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Cellulolytic Enzyme Production by *Trichoderma reesei* MTCC 164 Using Cattle Dung and Cattle Dung Slurry of Biogas as a Substrate

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ABSTRACT

Biological fuel production serves as a sustainable carbon neutral energy source in this era of decreasing fossil fuels. Biogas technology is an important alternative biological fuel. In this context, this paper present the use of cheap and easily available cattle dung and cattle dung slurry for producing cellulolytic enzymes using Trichoderma reesei MTCC 164. Being rich in lignocelluloses, cattle dung and slurry are suitable for cellulolytic enzyme production. In this study, upon optimization for fermentation conditions, it was found that sterilizing the sample by autoclaving do not show significant increase in enzyme production and non-autoclaved samples showed better enzyme production. For cattle dung, concentration of 75%, 10^6 spores/ml Cbase (185.81U/ml), Incubation period of 15 days showed higher activities i.e. 115.9U/ml Cbase, .While in case of digested biogas slurry, all parameters were more at 50% slurry concentration (i.e. 23.07U/ml Cbase) 10^6 spores/ml Cbase (17.79 U/ml), Incubation period of 15 days. But the incubation period for maximum enzyme production varies with the type of enzymes and their isozymes. Out of all the three components of cellulase complex produced by Trichoderma reesei MTCC 164, cellobiase was produced to the maximum, followed by Fpase and CMCase both in cattle dung and digested biogas slurry. These enzymes can further be purified for commercial purpose.

Key words: *Trichoderma reesei, Biogas, Cellulolytic Enzymes, Cattle Dung, Cattle Dung Slurry.*

INTRODUCTION

To make headway in the development efforts of a country, there should be adequate and regular sources of energy. Biogas technology provides an alternative source of renewable energy in today's world. Biogas is methane rich gas produced through anaerobic breakdown and fermentation of biomass. It contains 50-70% methane, 30-40% carbon dioxide, traces of hydrogen, hydrogen sulphide and nitrogen. Its calorific value is 23-28 MJ/m^3, [19]. In India, dung from cattle is mainly used for biogas production. The gas obtained from biogas plant is used as kitchen fuel.

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Slurry obtained from biogas plant has been proved as a good fertilizer which has more capability to improve the physical properties of soil. But being a viscous material, slurry is difficult to transport to the fields. Its direct application forms a layer on the soil surface, which is not desirable. However, being a lignocellulosic substance, slurry can be used as a substrate for enzyme production.

Various bacteria, actinomycetes and filamentous fungi produce extracellular enzymes, but moulds have generally higher enzyme activity[21]. Cellulases and hemicellulases such as xylanase have numerous industrial applications such as chemicals, fuel, food, brewery, animal feed, textile, laundry, paper and pulp industry[3,5,24,25,26]. It is estimated that approximately 20% of the more than one billion US dollars of world's sale of industrial enzymes consists of cellulases, hemicellulases and pectinases and the world market has increased in the range of 1.7-2.0 billion US dollars[5].

The production of cheap and active cellulase and xylanase in good yield on large scale is the key to the process of enzymatic hydrolysis of biomass for bioethanol production[23]. A cellulolytic enzyme system consists of three major components: endo-β-glucanase, exo-β-glucanase and β-glucosidase.The mode of action of each of these being: Endo-β-glucanase (1, 4-β-D-glucan glucanohydrolase,CMCase, C_X) is involved in "random" scission of cellulose chains yielding glucose and cello oligo saccharides. Exo- β -glucanase (1, 4-β-D-glucan cellobiohydrolase, Avicelase, C_1) is responsible for exo-attack on the non-reducing end of cellulose with cellobiose as the primary structure while β-glucosidase (cellobiase) cause hydrolysis of cellobiose units to glucose.

Commercial enzymes are very costly.e.g. 10 g of Cellulase Y-C from *Trichoderma viridae* costs Rs.18860. Similarily 0.1 U of Endo-β-galactosidase costs about Rs.88249, which is very uneconomical.[14]. Therefore, significant cost reduction is required in order to enhance the commercial viability of cellulase production technology. Filamentous fungi like *Aspergillus*, *Penicillium* and *Trichoderma* have demonstrated a great capability for secreting a wide range of cellulolytic enzymes.

