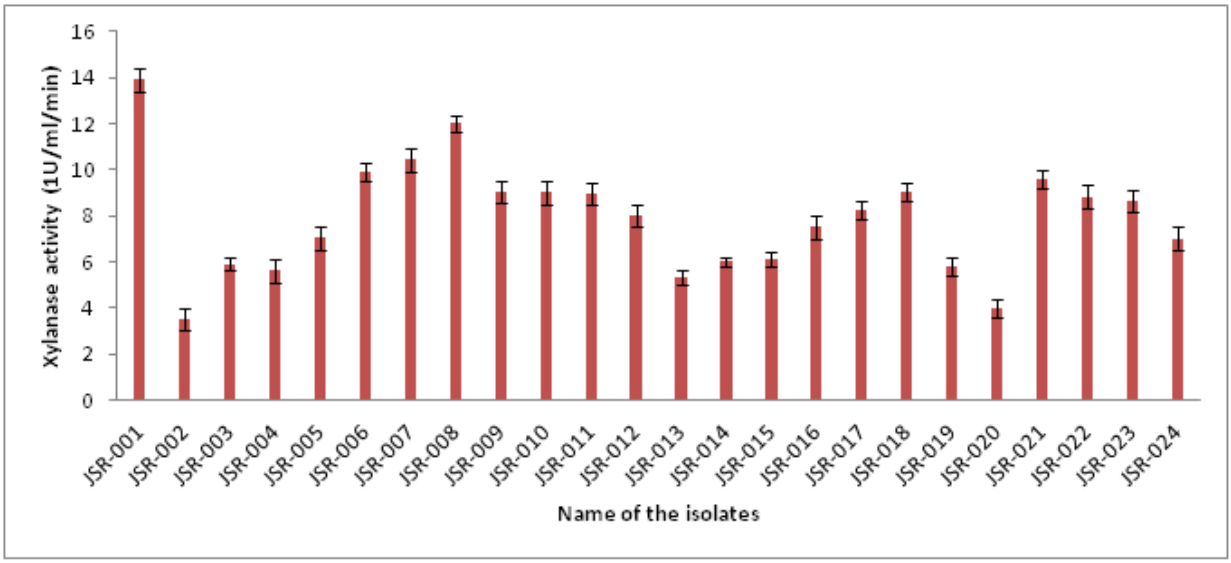

Fig. 3. Xylanase enzyme effect exerted by the biogas associated bacteria in their extracellular protein fractions and the xylose level denotes the mean value of triplicates with S.E.M.

