Microbial Enzymes from Cow Dung a Boon for Biofuel Production

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Abstract

Cow dung, composed of undigested food residues and urine, serves as a rich source of minerals and microbial diversity. This study explores the potential of cow dung microbes for sustainable agriculture and biotechnological applications. Various bacterial genera have been isolated from cow dung, with promising implications for enzyme and antimicrobial production. Our research focuses on isolating and characterizing bacteria from cow dung, screening for enzymatic activity, antibiotic susceptibility, and plant growth promotion. A microbial consortium exhibiting enzymatic activity was selected for organic waste degradation, demonstrating the practical utility of cow dung microbes. Additionally, we characterized the crude mannanase enzyme produced by isolated bacteria, highlighting its thermo stability and optimal pH range. Overall, this study sheds light on the untapped potential of cow dung as a bioresource for sustainable development and biotechnological innovation.

Key words: Cow dung, Biotechnological, Enzyme, Microbes and Sustainable Agriculture

Introduction

Cow Dung can be defined as the undigested residue of consumed food material being excreted by herbivorous bovine animal species. Being a mixture of faeces and urine in the ratio of 3:1, it mainly consists of lignin, cellulose and hemicelluloses. It also contains 24 different minerals like nitrogen, potassium, along with trace amount of sulphur, iron, magnesium, copper, cobalt and manganese. The indigenous Indian cow also contain higher amount of calcium, phosphorus, zinc and copper than the cross-breed cow. Cow dung harbours a rich microbial diversity, containing different species of bacteria, protozoa and yeasts. Many different bacterial genera such as Citrobacter koseri, Enterobacter aerogenes, Escherichia coli, Klebsiella oxytoca, Klebsiella pneumoniae, Kluyvera spp., Morgarella morganii, Pasteurella spp., Providencia alcaligenes, Providencia stuartii and Pseudomonas spp. have been isolated from cow dung [Gupta et al 2016]. Cow dung host a wide variety of microorganisms varying in individual properties. Exploitation of cow dung microflora can contribute significantly in sustainable agriculture and energy requirements. It is one of the bio-resources of this world which is available on large scale and still not fully utilised. An exciting area of research for future studies is developing microbial enzymes and antimicrobials.

The production of enzymes by microorganisms from this cheap bioresource can find wide applications in various fields such as agriculture, chemistry and biotechnology. The application of cow dung microflora with considerable antimicrobial potential can result in the promotion of human health; however, comprehensive screening of these microorganisms for the production of antibacterial, antifungal and antiviral metabolites needed to be investigated. It is certainly evident that more detailed studies of cow dung are needed, as there is still a tremendous scope for research and development to reach up to the industrial scale production of antibiotics and enzymes. In this way, cow dung may be considered as an easily available bioresource that holds a great potential for sustainable development in the near future. Our endeavour through this study is to isolate and characterize the bacteria from cow dung on different morphological and biochemical basis and study their usefulness with the preliminary biological screening of microbes Microorganisms from cow dung sample were screened for hydrolytic enzyme production, antibiotic susceptibility, IAA production, isolation of phosphate solubilizing bacteria and to exploit these

isolated microorganisms for potential biotechnological application.

An efficient microbial consortia from isolated bacteria with concomitant enzymatic activity was selected for the effective degradation of organic kitchen waste. Four bacterial strains concomitantly produced all these enzymes (protease, mannanase, and cellulase) that are responsible for the degradation of kitchen wastes. It was monitored for 30 days by gradual decrease in the volume of the kitchen wastes. Moreover, no comprehensive analysis of the frequency and activities of bacterial communities of cow dung to our knowledge, been performed to date.

