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Effect of Biological Pre-Treatment of Agricultural Waste on Biogas Production

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ABSTRACT

A total 12 samples of yak dung, compost and soil were collected and processed microbiologically for cellulose decomposer ability and identification of efficient isolates from high altitude from Lahaul-Spiti and Kinnaur districts. Based on the colony morphology, 34 bacterial isolates (08 from yak dung, 19 from soil and 07 from manure samples) recovered on nutrient agar. 24 isolates were of cellulose degrading nature further identified biochemically and 6 isolates were of *Bacillus* spp. And 01 isolate was of *Pseudomonas* spp. These isolates were employed for biological pretreatment of paddy straw for two weeks and then run in laboratory biogas digester along with control digester. The digester set was observed from the second day of run upto three weeks and it was observed that the there was high biogas production in test digester as compared to control digester. It was concluded from out study that the utilization of consortium of *Bacillus* spp and *Pseudomonas* spp found 10 percent higher biogas production at ambient temperature from rice straw.

Introduction

The increase in world's population with alarming speed, energy consumption has increased many folds over the last century. Fossil fuel has been the major source of energy that formed from fossils over millions of years the earth. Fossil fuels are considered as nonrenewable and depleting continuously with a predictable estimation from the 25 billion barrels to approximately 5 billion barrels by 2050 (Campbell and Laherrere, 1998). On the other hand, a huge amount of agro-industrial wastes and crop residues are produced all over the world and emerging many environmental problems if not utilized properly. Mainly three kinds of lignocellulosic biomass; woody biomass, agricultural residues, and energy crops are produced. Among the agricultural residues, rice straw is generated in abundance (667.6 million tons). In Asian countries every year and would serve as a great potential feedstock for biofuel and biogas production. After harvesting the rice, large amount of rice straw residues i.e., 40-50 cm of loose stubble and 50-60 cm of standing straw are usually left over the field and it is still burnt in the fields causing severe environmental and health issues (Liu *et al.,* 2011; Binod *et al.,* 2010).

Renewable energy plays important role to reduce global greenhouse gas emissions in our atmosphere. This step leads to the production of lots of energy resources like solar energy, wind energy, tidal energy, hydro electric energy, biomass, and biogas. The major advantage of biogas over fossil fuel includes electricity generation, potentially help to reduce global climate change. The major component of biogas are methane, CH_4 (50-70%), carbon dioxide, CO_2 (20-25%), nitrogen, N_2 (0-5%), hydrogen, H_2 (0-1%) and hydrogen sulfide, H_2S (0-3%). Anaerobic digestion is completed in four biological and chemical stages- hydrolysis, acidogenesis, acetogenesis sand methanogenesis. Hydrolysis is the process of breakdown of complex organic molecules into simple sugars, amino acids, and fatty acids with the action of hydrolytic bacteria.

The acidogenesis is the breakdown of the remaining components by acidogenic bacteria and again digested by acetogens and produce acetic acid. Methanogenesis converts into methane. Based on the solid content used for the process, systems can be categorized as high solid systems (>15%) and low solid systems (<15%). With regard to social and environmental sustainability, second-generation biofuel and biogas production from lignocellulosic material provides considerable potential, since lignocellulose represents an inexhaustible, ubiquitous natural resource, and is therefore one important step towards independence from fossil fuel combustion. However, the highly heterogeneous structure and recalcitrant nature of lignocelluloses such as the rice straw which consists of cellulose, hemicellulose and lignin that is strongly intermeshed and chemically bonded by non-covalent forces and by covalent cross linkages. It has been generally characterized to contain 28-36% of α cellulose, 12-16% of lignin, 15-20% of ash and 9-14% of silica, which is recalcitrant for degradation and restricts its commercial utilization in biogas plants. Improvements therefore depend on effective pretreatment methods to break down structural impediments, thus facilitating the accessibility and digestibility of lignocellulosic biomass during anaerobic digestion. However, chemical and physical pretreatment strategies exhibit inherent drawbacks including the formation of inhibitory products, biological pretreatment is increasingly being advocated as an environmentally friendly process with low energy input, low disposal costs, and milder operating conditions.

Biological pretreatment is an effective process which requires no preceding mechanical size reduction, preserves the pentose (hemicellulose) fractions, avoids the formation of degradation products that inhibit growth of fermentative microorganisms, minimizes energy demands, and limits costs. Therefore, a major objective of biological pretreatment is to break and remove the lignin seal and to disrupt the crystalline structure of cellulose to make it (more) inclined to an enzymatic or microbial attack, while minimizing the loss of carbohydrates for anaerobic digestion (Zheng *et al.,* 2014; Mosier *et al.,* 2005; Karimi and Taherzade, 2016). The delignification and the decomposition of hemicellulose enhance the availability of cellulose and resultant monomers, which can boost the overall anaerobic digestion process. The choice of application is mainly dependent on the chemical composition of the substrate; however, in practice, structural and economic factors like available facilities or excess energy can often play an equally important role. Biological pretreatment techniques for enhanced biogas production have mainly focused on the use of fungal and bacterial strains or microbial consortia under both aerobic and anaerobic conditions, as well as on enzymes, with the latter being less important.

