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# Isolation and Screening of Cellulolytic Bacteria from Yak (*Bos grunniens*) Excreta from Lahaul Valley Region for Pre-treatment of Paddy Straw in Himachal Pradesh

**Rajinder Kumar and Dinesh Kumar Vatsa**

Dept. of Agricultural Engineering, College of Agriculture, Himachal Pradesh Krishi Vishvavidyalaya, Palampur, Himachal Pradesh (176 062), India

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**Corresponding** rajinder.kumar226@gmail.com

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## ABSTRACT

The present study was conducted during August, 2021 at Department of Agricultural Engineering, College of Agriculture, CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur to isolate and identify the cellulolytic bacteria from yak dung, yak dung manure and soil samples from spiti region in Lahaul-Spiti district of Himachal Pradesh. The bacterial isolates were identified by using morphological and biochemical standard methods, and identification based on Bergey's Manual of Determinative Bacteriology. Cellulolytic bacteria from mentioned samples were isolated and cultured on CMC (Carboxymethyl cellulose) agar medium. The activity of cellulolytic bacterial was conducted based on halo area and cellulolytic index on CMC agar medium. Among 24 isolates of bacteria, 08 isolates were identified as cellulolytic bacteria. Furthermore, our isolates with higher cellulolytic index were identified as the Bacillus and Pseudomonas genus. Out of eight efficient cellulolytic isolates were identified as *Bacillus* spp. (06) three from yak dung manure, two from yak dung and one from soil sample and *Pseudomonas* spp. (02) one from yak dung manure and one from soil sample. Most Efficient cellulose degrading isolates of *Bacillus* spp. and *Pseudomonas* spp. were employed for biological pretreatment of paddy straw for two weeks and then run in laboratory biogas digester along with control digester. These digester sets were under observation from the second day of run upto three weeks and it was observed that the there was 10% hike in biogas production as compare to control digester on 22$^{nd}$ days at laboratory condition.

**KEYWORDS:** Bacillus, cellulose, CMC, degradation, dung, energy, pseudomonas, yak

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***Data Availability Statement:*** Legal restrictions are imposed on the public sharing of raw data. However, authors have full right to transfer or share the data in raw form upon request subject to either meeting the conditions of the original consents and the original research study. Further, access of data needs to meet whether the user complies with the ethical and legal obligations as data controllers to allow for secondary use of the data outside of the original study.

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## 1. INTRODUCTION

In India, agricultural waste is generated more than 620 mt annually (Choudhary, 2018), with plant organic waste (mainly composed of cellulose) accounting for 70% of the waste. Out of which only 25–30% is utilized as livestock fodder and energy production and the remainder is waste. Cellulose is a polymer with several glucose units linked via-1,4-glycosidic bonds that synthesize plant cell walls their rigid structure. Cellulose is the most common element in lignocellulosic biomass bioresources (Hussain et al., 2017) which is resistant to degradation (Abot et al., 2016). The inappropriate use of cellulose-rich waste leads to resource loss and is not environmental friendly. Therefore, the need to achieve its effective use has become a longstanding issue. Currently, composting of wastes is considered to be the main method for the recycling of such cellulose waste (Wang et al., 2020; Xuejiao et al., 2021). However, the abundance of refractory cellulose is a bottleneck restricting the efficient recycling of agricultural waste, which seriously limits the composting efficiency of such waste.

Cellulases produced by microorganisms such as bacteria (Das et al., 2010; Rajeeva et al., 2015) and fungi play the most important role in cellulose biodegradation. The process of cellulose biodegradation is a complex enzymatic process in which cellulose is degraded into glucose units via the action of various cellulases. It can be classified into endo-1,4-glucanase, exo-1,4-glucanase, and-1,4-glucosidase (Bajpai, 2018; Kumar and Murthy, 2017; Liu et al., 2022). High-yield cellulase-degrading bacteria are often used as exogenous microbial agents and are widely employed for treating cellulose-rich agricultural waste. These bacteria exhibit the advantages of being environmentally friendly, cheap, convenient, and not causing secondary pollution. Therefore, the screening of functional strains with high cellulase production has attracted immense attention (Egwuatu and Appeh, 2018; Harnvoravongchai et al., 2020; Kim and Yu, 2020; Paul et al., 2021).