Environmental and nutritional factors are known to have marked effects on enzyme production by microorganisms. These factors include moisture level, inoculum size, incubation time, temperature and pH etc. e.g. increasing the moisture level reduces the porosity of substrate, thus limiting the oxygen transfer into the substrate[22]. Likewise, low moisture ratio leads to reduced solubility of the nutrients of solid substrate, lower degree of swelling and higher water tension[10]. Similarily, appropriate inoculum size is very essential to achieve maximum production of enzymes. Decrease in inoculum size leads to insufficient utilization of substrate, while increase in inoculum size leads to more utilization of substrate for growth of microorganism and the substrate gets exhausted very fast[22]. The effect of incubation time on cellulase production by various fungi on cassava waste was examined by Pothiraj *et al*[21]. Carboxy methyl cellulase activity of *Rizopus stolonifer* declined from second day till sixth day of fermentation and the activity again increased till the tenth day of fermentation. While in case of CMCase activity of *Aspergillus niger* and *Aspergillus terreus* the build up was slow, to reach the maximum on the eight day of fermentation (0.12 IU/ml). In case of temperature, if culture temperature is too high, microorganisms grow faster, but easy to age and enzyme production is low. If temperature is too low, microbial growth is slow, resulting in long production cycle. The optimal temperature for incubation was 30°C for CMCase and FPA[9]. The optimal pH for fungal cellulases vary from species to species, though in most cases optimum pH ranges from 3.0 to 6.0[8,20].

Due to high production cost of cellulolytic enzymes, extraction of cellulases from spent slurry can prove to be beneficial. Till date there is no efficient method to utilize digested cattle dung slurry from biogas plants. The application of microorganisms to extract

cellulolytic enzymes using cattle dung slurry as a substrate is not reported in literature. Keeping in mind, the importance of digested slurry as a potential substrate for value added products; the present work has been done to extract cellulolytic enzymes from digested biogas slurry using *Trichoderma reesei* MTCC 164.

Hence the present study is aimed to accomplish the following objective:

- To extract cellulolytic enzymes from digested cattle dung slurry using *Trichoderma reesei* MTCC 164.

MATERIAL AND METHODS

Procurement of substrates, chemicals and microbial culture

Cattle dung was procured from Dairy farm, GADVASU (Guru Angad Dev Veterinary and Animal Science University), Ludhiana. Digested cattle dung slurry was procured from a working biogas plant in biogas field laboratory of School of Renewable Eneergy Engineering, PAU, Ludhiana. All the chemicals used for proximate analysis, media and solutions preparation were of analytical grade and were purchased from Hi-Media, SRL, Sigma and S.D fine chemicals Pvt. Ltd. Standard culture of *Trichoderma reesei* MTCC 164 was procured from MTCC (Microbial Type Culture Collection), Institute of Microbial Technology, Chandigarh and was maintained on malt extract Blakeslee`s agar (composition given in Annexure I) slants at 30 ± 2^0C by monthly transfers. The culture was stored in refrigerator after sub-culturing.

Proximate and Chemical Analysis of Cattle Dung and Digested Cattle Dung Slurry

Cattle dung and digested slurry were analyzed for pH, proximate and chemical composition, total solids, volatile solids, cellulose, hemicelluloses, lignin and silica were determined by standard methods of AOAC[2]. Total sugars were estimated by Phenol-sulphuric acid method of Dubois *et al*[7] using glucose as standard. Reducing sugars were estimated by the method of Miller[17]. Total protein content was determined by the method of Lowry *et al*[13].