Beside the hydrolysis of lignocellulose during pretreatment, microorganisms can further be used to upgrade the quality of certain substrates by removing undesired, potentially inhibitory substances. However, the efficiency of biological pretreatment is limited by the rate of microbial growth and the utilization of readily available sugars by the engaged organisms.

In order to permit degradation of agricultural materials in an anaerobic digester, the structure has to open up and/or the lignin has to be degraded or removed. Decreased lignin content leads to increased biogas yield. Fernandes *et al.,* (2009) indicated that the biodegradability of rice straw increased with decreasing lignin content, i.e., the higher the lignin content, the lower the biogas product order to improve biogas production from rice straw, a pretreatment process is necessary to disrupt the naturally recalcitrant carbohydrate-lignin shields that impede the accessibility of enzymes and microbes to cellulose and hemicellulose. Among different pretreatment methods, biological pretreatment method is one of important methods that not too costly and not cause environmental pollution. Thus, the alternative approach of microbial pretreatment which employs the use of microorganism to increase digestibility of rice straw is the most reliable approach.

Materials and Methods

Collection Samples

A total 12 samples were collected and processed microbiologically for cellulose decomposer ability and identification of efficient isolates from high altitude from Lahaul-Spiti and Kinnaur districts which were comprised 4 each of yak dung, compost and soil. Samples were transported to the laboratory and stored at 48 C until used.

Isolation of Bacteria

Ten-fold serial dilutions of each sample were prepared in sterilized distilled water, and 0.1 ml diluted sample was spread on the surface of nutrient agar medium for bacteria (Hi Media, India). Plates were incubated at 28^{o}C for 48hr.The colonies appeared were segregated and kept on agar slants for subsequent use.

Screening and Identification of Cellulase Producing Bacteria

Each colony was screened for cellulose degrader on CMC Congo red agar. Five microlitres of overnight grown culture of selected colonies of bacteria from nutrient broth were spot plated on CMC Congo red agar. The plates were incubated at 28^{o}C for 48 hours. Then plates were flooded with 0.1% Congo red for 15 to 20 minutes and then with 1M NaCl for 15 to 20 minutes clear zone of cellulose degradation.

Screening and Characterization of Cellulose Degrading Bacteria

Each cellulose degrading bacterial colonies were selected from CMC Congo red agar showing cellulose degrading ability and were selected for identification on the basis of morphological, Gram's staining, motility and biochemical characterization.

Biochemical characterization catalase, oxidase, urease, Nitrate, motility, hydrogen sulfide production, sugar fermentation and IMViC tests were carried out for biochemical analysis, as per Bergy's manual.

Pre-treatment of Agricultural waste with effective cellulose degrading bacteria

Paddy straw was chopped in to very small pieces and 500g of this biomass was mixed with consortium composed of *Bacillus* spp. and *Pseudomonas* spp. culture (mixed 1:1 ratio) of 900ml in nutrient broth (grown in liquid culture constantly 30^{o}C, 120 rpm for two days) and the biomass was pretreated for two weeks at room temperature in a tray covered with polythene sheet.

After pre-treatment of paddy straw was digested anaerobically in 2.5 L digester by feeding 500g biological pretreated rice straw (wt weight basis) and 500gm cow dung which was inoculated with 10% inoculum in the form slurry obtained from continuous operated biogas plant and run the test digester as well as control digester (1000g cow dung) in laboratory scale at room temperature for four weeks. The biogas production was recorded from second day.

Results and Discussion

Bacterial Isolation

A total of 12 samples comprised four each from yak dung, compost of cow dung and soil samples from high altitude areas were processed for primary isolation of on nutrient agar by serial dilution of samples. Based on the colony morphology, 34 bacterial isolates (08 from yak dung, 19 from soil and 07 from manure samples) recovered on nutrient agar.

Screening of Cellulase degrading isolates

24 isolates were screened as cellulose degrader on CMC Congo red as a clear zone around the colonies.

Gram staining

All the 24 cellulose hydrolytic bacterial isolates screened for Gram staining reaction. The Gram staining revealed small rods, long rods and coccobacilli were observed. Long rods Gram positive (06) and negative bacteria (02) observed from yak dung.

Out of 09 cellulose degrading bacteria from soil samples, 06 were Gram positive and 03 Gram negative. Manure samples showed 03 Gram negative and 04 Gram positive.

Biochemical Identification

Out of 24 cellulose hydrolytic bacteria, 07 isolates were identified biochemical basis as *Bacillus* spp (06). and *Pseudomonas* spp.(01). Bacillus spp. isolates were found oxidase positive, catalase positive, motile, methyl red negative, voges proskaur positive, citrate positive, urease positive, nitrate negative, glucose positive, fructose positive, lactose negative, maltose positive and Mannitol negative.