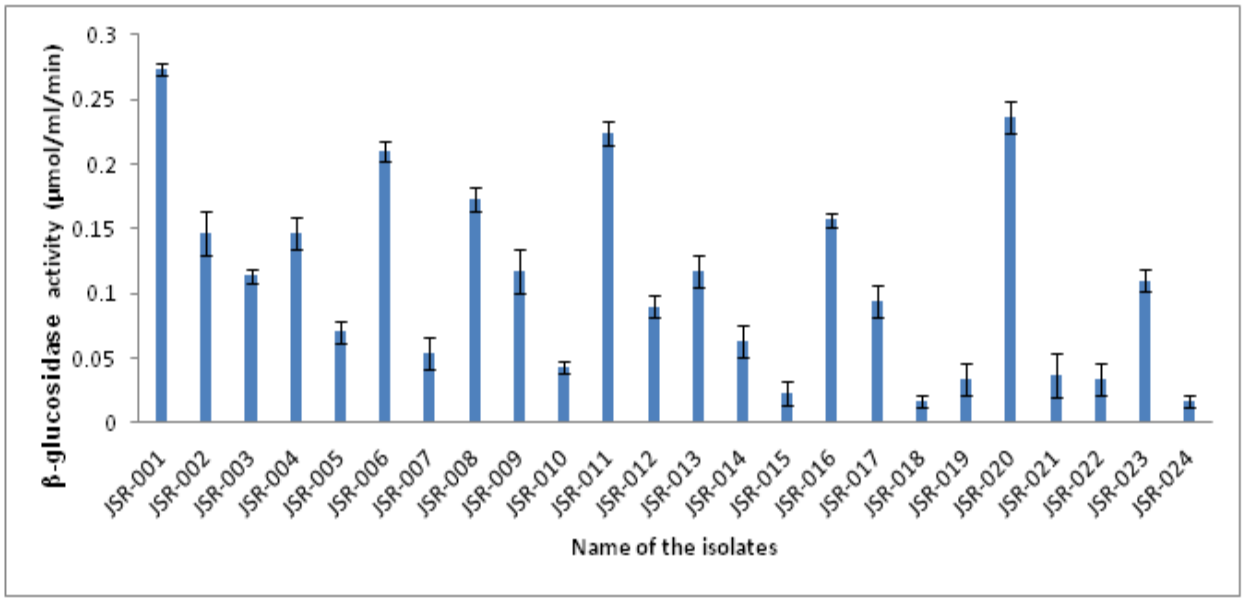

Fig. 4. β-Glucosidase enzyme effect exerted by the biogas associated bacteria in their extracellular protein fractions and the measurements were represented as average enzyme units and error bar denotes the standard error (S.E.M) of an enzyme unit level calculated from each triplicate experiments.

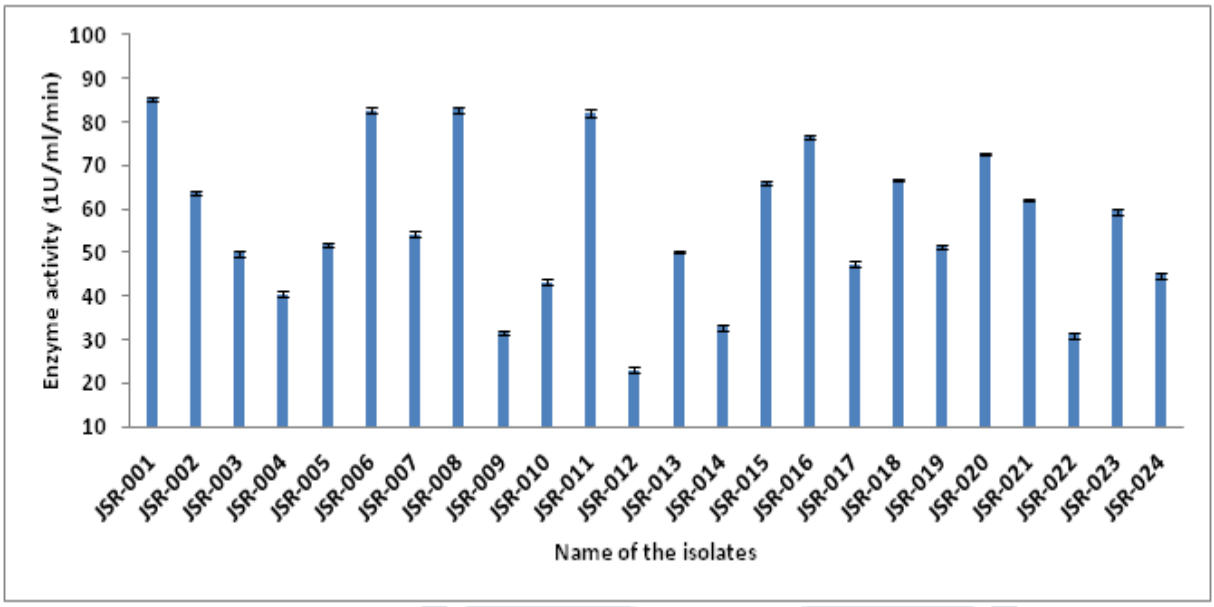

Fig. 5. Lignin peroxidase enzyme effect exerted by the biogas associated bacteria in their extracellular protein fractions and the measurements were represented as average enzyme units and error bar denotes the standard error (S.E.M) of an enzyme unit level calculated from each triplicate experiments.

3.5 Saccharification of lignocellulosic biomass by enzyme extract:

The ability of bacterial saccharification on inexpensive lignocellulosic materials such as sugarcane, rice bran, wheat straw and corn stover has been already reported by several researchers (Harshvardhan *et al.*, 2013; Lee *et al.*, 2008; Dantur *et al.*, 2015; Santhi *et al.*, 2014b). The cellulose and hemicellulose compounds present in pre-treated and non-pretreated lignocellulosic plant materials can be effectively hydrolyzed into fermentable sugars by the synergistic action of different glycoside hydrolases and this combined activities of enzymatic cellulolytic action are known as 'synergistic enzyme interaction' effect (Hu *et al.*, 2011). Many studies have looked at the synergistic interactions among the major cellulase components like that in the present study also; enzyme activities like lignin peroxidase, hemicellulases and cellulases were present in all 24 biogas plant-associated microbial isolates. Hydrolytic effect of these enzymes secreted by the isolates was compared for AFEX treated and non treated biomasses. Figure 6 illustrates the percentage (%) of saccharification obtained for different plant biomasses such as pretreated paddy straw (RP), non-pretreated paddy straw (RN), pretreated sorghum stubbles (SP), non-pretreated sorghum stubbles and AFEX pretreated paddy straw from Mumbai used as a control. Its enzymatic saccharification level varies from 50.2 to 95.1 % for different substrates. When compared to non-pretreated paddy straw, AFEX pretreated substrates yield 1.5 fold high sugar yields. Whereas in sorghum stubbles 1.3 fold high sugar yield was observed in ammonia pretreated substrates compare than not pretreated.