Domestic yaks (*Bos grunniens*) are important livestock that can provide food and livelihood for millions of people living in the Qinghai-Tibet Plateau (Zhang et al., 2016). Yaks feed on grasses, straw, and lichens, which are plant materials rich in lignocellulosic biomass, such as cellulose, hemicellulose, and starch particles (Dai et al., 2012; Park and Kong, 2018). Digestion of complex dietary fiber composed of plant cell wall polysaccharides and resistant starch is essential for preserving numerous physiological processes and host energy metabolism. Since the mammalian genomes generally encode few enzymes linked to digestion (El Kaoutari et al., 2013), a consortium of gastrointestinal microorganisms that harbor multiple carbohydrate-metabolizing enzymes play a significant role in the breakdown of structural polysaccharides, particularly for those found in the plant cell wall (Lee et al., 2014; Sathya and Khan, 2014).

Transformation of dietary carbohydrates into soluble oligosaccharides and fermentable monosaccharides for further energy production is a crucial biological process, which requires synergism of microbial carbohydrate-degrading enzyme activities, including glycoside hydrolases, pectatelyases and carbohydrate esterases Flint et al., 2012. Gong et al., 2020 revealed a great diversity of carbohydrate-degrading enzymes in the yak gut microbial community and uncultured species, which provides a useful genetic resource for future studies on the discovery of novel enzymes for industrial applications.

Due to increased concern about the greenhouse effect, depleting oil reserves and rising global oil prices, as well as the focus on utilising renewable fuels such as bioethanol, cellulase enzymes have become quite important to keep the environment active and interactive (Mohanappriya and Kapilan, 2018). The present study was aimed to isolate cellulase degrading bacteria from yak dung, soil and yak dung manure of hostile cold region of Lahaul and spiti of Himachal Pradesh keeping in view their applicability in various aspects of energy production and industrial use .

## 2. MATERIALS AND METHODS

The present study was conducted during August, 2021 at Department of Agricultural Engineering, College of Agriculture, CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur to isolate and identify the cellulolytic bacteria from yak dung, yak dung manure and soil samples from spiti region in Lahaul-Spiti district of Himachal Pradesh.

### *2.1. Samples collection*

Samples for isolation and identification of cellulytic bacterial was collected from yak dung, yak dung manure and soil from Lahaul-spiti district of Himachal Pradesh, India during August, 2021 in sterile polythene bags and transported to microbiology laboratory and kept at 4°C in refrigerator till further use.

### *2.2. Isolation of bacteria*

Ten-fold serial dilutions of each sample was prepared in sterilized distilled water and 0.1 ml diluted sample (dilution $10^{-2}$ and $10^{-3}$) was spread on the surface of nutrient agar medium (0.3% beef extract, 0.5% peptone, 0.5% NaCl, and 1.7% agar, pH 7.0) plates and incubated at 28±2°C for 48 hours for bacterial growth. Morphologically different colonies appeared on nutrient agar medium were further purified and preserved at 4°C for further screening process.

*2.3. Screening of cellulose degrading bacteria*

Five microlitres of overnight grown culture of pure isolates from nutrient agar medium in nutrient broth was spot plated on Carboxymethylcellulose (CMC ) agar medium (0.2% $NaNO_3$, 0.1% $K_2HPO_4$, 0.05% $MgSO_4$, 0.05% KCl, 0.2% carboxymethyl cellulose (CMC) sodium salt, 0.02% peptone and 1.7% agar) (HiMedia, India). Plates were incubated at 28±2°C for 48 hours. After incubation, colonies growth on CMC agar medium plates were observed for zone of clearance around the colony by flooding plates with 0.1% congo red for 15 to 20 minutes and then with 1M NaCl solution for 15 to 20 minutes.

*2.4. Identification of cellulose degrading bacteria*

After screening of cellulose degrading bacteria on CMC medium were further identified on the basis of morphological characteristics and biochemical tests as per identification scheme given in Bergey's manual of systematic Bacteriology (Bergey, 1957). Identification included shape and size of colony, Gram's staining, Indole test, Methyl red test, Voges-Proskauer test, Citrate utilization test, Catalase test, Oxidase test, Motility test, Nitrate reduction test, Carbohydrate fermentation test by standard methods.