Production of Cellulolytic Enzymes from *Trichoderma Reesei* MTCC 164 Inoculated Cattle Dung and Digested Slurry

Initially cattle dung and cattle dung slurry was examined for cllulolytic enzymes without any external microbes. Supernatant of diluted substrates was used as crude enzyme extract and was analyzed for activities of carboxymethylcellulase, filterpaperasee and cellobiase by Mandels *et al*[15] method and protein content by Lowry *et al* [13] method. Enzyme activities (U/ml of sample) and protein (mg/ml of sample) was determined spectrophotometrically using UV-VIS spectrophotometer 2800 model.

After initial examination, the substrates were inoculated with 2ml spore suspension of *T.reesei* spores @ 10^6spores/ml and were incubated at 30±2°C for enzyme production. After 10 days, crude enzyme was extracted by centrifugation and supernatant was analyzed for all the cellulolytic enzyme activites.

Optimization of Process Parameters for Cellulolytic Enzyme Production

Various parameters viz. sterilization (autoclaved and non autoclaved sample), slurry concentration (25-75%), spore concentration (10^6-10^9 spores/ml) and varying incubation period (5-15 days) were studied for optimization of cellulase production from *T.reesei* inoculated cattle dung and digested slurry. For sterilization effect, diluted and sterilized as well as non-sterilized samples of both cattle dung and digested biogas slurry were inoculated with 10^6spores/ml of *T.reesei* using 2ml of spore suspension, incubated at 30±2°C for 7 days and then crude enzyme was extracted by centrifugation at 10000 rpm for 15 minutes and used for analyzing cellulase activities, protein content and total reducing sugars[17]. Similarily, for effect of spore count, 75% concentration of substrates was inoculated with different spore sizes i.e. 10^6,10^7,10^8 and 10^9 spores/ml spore suspension and after 7 days incubation at 30±2°C crude enzyme was extracted and analyzed for CMCase, Cbase, Fpase activities, protein content and total reducing sugars. For effect of incubation period on enzyme

production 50% concentration of substrates was incubated for 5, 10, 15 and 20 days. Enzyme production was analysed after 5, 10, 15 and 20 day incubation period.

Enzyme units

The cellulase enzyme activity is expressed in terms of Internatinal units and international unit of cellulase may be defined as 1 micromole of reducing sugar released per minute per mililitre of enzyme extract, measured as glucose. Appropriate dilution factors were used as and when followed during estimation of enzyme activity

$$\text{Reducing sugar } \mu \text{ mole/ml/min} = \frac{\text{mg of reducing sugar produced/ ml}}{0.18\times \text{Incubation Period(min)}}$$

Statistical Analysis

Critical difference at 5% level was performed for cellulolytic enzyme production data using CPCS1 software (developed by Department of Statistics, PAU, Ludhiana). Standard error was calculated manually for all the experiments. Optimization studies were performed using Statgraphics Centurian XVI software. All treatments were completed in triplicate.

RESULTS AND DISCUSSION

The present study was conducted to extract and optimize cellulolytic enzymes from cattle dung and digested cattle dung slurry using a mesophilic, filamentous fungi *Trichoderma reesei* MTCC 164.

Proximate and chemical composition of cattle dung and digested slurry

Firstly the cattle dung and cattle dung slurry was analyzed for their proximate and chemical composition. Results from the Figure 1 indicate that cattle dung is of very weak acidic nature showing a pH value of 6.97 while digested slurry is nearly neutral with a pH of 7.03.Total solid concentration in cattle dung is much higher (15%) than that of digested slurry (5%). The volatile solid content in cattle dung is 84% in comparison to 66.67% in digested slurry. Cellulose and hemi-cellulose content is more in cattle dung as compared to digested slurry. This might be due to the fact that part of organic matter like cellulose and hemicelluloses are consumed by consortium of microorganisms in biogas plant for biogas production. Moreover, cattle dung undergoes single digestion in ruminants while digested slurry undergoes further anaerobic digestion in biogas plant. Results have shown higher concentration of lignin in digested slurry (19.8%) than in cattle dung (10.8 %). Silica content in both samples is nearly same i.e. 9.4% in dung and 9.2% in slurry.

Anonymous[1] too reported that pH of dung and slurry is 6.85 and 7.27 respectively. The average total solid concentration of the feed and the digested slurry was 18.62 % and 12.38 % respectively and volatile solids content was 84 % and 66.5 % respectively.