One Pseudomonas spp was recovered from soil samples and found oxidase positive, catalase positive, motile, methyl red negative, voges proskaur negative, citrate positive, urease negative, nitrate positive and negative for carbohydrates like glucose, fructose, lactose, maltose and mannitol.

Biogas Production

Biogas production is one way that can be used to treat waste into something more useful. The anaerobic digestion biological pretreated paddy straw results in the production of biogas. The biogas production was observed from the second days of incubation and calculated by saline water displacement method and it was accumulated maximum at 22nd days in pre-treated biomass with cellulose degrading bacterial isolates digester as compared to control digester. In our findings, the maximum gas production in test digester was observed to 790ml as compare to 650ml in control. Here the employment of pre-treatment along with cellulose bacterial culture for biomass treatment has increased the production of biogas observed upto fourth week at ambient temperature and it was always higher in treated digester and was about 10% hike in biogas production in test digester when compare with control. After 28 days, the biogas production in both the digesters found decreased. The results of the biogas production test with biological pre-treated paddy straw with consortium of *Bacillus*+*Pseudomonas* from soil samples showed an enhancement biogas production.

Raj *et al.*, (2022) revealed that out of four isolates, only the AMB-CD-1 isolate performed the best in all the screening analyses. The results concluded that the AMB-CD-1 culture inoculated straw had a low level of cellulose and hemicellulose, indicating these particles' degradation.

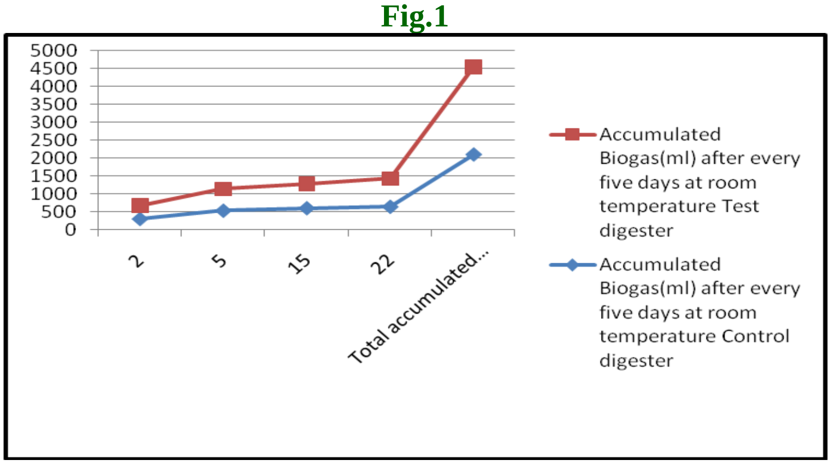
Table.1 Biochemical and Carbohydrates utilzation of cellulose degrading isolates

Biochemical and Carbohydrates utilzation Tests	Gram positive isolates						Gram negative isolate
	1	2	3	4	5	6	
Oxidase	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
SIM (H_2S, Indole production and motility)	-/-/+	-/-/+	-/-/+	-/-/+	-/-/+	-/-/+	-/-/+
MR	-	-	-	-	-	-	-
VP	+	+	+	+	+	+	-
Citrate Utilization	+	+	+	+	+	+	+
Urease	+	+	+	+	+	+	-
Nitrate	-	-	+	-	+	+	+
Glucose	+	+	+	+	+	+	-
Fructose	+	+	+	+	+	+	-
Lactose	-	-	-	-	-	-	-
Maltose	+	+	+	+	+	+	-
Mannitol	-	-	-	-	-	-	-
	***Bacillus* spp.**						***Pseudomonas* spp.**

Table.2 Utilization of effective strain of *Bacillus* spp. and *Pseudomonas* spp. for pre-treatment of paddy straw for Biogas production

Incubation Days	**Accumulated Biogas(ml) after every five days at room temperature**	
	Control digester	**Test digester**
2	**310**	**370**
5	**540**	**610**
15	**610**	**680**
22	**650**	**790**
Total accumulated Biogas(ml)	**2110**	**2450**

Fig.1



The isolated AMB-CD-1 was identified as *Pseudomonas aeruginosa* through the Sanger sequencing technique of the 16S rRNA gene. Zhang *et al.,* (2016) observed that the rice straw was pretreated with the rumen fluid at 39°C for 120 h under anaerobic conditions. The results indicated that the optimal pretreatment time for anaerobic digestion was 24 h, resulting in a biogas production increase of 66.5%, a methane yield increase of 82. 6% and a technical digestion time decrease of 40.0%, compared with the control.

The consortium of *Bacillus* spp and *Pseudomonas* spp found 10 percent more biogas production at ambient temperature after three weeks AD of rice straw.

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