*2.5. Cellulolytic activity assay*

Cellulolytic activity of bacterial isolates was measured in terms of diameter of clear zone on carboxymethyl cellulose agar medium plate which were flooded with 0.1% Congo red followed by sodium chloride (1M) solution. Cellulolytic index was calculated using formula as follows (Ferbiyanto et al., 2016):

Cellulolytic index=(Diameter of zone-Diameter of Bacterial colony)/Diameter of bacterial colony

*2.6. Pre-treatment of paddy straw with effective cellulytic consortium*

Paddy straw was chopped in to very small pieces and 500 g of this biomass was mixed with consortium composed of *Bacillus* spp. and *Pseudomonas* spp. culture (mixed 1:1 ratio) of 900 ml in nutrient broth (grown in liquid culture constantly 30°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, paddy straw was digested anaerobically in 2.5 l digester by feeding 500 g biological pretreated rice straw (wt weight basis) and 500 g 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 (1000 g cow dung) in laboratory scale at room temperature for four weeks. The biogas production was recorded from second day of running the experiment.

## 3. RESULTS AND DISCUSSION

*3.1. Isolation and screening of cellulytic bacterial isolates*

The isolation of cellulytic bacteria was done from 12 samples from yak excreta, yak manure and soil from cold region of Lahaul-spiti on CMC agar medium and 24 isolates (out of 34 colonies appeared on nutrient agar) were screened by flooding the colonies growing with congo red followed by sodium chloride application. Eight efficient cellulytic isolates out of 24 isolates having greater cellulytic index were further identified. As we know that cellulose are the key building blocks of plants and have major fraction of organic carbon in soil. Microorganisms, which live in soil, are accountable for recycling of this organic carbon to the Environment (Wang et al., 2008). Degradation of cellulosic materials is a complex process and requires participation of microbial cellulolytic enzymes. Habitats where these substrates are present, the best sources for isolation of cellulolytic microorganisms (Das et al., 2010). Several microorganisms have been discovered for decades which have capacity to convert cellulose in to simple sugars (Perez et al., 2002) but need for newly isolated cellulose degrading microorganisms still continues (Nirajane et al., 2007) and in our study the yak excreta was one of the source for cellulytic bacterial isolation. Li et al., 2023 isolated the acid-resistant thermophilic cellulolytic bacterium *Bacillus subtilis* DC-11 from silkworm excrement having good CMCase, CXase, and FPase activities. Furthermore it has immense economic potential for the efficient resource treatment of cellulose-rich agricultural waste.

*3.2. Morphological and Biochemical characteristics of cellulolytic bacterial isolates*

24 cellulose degrading bacteria were screened out of 34 isolates from 12 samples of yak dung, yak dung manure and soil samples from cold region of Lahaul-spiti and identified 6 isolates of *Bacillus* spp. and 02 *Pseudomonas* spp. These eight cellulose degrading bacteria were identified by studying their morphological characteristics and biochemical characteristics. The Gram staining of 24 cellulytic bacteria revealed small rods, long rods and coccobacilli were observed. Long rods Gram positive (06) and negative bacteria (02) observed from yak dung (08 isolates). Yak dung manure recovered 07 cellulose degrading bacteria showed Gram positive(04) and Gram negative (03). Soil samples showed 04 Gram negative and 05 Gram positive (Figure 1).

Eight best cellulytic bacterial isolates were identified biochemically out of 24 cellulytic bacteria isolates on CMC medium as *Bacillus* spp. (06) and *Pseudomonas* spp. (02). These isolates were recovered as three isolates from yak dung manure, one from yak dung and two from soil samples characterized on the basis of biochemical tests