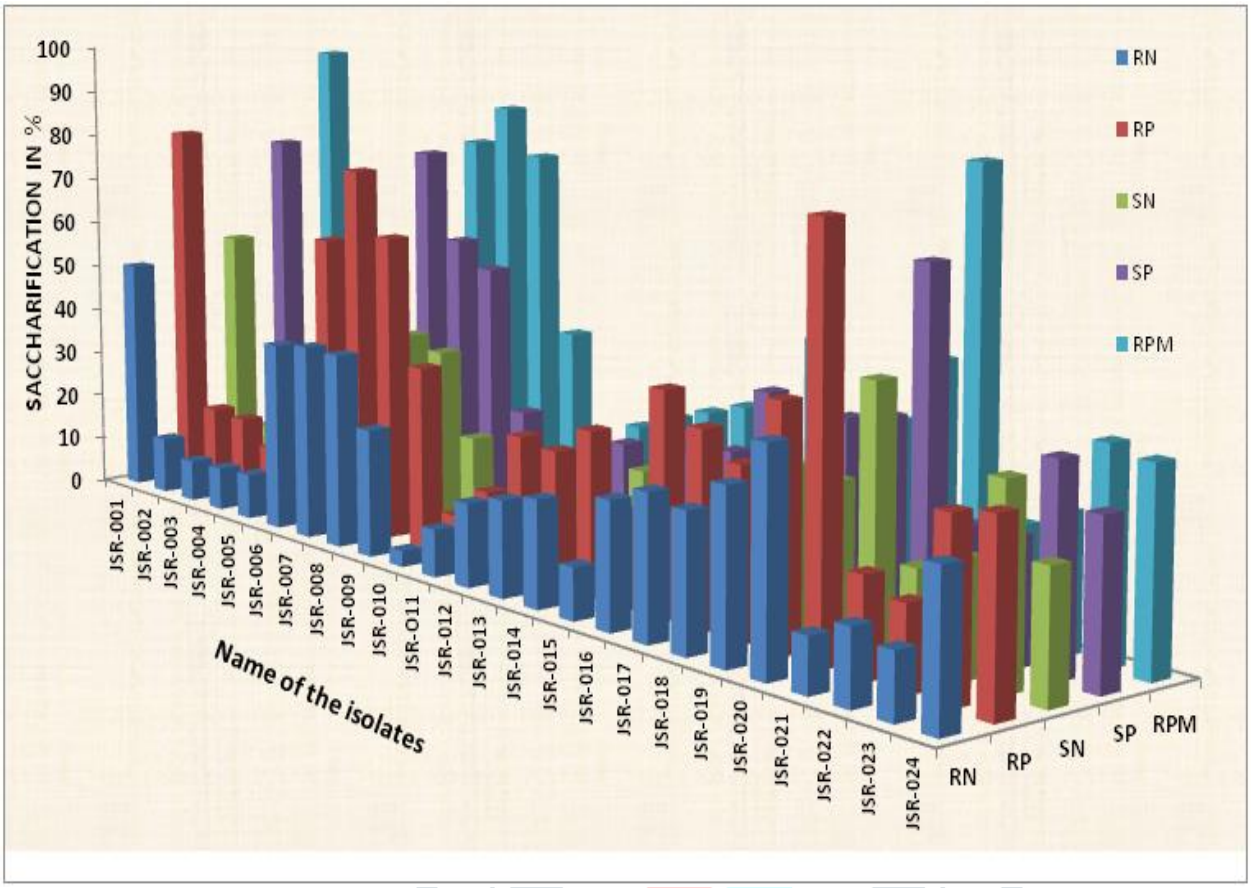

Fig. 6. Saccharification of plant biomass substrates by biogas plant-associated bacterial isolates.