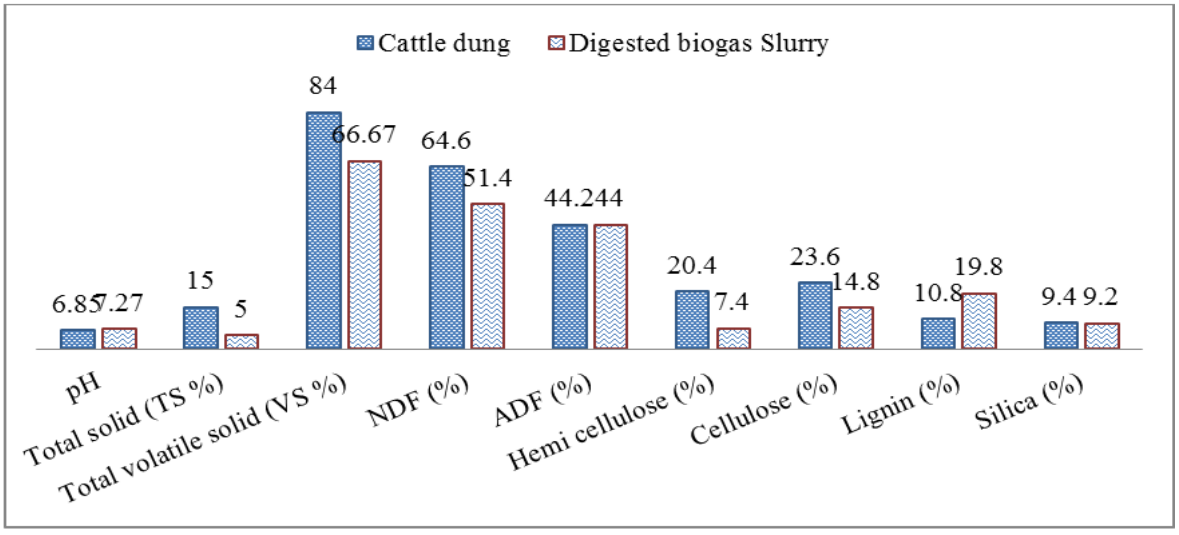


Fig. 1: Proximate and chemical analysis of cattle dung and cattle dung slurry

Production of cellulolytic enzymes

Cellulolytic enzyme activities, total protein and reducing sugar content were determined in cattle dung and digested biogas slurry (50% concentration). Results from Table 1 show a comparison in enzyme profile studies of both. Cattle dung showed relatively more enzyme units and reducing sugar as compared to digested slurry. Among all enzymes, cellobiase activity was highest both in cattle dung (116.30U/ml) and digested biogas slurry (94.8U/ml). Activity of carboxymethylcellulase was calculated as the lowest value i.e. 5.0U/ml in cattle dung and 1.39U/ml in slurry. Fine particles of slurry are a reason for lower enzyme activity in digested slurry, because smaller particle size of the substrate increases the available surface area for enzyme binding. As more enzymes bind to the substrate, the enzyme yield in digested slurry decreases[6]. Protein content of both samples is nearly same i.e. 73.6 mg/ml in cattle dung and 73.58 mg/ml in digested biogas slurry.

Table 1: Cellulolytic enzymes and biochemical constituents of cattle dung and digested biogas slurry

Cellulolytic enzymes and Biochemical constituents	Cattle dung	Digested Biogas Slurry from semi-continuous plant
Carboxy Methyl Cellulase (U/ml)	5.00 ± 0.46	1.39 ± 0.17
Cellobiase(U/ml)	116.30 ± 9.30	94.80 ± 8.23
Filter Paperase (U/ml)	9.66 ± 0.63	6.30 ± 0.49
Protein(mg/ml)	73.60 ± 5.21	73.58 ± 6.13
Reducing sugars(mg/ml)	92.20 ± 7.34	80.91 ± 6.57

The data represents the mean of three determinations each; ±values indicate standard error.