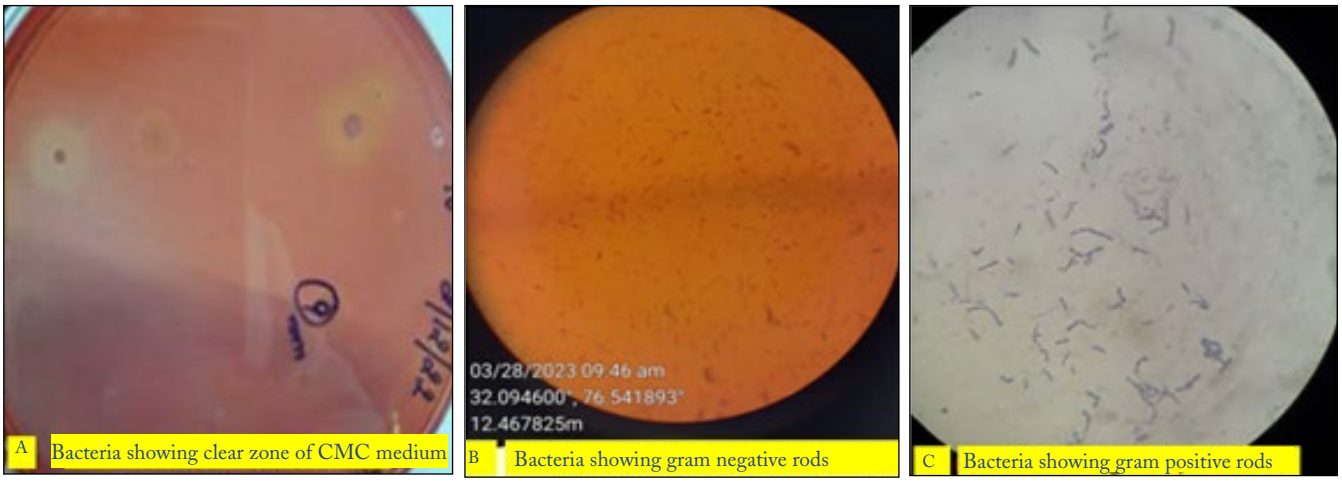


Figure 1: Growth of cellulytic bacteria on CMC medium and gram staining reaction

such as 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. Two *Pseudomonas* spp. isolates were recovered one each from yak dung manure and soil sample 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 out of nine Gram negative bacterial isolates (Table 1).

### *3.3. Cellulolytic activity of bacterial isolates*

Cellulolytic activity of 08 best bacterial isolates was done on basis of clear zone formed by colonies on CMC medium upon congo red flooding followed by sodium chloride solution application. Cellulolytic activity test showed that isolate YAKM1 and isolate YAKM4 has the largest cellulolytic index 2.3 mm and 2.0 mm isolate YAKD1 has the smallest cellulolytic index (1.1 mm) respectively. Based on cellulolytic index and growth isolate YAKM1 and isolateYAKM4 were potential isolates (Table 2).

Table 1: Biochemical and carbohydrates utilization of cellulose degrading isolates from yak dung, yak dung manure and soil samples

| Biochemical tests | Gram positive rods | | | | | | Gram negative coccobacilli | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 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: Cellulolytic index of bacteria isolated from yak dung, yak dung manure and soils of Lahaul-spiti

| Isolate | Diameter of colony (mm) | Diameter of cellulolytic zone (mm) | Cellulolytic index |
|---|---|---|---|
| YAKM1 (*Bacillus* sp.) | 7.5 | 24.4 | 2.3 |
| YAKM2 (*Bacillus* sp.) | 6.5 | 17.3 | 1.7 |
| YAKM3 (*Bacillus* sp.) | 8.0 | 21.5 | 1.7 |
| YAKD1 (*Bacillus* sp.) | 7.0 | 14.4 | 1.1 |
| SOILA (*Bacillus* sp.) | 6.4 | 16.8 | 1.6 |
| SOILB (*Bacillus* sp.) | 4.2 | 12.2 | 1.9 |
| YAKM4 (*Pseudomonas* spp.) | 7.8 | 12.4 | 2.1 |
| SOILC (*Pseudomonas* spp.) | 6.8 | 14.8 | 1.2 |

*3.4. Employment of cellulytic consortium for 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 22$^{nd}$ 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 790 ml as compare to 650 ml in control. Here the employment of pre-treatment along with cellulose bacterial culture for biomass treatment has increased the production of biogas observed upto 4$^{th}$ 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. Zhang et al. in 2016 observed that the rice straw was pretreated with the rumen fluid at 39$^{o}$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.

## 4. CONCLUSION

Eight (08) best cellulose degrading bacterial isolates were isolated and identified on morphological and biochemical basis as *Bacillus* spp. (06) and *Pseudomonas* spp. (02) from yak dung,yak dung manure and from soil samples from Lahaul-spiti valley. Bacterial isolates from yak dung manure (YAKM1) and (YAKM4) showed the highest cellulytic index values. Most Efficient cellulose degrading isolates of *Bacillus* spp. and *Pseudomonas* spp. were employed for biological pretreatment of paddy straw and it was concluded that there was 10% increase in biogas production as compare to control digester on 22$^{nd}$ days at laboratory condition.

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