Similar kind of results was already observed by the researcher (Santhi *et al.,* 2014a) with same lignocellulosic substrates. The results revealed that the strain JSR-001 has the ability to saccharify all the tested biomass substrates compare than other isolates. Highest saccharification (glucose yield) was observed for 95.1% for RPM (Rice Pretreated Mumbai) after 72 hours of incubation. Next, to JSR-001 bacterial strain JSR-020, JSR-006, JSR-007, JSR-008 strains showed better saccharification efficiency on different biomass substrates. The degradation capacity of these biogas plant-associated strains on plant substrates even without treatment depicted that, these strains have complex enzyme activity with lignocellulolytic effect and good saccharification potential.

3.6 Phylogenetic analysis:

Among 24 isolates, one bacterial isolate was found to be a potential cellulase producing strain showing very high enzymatic activity and saccharification efficiency. The culture was further identified by molecular approach 16S rRNA gene sequencing. For predicting the phylogenetic position of the isolate JSR-001, the phylogenetic tree (Fig.7) with its closely related type and non-type strains were analyzed using the Ribosomal Database Project. The culture was found to be closely similar with *Stenotrophomonas maltophilia* strain NBRC 14161. This *S. maltophilia* strain was already reported as cellulolytic by Wang *et al.,* 2011, from mesophilic microbial community BYND-8. The sequence has been submitted to NCBI GenBank with an Accession No. MG551551.

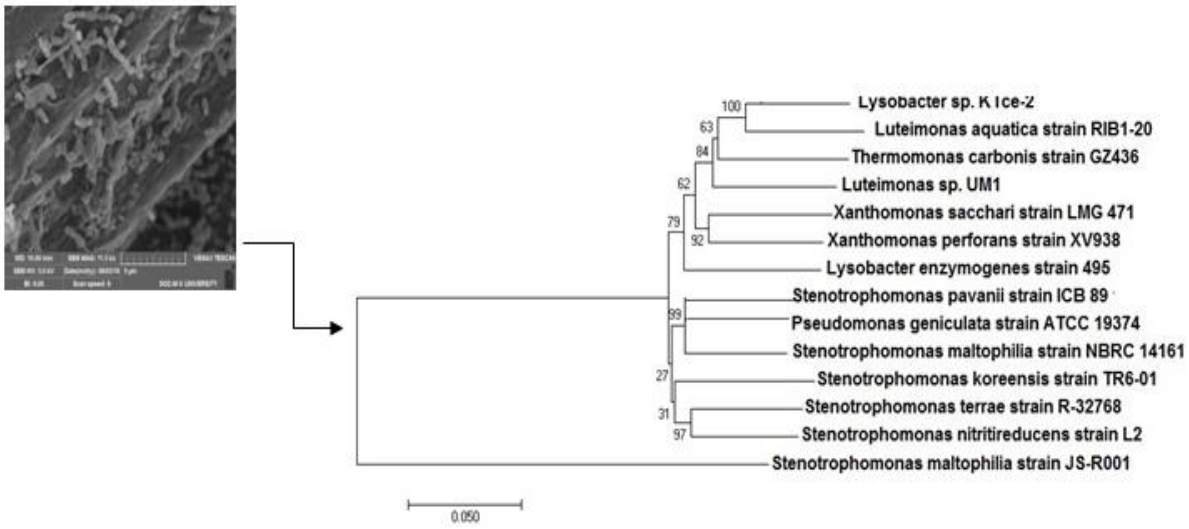

Fig. 7: Phylogenetic tree based on 16S rRNA gene sequences of bacterial isolate JS-R001 and related taxa belonging to the phylum Proteobacteria. The tree was constructed by the neighbor-joining method using the Ribosomal Database Project, Mega software tools. The numbers represent bootstrap values for each branch, based on data for 1000 trees.

Conclusion:

The present study focused only on the cellulolytic bacterial communities in the outlet tanks of the biogas plant. Results from this study clearly showed that the bacterial isolate identified as *Stenotrophomonas maltophilia* has the potential celluloytic effect on breaking down the lignocellulolytic substrates into fermentable sugar components. Further deeper exploration of all microbial communities in this kind of energy tapping systems will provide a handful of novel ideal bacterial candidates to increase the yield of biofuels by making the process of pretreatment of lignocellulosic substrates trouble-free.

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Conflict of Interest: The authors do not have any conflict of interest.

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