Material and methods

Biochemical tests are mainly done to identify bacteria capable of producing various exoenzymes which explore their properties of hydrolyzing waste material. The biochemical tests were done to identify the secretion of three exoenzymes viz. protease, cellulase and hemicellulase. Agar plates were prepared containing skimmed milk, cellulose and locust bean gum for testing protease, cellulase and hemicellulase activity, respectively. If the inoculated bacterium secretes the respective exoenzymes, a clear zone of hydrolysis is observed around the growth. The relative cellulase and hemicellulase activities were observed by staining the plates with Congo Red. The plates were flooded with 0.5 % Congo red for 15 minutes and then washed with 1M NaCl to see the cellulolytic activity of isolated strain. The formation of a clear zone of hydrolysis indicated the cellulose/hemicellulose degradation [Mistry et al 2016]. Assay for production of growth promoting hormones: All isolates were screened for indole acetic acid (IAA) production. Colorimetric method was used for quantitative measurement of IAA [48]. Composition of Media and Reagents: Yeast Malt Dextrose Broth: Yeast- 4 g/l, Malt- 10 g/l, Dextrose- 20 g/l. Salkowski's Reagent: 1ml $FeCl_3$ (0.5M, 2%) dissolved in 50ml $HClO_4$ (35%, v/v). To determine the amounts of IAA produced by each isolate, a colorimetric technique was performed using the Salkowski's method. The isolates were grown in yeast malt dextrose broth (YMD broth) in duplicates and incubated at 37° for 24- 48 hours. The broth was centrifuged after incubation. 1ml Supernatant was taken and was mixed with 2 ml of Salkowski's reagent (2% of 0.5 M $FeCl_3$ in 35% $HClO_4$ solution) and kept in dark. The optical density (O.D.) was recorded at 530 nm after 30 minutes and 120 minutes using spectrophotometer [B. Mohite 2013]. Auxin quantification value was recorded by extrapolating calibration curve made by using IAA as standard (10 - 100µg/ml).

Enzyme Assay for extracellular hydrolytic enzymes: The protease producing bacterial colony (sample C) was inoculated in LB broth and incubated at 37°C for 48 hours. After incubation, the cultures were centrifuged at 8000 rpm for 6-8 minutes and supernatants were used as source of crude enzyme. The crude enzyme solution was utilized for determination of enzyme activity [Dalal, 2015]. Freshly prepared 0.65% (W/V) casein solution by mixing 0.13gm casein in 20ml phosphate buffer. Trichlroaceticacid (TCA) by diluting 1.8ml to 100 ml distilled water. A 500mM sodium carbonate solution (53mg/ml) ,prepared by dissolving
5.3gm of anhydrous sodium carbonate in 100 ml purified water. An enzyme diluents solution, which consists of phosphate buffer (pH-7) were prepared to dissolve protease samples. Follin's reagent

Assay for protease activity: 0.5ml enzyme, 5.0ml substrate and 5.0ml TCA was added in test tubes and were incubated at 37°C for 30 minutes. No enzyme was added in blank.After incubation at 37 C took 2ml filtrate from all the test tubes was transferred to new test tubes. 5ml of sodium carbonate and 1ml of Follin's reagent were added to all the test tubes and were incubated at 37°C for 30 minutes. Appearance of blue colour after incubation, confirms the presence of active protease enzyme.

Test For IAA Production

IAA production test was done to find out whether these isolates produced auxins that promote plant growth. For this auxin quantification was recorded by extrapolating calibration curve made by using IAA as standard (10-100µg/ml) and measuring optical density at 530 nm. Then IAA production test was performed. After growth under dark and shaking conditions, the presence of IAA in the medium was detected. Out of 5 isolates, 3 i.e. A, B and E produced IAA more than 25 µg /ml in the presence of the precursor tryptophan. Of these isolates, two isolates did not produce any IAA, even when amended with tryptophan.
Our results agree in few respects with (Vijayaraghavan *et al*. 2012) who found that bacteria from 'cow dung' were capable of P solubilization and production of growth promoting substances like IAA and GA. But they also found the bacteria to be capable of N_2-fixation and suppressing *Sclerotium sp.*

Production of Hydrolytic Enzymes:

When the isolated bacteria were inoculated at 37^0C in substrates like skimmed milk, Locust bean gum and cellulose containing plates for 24-48 h, when these plates are screened for enzymatic activities then it was found that strain E and C gave positive result for cellulase activity. Strains B and C isolated from cow dung were able to produce protease. Isolate E was the only one that gave positive result for mannanase activity.

Table 5: Screening for hydrolytic enzymes in isolated bacteria

Isolate	Protease	Cellulase	Mannanase
A	-	-	-
B	++	-	-
C	+++	+++	-
D	-	+	-
E	-	++	++++

According to the studies conducted on cow dung isolates it is revealed that *Bacillus spp.* known to produce α-amylases which have wide application in industrial processes, especially in starch industry [Sadhu, 2014]. Besides these studies sulphur oxidizing- *Pseudomonas sp.PRK786*, cellulase producing bacterial strains were isolated and characterized biochemically and on molecular basis [Illavarasi, 2014].