Optimization of production parameters for cellulolytic enzymes

Different cultural conditions like incubation temperature, size of inoculum, incubation period and slurry concentration etc. affects the production of enzymes.

Effect of sterilization and slurry concentration

Substrate concentration is the key element for regulating and optimizing the fermentation process. Hence effect of different slurry concentrations i.e. 25, 50 and 75 % was studied for all autoclaved as well as non-autoclaved samples.

Table 2: Effect of sterilization and slurry concentration on cellulolytic enzyme production from *Trichoderma reesei* MTCC 164 inoculated cattle dung

Concentration (%)	CMCase (U/ml)		Cbase (U/ml)		Fpase (U/ml)		Protein Content (mg/ml)		Reducing sugars (mg/ml)	
Sterilization Condition →	**A**	**NA**	**A**	**NA**	**A**	**NA**	**A**	**NA**	**A**	**NA**
25	5.39 ± 0.14	13.62± 0.11	22.11± 0.97	41.6± 0. 53	3.45± 0.04	6.34± 0.03	46.72± 0.08	52.00± 0.53	205.60± 0.85	192.40 ± 1.36
50	20.84± 0.06	17.68± 0.07	32.31± 0.64	48.59± 0.01	5.34± 0.25	6.91± 0.09	65.96± 0.17	34.52± 0.03	268.04± 0.78	206.80 ± 0.06
75	11.19± 0.90	**47.42 ± 0.02**	19.88± 0.55	**115.9± 2.46**	3.78± 0.05	**21.28± 0.16**	**73.28± 0.55**	24.67± 0.05	277.04± 0.59	**308.16 ± 3.12**
C.D. (5%) A (Condition)	1.32		0.97		1.27		1.83		3.08	
C.D. (5%) B (Dilution)	1.08		0.79		1.03		1.49		2.51	
C.D. (5%) AB (Condition × Dilution)	1.87		1.38		1.79		2.58		4.35	

#Cultural conditions: Incubation period: 7 days; Incubation temperature: 30±2◦C; Spore concentration: 10^6 spores/ml of suspension; The data represents the mean of three determinations each; ±values indicate standard error;
C.D: Critical difference at 5% level; NS: Non-significant; A: Autoclaved; NA: Non-Autoclaved.

Results from Table 2 shows that sterilizing the samples does not affect the cellulolytic enzyme production to much extent. In cattle dung, all the three activities were higher in non-autoclaved samples as compared to autoclaved samples i.e. CMCase activity at 75% concentration was 47.42 U/ml vs. 11.19 U/ml, Cbase 115.9 U/ml vs. 19.88 U/ml and

Fpase 21.28 vs. 3.78 U/ml which means that the samples can be used without autoclaving, thus saving energy. Similar effect was also noted on enzyme production in digested biogas slurry (Table 3). In digested biogas slurry autoclaved sample showed CMCase 6.1U/ml, Cbase 20.1U/ml and Fpase 2.42U/ml while non-autoclaved sample showed CMCase 5.97U/ml, Cbase 23.07U/ml and Fpase 3.33U/ml. Thus the step of autoclaving the sample was omitted from further experiments.It was also noticed that in non-autoclaved cattle dung, enzyme activities for CMCase, Cbase and Fpase were maximum at 75% concentration i.e. 47.42U/ml, 115.9U/ml and 21.28U/ml respectively (Table 2). Table 3 also indicates that all parameters were more at 50% slurry concentration except CMCase activities. In non-autoclaved samples, digested biogas slurry showed maximum CMCase activity of 7.60 U/ml at 75% slurry concentration, whereas maximum Cbase and Fpase activities were recorded at 50% concentration (23.07U/ml and 3.33U/ml respectively).