Characterization of crude mannanase enzyme:

Mannanase production by the isolate E in MEM-LBG medium was extra-cellular as no activity could be detected in intact cells. The increase or decrease in mannanase activity present in the cell free supernatant of bacterial isolates was checked by adjusting pH of the reaction mixture between 4.0-9.0 and incubation temperature of medium between 45-75°C. **Optimum temperature**

The optimum temperature for mannanase activity of the crude enzyme with LBG as substrate was determined to be 55°C. The enzyme demonstrated more than 80% activity at temperatures between 55°C and 75°C (Fig. 3). Earlier, bacterial mannanases, reported to be active in the temperature range of 50-60°C, have been isolated from various *Bacillus* sp. [Dhama *et al.* 2004].

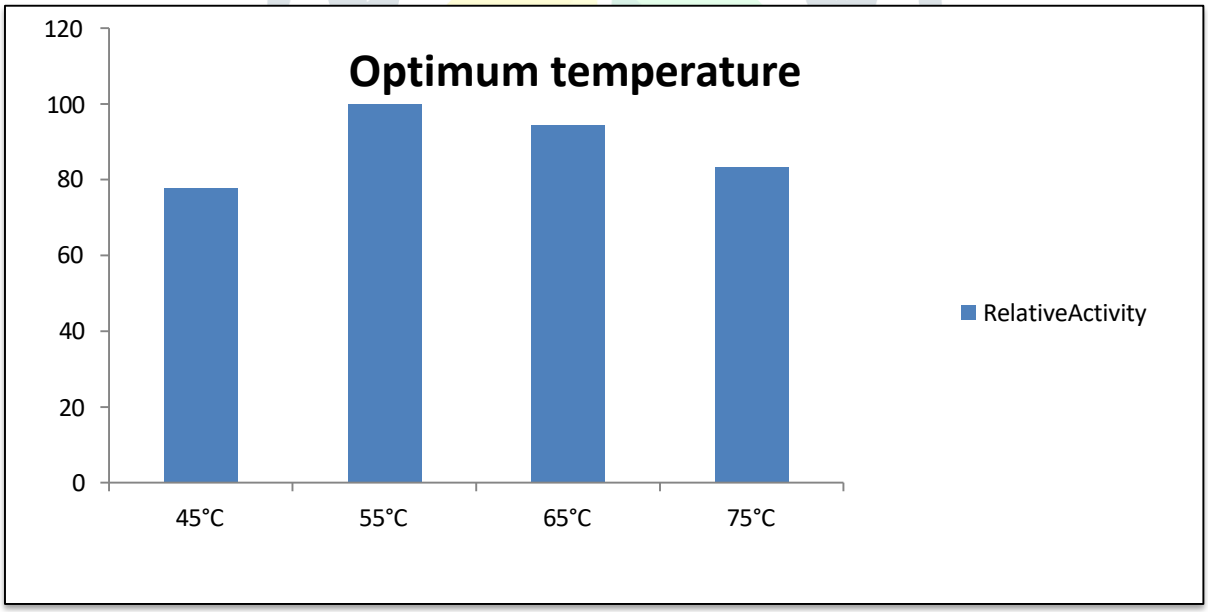

Fig.3: Optimum temperature for crude enzyme

To examine thermo stability, the crude enzyme was incubated at various temperatures and the residual activities were then assayed. The enzyme was 100% stable up to 2 h at 55°C and retained more than 80% of its activity at 65°C after 2 h of incubation, but its stability rapidly decreased at 70°C and above. This thermo stability is comparable to that reported for several mannanases from the thermophilic fungi and significantly higher than that of other bacilli strains viz. *Bacillus licheniformis, Bacillus circulans* CGMCC, *Thermoascus aurantiacus* [Vijayaraghavan et al 2012]. The stability of β-mannanase for prolonged periods of time at elevated temperature is a desirable property for enzyme to be used in commercial processes

involving either paper & pulp industry or food and feed industry (Dorothy and Frisvad 2002).