Table 3: Effect of sterilization and slurry concentration on cellulolytic enzyme production from *Trichoderma reesei* MTCC 164 inoculated digested biogas slurry

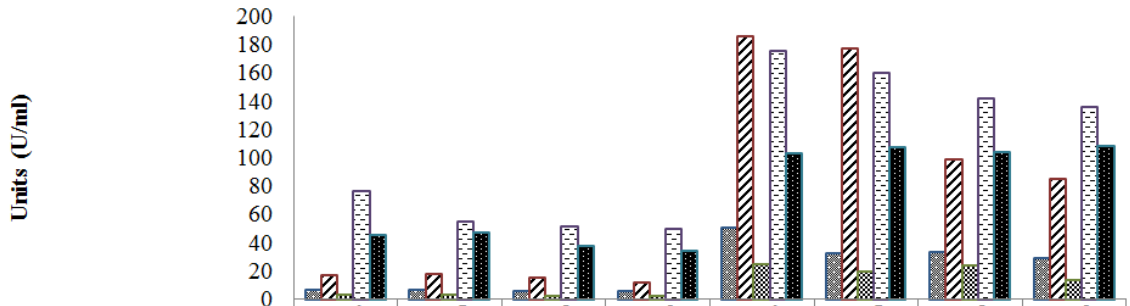
Slurry concentration (%)	CMCase (U/ml)		Cbase (U/ml)		Fpase (U/ml)		Protein Content (mg/ml)		Reducing sugars (mg/ml)	
Sterilization condition →	A	NA	A	NA	A	NA	A	NA	A	NA
25	6.21 ± 0.04	6.30 ± 0.11	19.82 ± 0.01	16.75 ± 0.34	**4.06 ± 0.33**	2.37 ± 0.04	33.80 ± 8.39	22.61 ± 6.14	122.04 ± 0.67	76.84 ± 0.89
50	6.10 ± 0.12	5.97 ± 0.06	20.10 ± 0.56	**23.07 ± 0.28**	2.42 ± 0.07	**3.33 ± 0.67**	**37.24 ± 5.35**	**26.78 ± 2.42**	116.74 ± 0.96	**134.20 ± 0.05**
75	6.12 ± 0.09	**7.60 ± 0.34**	17.96 ± 0.05	18.04 ± 0.21	3.14 ± 0.91	2.25 ± 0.47	22.47 ± 3.65	22.69 ± 3.56	**139.21 ± 0.57**	105.62 ± 0.11
C.D. (5%) A (Condition)	NS		0.89		NS		1.49		1.77	
C.D. (5%) B (Dilution)	0.66		0.73		0.89		NS		1.45	
C.D. (5%) AB (Condition × Dilution)	NS		1.27		NS		2.11		2.51	

#Cultural conditions: Incubation period: 7 days; Incubation temperature: 30±2◦C; Spore concentration: 10^6 spores/ml of suspension;
NA: Non-autoclaved; A: Autoclaved;The data represents the mean of three determinations each; ±values indicate standard error;
C.D: Critical difference at 5% level; NS: Non-significant.

Moisture content is a critical factor as this variable influences fungal cell growth, the biosynthesis and secretion of fungal enzymes. Lower moisture content causes the reduction in the solubility of the substrate nutrients, low degree of swelling and high water tension[12,18]. Kundu *et al*[11] observed that moisture level for solid state culture below the determined optimal value, leads to enzyme inhibition, whereas above the optimum level, greater enzyme diffusion away from the substrate take place. Optimum moisture level varies with the substrate used, as the various type of substrate have different water holding capacity. Maurya *et al*[16] reported that the maximum yield of enzyme (2.29 U/ml) was obtained at 70% moisture level. However, a further increase to 80% had a negative effect on the production of the cellulolytic enzymes.

Effect of spore concentration

Proper inoculum size is required for efficient enzyme production. Results from Figure 2 indicate that in digested biogas slurry, maximum i.e. 6.99 U/ml of CMCase, 3.31 U/ml of Fpase and 76.55 mg/ml of protein was observed at the spore concentration of 10^6 spores/ml, whereas maximum Cbase i.e. 17.79 U/ml and reducing sugars (47.69 mg/ml) were observed at 10^7 spores/ml. While in cattle dung, maximum cellulase activity i.e 50.38U/ml CMCase, 185.81U/ml Cbase and 24.8 U/ml Fpase was observed at a spore concentration of 10^6 spores/ml and activities decreased at higher concentrations.



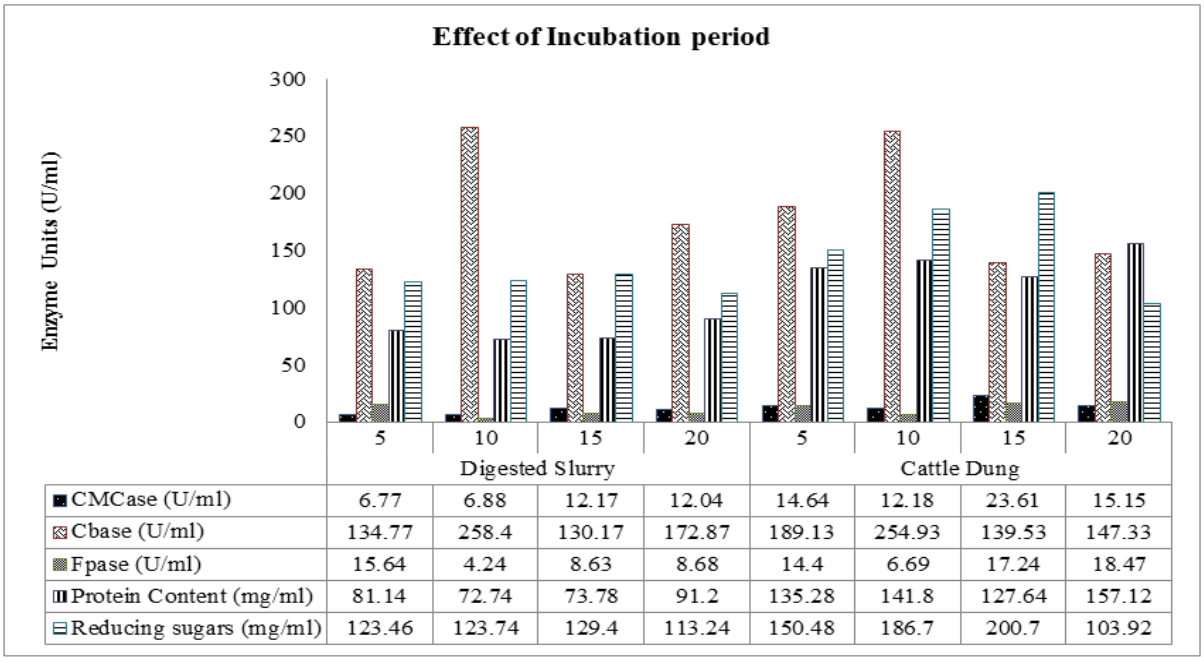
	Digested Slurry				Cattle Dung			
CMCase (U/ml)	6.99	6.77	6.21	5.76	50.38	33.08	33.29	29.42
Cbase (U/ml)	17.39	17.79	15.24	12.28	185.81	177.15	99.43	85.65
Fpase (U/ml)	3.31	3.29	2.8	2.61	24.8	19.7	23.71	13.38
Protein Content (mg/ml)	76.55	55.09	51.79	50.06	176	160.24	141.96	136.36
Reducing sugars (mg/ml)	45.43	47.69	37.72	34.187	103.27	107.48	104.59	109.01

#Cultural conditions: Incubation period: 7 days; Incubation temperature: 30±2◦C; Sterilization: Non-autoclaved; Slurry concentration: 75%; The data represents the mean of three determinations each;

Fig. 2: Effect of spore count on enzyme production

Effect of incubation period

Incubation period plays an important role in substrate utilization and enzyme production. From Figure 3 it was observed that the maximum yield of CMCase in both slurry and cattle dung (12.17U/ml and 23.61 U/ml, respectively) was obtained on 15th day of incubation while maximum Cbase in both slurry and cattle dung (258.4U/ml and 254.93 U/ml, respectively) was obtained on 10th day of incubation. Digested biogas slurry showed maximum Fpase activity i.e. 15.64 U/ml on 5th day of incubation while cattle dung showed maximum activity i.e. 18.47 U/ml on 20th day of incubation. Thus the incubation time period varies with the type of enzyme and Fpase production took maximum time i.e. of 20 days.