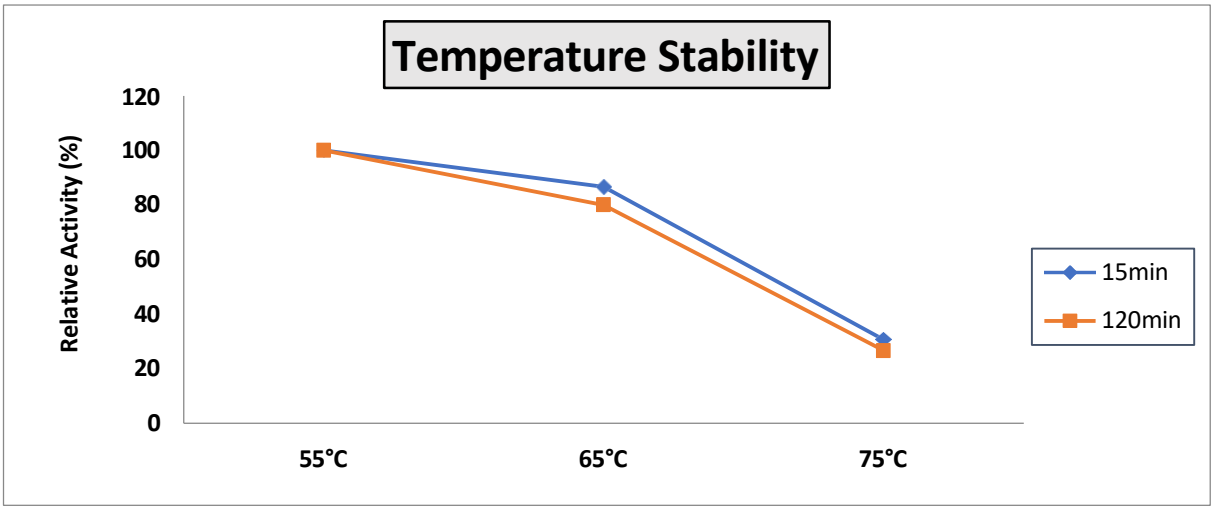

Fig 4: Temperature stability for crude enzyme Optimum pH

for the activity of crude mannanase

Importance of pH in enzyme activity has already been well established. Varying the pH values of reaction mix in the range of pH 4.0- pH 9.0, the optimum pH for crude mannanase activity (at 60°C) was determined to be 5.0. Microbial mannanases are known to

exhibitmaximal activities in the diverse pH ranges of 5.0-6.5 [Li et al. 2009).], 6.5-9.0 [Umanu et al. 2013] or 9.0-10.0 [Haritash and Kaushik, 2009]. The enzyme was observed to be very stable at pH 5. Between pH 6.0-7.0 retained more than 80% of its activity in this pH range. More than 80% and 50 % of the enzyme activity was retained after incubation at pH 5.0 and 8 .0 respectively, while enzyme activity decreased drastically below pH 4.0 and above pH 10.0.

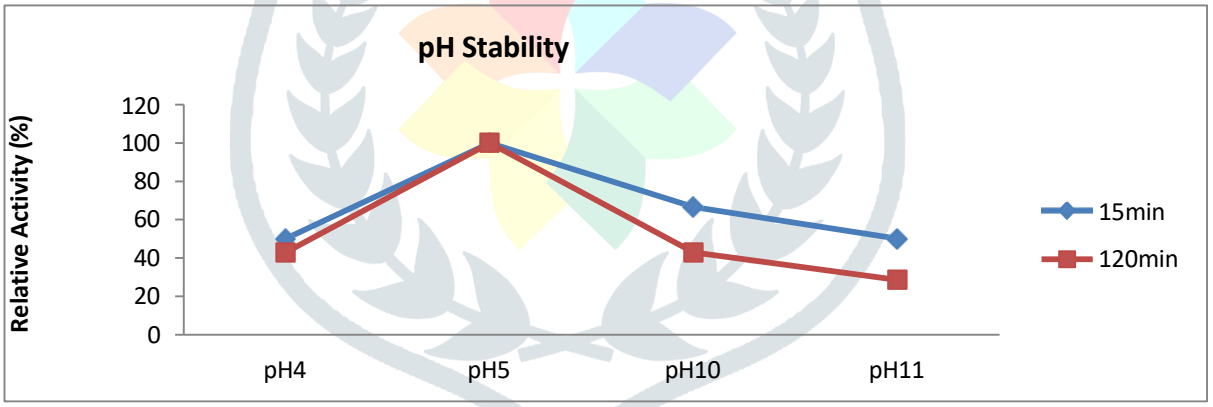

Fig. 6: pH stability for crude enzyme

Partial Purification The highest activity was observed from ammonium sulphate fraction of 80 % saturation. The enzyme activity increases from one purification step to another as follows; 1.5 times for ammonium sulphate fraction of 50 %; 3.6 times for ammonium sulphate fraction of 70 % and 5.0 times for ammonium sulphate fraction of 80%. The enzyme assay was performed to determine the mannanase activity in ammonium sulphate fractions. Localization of mannanase activity was seen in the 80% fraction of supernatant obtained from cut compared to the enzyme activity from 0–30% fraction. There is an approximate 5-fold increase in enzyme activity compared to crude enzyme. The increase of enzyme activity for each purification process was an indication that the step of purification used was effective.