	Digested Slurry				Cattle Dung			
CMCase (U/ml)	6.77	6.88	12.17	12.04	14.64	12.18	23.61	15.15
Cbase (U/ml)	134.77	258.4	130.17	172.87	189.13	254.93	139.53	147.33
Fpase (U/ml)	15.64	4.24	8.63	8.68	14.4	6.69	17.24	18.47
Protein Content (mg/ml)	81.14	72.74	73.78	91.2	135.28	141.8	127.64	157.12
Reducing sugars (mg/ml)	123.46	123.74	129.4	113.24	150.48	186.7	200.7	103.92

#Cultural conditions: Incubation temperature: 30±2◦C; Non-autoclaved sample; Dilution rate: 50% dilution; Spore concentration: 10^7spores/ml of suspension; The data represents the mean of three determinations each

Fig. 3: Effect of incubation period on enzyme production

CONCLUSION

This research paper presents work on extraction and optimization of cellulolytic enzymes from cattle dung and digested cattle dung slurry using celluloytic fungi *Trichoderma reesei* MTCC 164. Proximate and chemical compositions of both the substrates *viz.* cattle dung as well as slurry shows that these are rich in lignocelluloses and thus are suitable for cellulolytic enzyme production using some cellulolytic microorganism. *Trichoderma reesei* MTCC 164 was used to extract cellulolytic enzymes from dung and slurry. Enzyme activities from cattle dung and slurry were reported to be 9U/ml CMCase, 116.3 U/ml Cbase, 9.66 U/ml Fpase and 1.39 U/ml CMCase, 94.8 U/ml Cbase, 6.3 U/ml Fpase, respectively. Cellobiase activity was measured to be maximum in both cattle dung as well as in digested biogas slurry i.e. 116.30 and 94.8 U/ml of sample respectively. Carboxymethylcellulase content was found to be minimum in both cases. Upon optimization of fermentation conditions like sterilization, slurry concentration, spore concentration and incubation period using one variable at a time, it was found that sterilizing the sample by autoclaving do not show significant increase in enzyme production and non-autoclaved samples showed better enzyme production as in cattle dung, at 75% concentration. Thus, it was concluded that samples can be used without autoclaving, which saves energy and enhance enzyme production due to presence of natural consortia in substrate. Slurry concentration of 75% showed higher activities in case of cattle dung. While in case of digested biogas slurry, all parameters were more at 50% slurry concentration (i.e. 23.07U/ml Cbase and 3.33U/ml Fpase) except CMCase activity at 75% slurry concentration (7.60 U/ml). In digested biogas slurry, spore concentration of 10^6 spores/ml showed maximum CMCase (6.99 U/ml), Fpase (3.31 U/ml) and protein (76.55 mg/ml), whereas Cbase (17.79 U/ml) and reducing sugars (47.69 mg/ml) were maximum at 10^7 spores/ml. For cattle dung maximum CMCasse (50.38U/ml), Cbase (185.81U/ml) and Fpase (24.8 U/ml) was recorded at a spore concentration of 10^6 spores/ml. Incubation period of 15 days showed maximum yield of CMCase in both slurry and cattle dung (12.17U/ml and 23.61 U/ml, respectively) while Cbase was higher (258.4U/ml in slurry and 254.93 U/ml in cattle dung, respectively) on 10^{th} day of incubation. Maximum Fpase activity (15.64 U/ml) in digested biogas slurry was noticed on 5^{th} day while in case of cattle dung maximum Fpase was recorded on 10^{th} day of incubation (18.47 U/ml). Thus the incubation period for maximum enzyme production varies with the type of enzymes and their isozymes. Further research is required to get insight into the purification of cellulolytic enzymes to match up with commercial enzyme production, using new technologies.

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