Conclusion

The rich microbial diversity present in cow dung offers promising opportunities for various applications, including enzyme production, antimicrobial development, and organic waste degradation. Through our research, we have isolated and characterized bacteria from cow dung, demonstrating their enzymatic activity, antibiotic susceptibility, and plant growth- promoting capabilities.Furthermore, the microbial consortium identified in this study shows promising results in degrading organic kitchen waste efficiently, highlighting the practical applications of cow dung microbes in waste management. Additionally, the characterization of the crude mannanase enzyme produced by isolated bacteria reveals its stability and optimal conditions for activity, further emphasizing the potential for industrial applications.

Overall, our findings underscore the need for further exploration and utilization of cow dung as a renewable bioresource. By harnessing the microbial diversity present in cow dung, we can unlock new opportunities for sustainable agriculture, waste management, and biotechnological advancements. Moving forward, continued research in this area is essential to fully exploit the untapped potential of cow dung for the benefit of society and the environment.

References

B. Mohite [2013] Isolation and characterization of indole acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth, Journal of Soil Science and Plant Nutrition , Vol.13(3): 638-649.

Dalal Rupali [2015]. Screening and Isolation of Protease Producing Bacteria from Soil Collected from Different Areas of Burhanpur Region (MP) India, International journal of current microbiology and applied sciences. Vol. 4(8).597-60.

Dhama K, Chauhan RS, Singhal L (2005). Anti-cancer activity of cow urine: current status and future directions, *International Journal of Cow Science*, Vol.1: 1–25.

Dorothy ET, Frisvad JC (2002). Eupenicillium bovifimosum, a new species from dried cow manure in Wyoming. Mycologia, Vol 94: 240–246.

Gupta.K, Aneja.R. Kand Rana.D [2016]. Current status of cow dung as a bioresource for sustainable development. Bioresources and Bioprocessing Vol. 3 (28).

Haritash AK, Kaushik CP [2009]. Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review. Journal of Hazard Mater , Volume-169: 1–15.

Illavarasi S. Isolation and identification of cellulase producing bacteria from cow dung. [2014]. Scrutiny International Research Journal of Microbiology and Bio Techniques.Vol.1 (1): 12-18.

Li R, Chen S, Li X (2009). Anaerobic co-digestion of kitchen waste and cattle manure for methane production. Energy Source Part A Recovery Utilisation Environment Effect, Vol 31: 1848–1856.

Mistry.T, Shaikh.N.M, Rana.N.H and Patel.D.N [2016]. Isolation and Screening of Cellulase Producing Thermophilic Bacteria from Compost Piles and Optimization of Cellulase Production, International Journal of Advanced Biotechnology and Research,Vol-7, Issue-1: 64-76.

Sadhu S, Ghosh PK, Aditya G, Maiti TK (2014). Optimization and strain improvement by mutation for enhanced cellulase production by *Bacillus* sp. (MTCC10046) isolated from cow dung. *Journal of King Saud University*-science. Vol.26: 323–332.

Umanu G, Nwachukwu SCU, Olasode OK [2013]. Effects of cow dung on microbial degradation of motor oil in lagoon water. Global Journal of Bio-Science and Biotechnology, Vol-2: 542–548.

Vijayaraghavan P, Vijayan A, Arun A, Jenisha J, Vincent SGP (2012). Cow dung: a potential biomass substrate for the production of detergent-stable dehairing protease by alkaliphilic *Bacillus subtilis* strain VV. Springer plus .Vol.1.(76)

Vijayaraghavan P, Vincent SGP (2012). Cow dung as a novel, inexpensive substrate for the production of a halo-tolerant alkaline protease by *Halomonas* sp. PV1 for eco-friendly applications. *Biochemical Engineering Journal*.Vol.69: 57–60.