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Evaluation of poultry dropping based biodigested slurry as a substrate for the cellulolytic enzymes production using *Penicillium roqueforti* NCIM 712

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Abstract
Evaluation of poultry dropping based biodigested slurry as a substrate for cellulolytic enzyme production using *Penicillium roqueforti* NCIM712 was the main objective of this study. Poultry droppings and poultry dropping based biodigested slurry were examined for cellulolytic enzyme activity. Production of cellulolytic enzymes from poultry dropping based biodigested slurry was estimated. Endoglucanase activity was highest in both unautoclaved and autoclaved biodigested slurry i.e. 60.05 and 43.81 U/ml of BDS respectively. Exoglucanase activities were found to be 21.15 and 9.06 U/ml of BDS in unautoclaved and autoclaved respectively. Out of all the three enzymes of celluase complex, activities of β glucosidase were found to be minimum viz. 4.81 and 2.13 U/ml of BDS in unautoclaved and autoclaved slurry respectively. Protein content was also high in unautoclaved (4.53 mg/ml) than autoclaved (2.13 mg/ml) biodigested slurry. As biodigested slurry is rich in organic matter along with consortium of microorganisms of diverse groups, thus contributing towards higher activities of endoglucanse, exoglucanase and β glucosidase. However after autoclaving, reduction in enzyme activities was reported which might be due to the fact that high temperature (121°C) would degrade and denature some of the proteins and thus affecting catalytic activities of enzyme. These results also suggest that use of unautoclaved slurry for enzyme production will save the cost of autoclaving which is energy consuming process.

Keywords: Biodigested slurry, cellulases, *Penicillium roqueforti* NCIM 712**,** endoglucanse, exoglucanase and β glucosidase

1. Introduction
Cellulose is a naturally occurring renewable biopolymer in the biosphere, degraded by synergistic action of multicomponent hydrolytic enzymes i.e. cellulases. The enzyme system of cellulase consists of three parts: endo-1, 4-β D-glucanase (CMCase, EC 3.2.1.4), exo-1, 4-β D-glucanase (CBH, EC 3.2.1.91), and β- glucosidase (EC 3.2.1.21) [1]. Cellulase can be produced by various actinomycetes, bacteria, and fungi but the most common and efficient producer of cellulase is fungi [2]. Fungal species such as *Aspergillus, Trichoderma, Humicola and Penicillium* are most efficient producers of cellulase enzyme [3].
In India about 1700 million broilers and 150 million hens are present. About 3.6 million tons poultry waste is generated per annum in India, thus generating significant forces on its disposal and management. Poultry droppings are rich in cellulose, hemicelluloses, nitrogen potassium, phosphorous thus, poultry droppings can be used an effective substrate for production of biogas. The biodigested slurry is generated as a by-product from biogas plants which is rich in organic matter including cellulose, micronutrients and macronutrients and bioactive compounds of low molecular weight like vitamins, hormones, hemicelluloses and humic acids etc. Thus biodigested slurry can be used as a substrate for production of value added products utilizing hydrolytic efficiency of filamentous fungi, actinomycetes and bacteria and better management of slurry along with controlling environmental pollution. Therefore in the present paper an attempt has been made to evaluate poultry dropping based biodigested slurry as a substrate for the cellulase production using *Penicillium roqueforti* NCIM 712.

2. Materials and methods
2.1 Culture Collection and Maintenance
Standard culture of *Penicillium roqueforti* NCIM 712 was procured from NCIM (National Collection of Industrial Microorganisms) Pune and was preserved on Potato Dextrose Agar ((g/l: potatoes infusion; 250, Dextrose; 10, Agar; 20, pH 5.5) at 30±2^{0}C by monthly transfer

and after sub culturing, culture was stored in refrigerator.

2.2 Poultry droppings and poultry dropping based biodigested slurry

Poultry droppings was procured from poultry farm, GADVASU (Guru Angad Dev Veterinary and Animal Science University), Ludhiana and poultry dropping based biodigested slurry was obtained from a working biogas digester in biogas field laboratory of School of Renewable Energy Engineering, PAU (Punjab Agricultural University), Ludhiana.

2.3 Chemicals

All the chemicals required for preparation of media, solutions, and utilized for proximate analysis were of analytical grade and were purchased from Hi- Media, SRL, Sigma and S.D fine chemicals Pvt. Ltd.

2.4 Proximate and Chemical Analysis

Poultry droppings and biodigested slurry (sterilized and non-steriltzed) were analyzed for pH, proximate (total solids, volatile solids and ash) and chemical (cellulose, hemicellulose, lignin and silica) composition by the standard methods of AOAC (2000) [4]. Nitrogen content was measured by Kjeldahl method (1883) [5].

2.5 Protein Content Estimation

Protein content of poultry droppings and biodigested slurry (sterilized and non-steriltzed) were measured by Lowry *et al.* method (1951) [6] bovine serum albumin (BSA) as a standard. Protein content (mg/ml) was measured measured spectrophotometrically with the help of UV-VIS spectrophotometer 2800 model.

2.6 Cellulolytic enzymes production from poultry dropping based biodigested slurry

2.6.1 Crude Enzyme Extraction

Hundred milliliters sample of biodigested slurry (autoclaved and unautoclaved) were centrifuged at 10,000 rpm for 15 minutes at 4 °C to obtain clear supernatant. This supernatant were used as crude enzyme extracts and were analyzed for endoglucanase and exoglucanase activities by method of Mendels *et al.* (1976) [7]. Method of Toyama and Ogawa (1977) [8] was used for measuring β glucosidase activity.

2.6.2 Cellulolytic enzymes production from poultry dropping based biodigested slurry inoculated with spores of *Penicillium roqueforti* NCIM 712

Erlenmeyer flasks, 250 ml capacity, each containing 50 ml biodigested slurry and 50 ml distilled water were inoculated with spore suspension of *P. roqueforti* NCIM 712 @ 10^6 spores/ ml and were incubated at 30 ± 2^0C for 7 days. After incubation, separation of crude enzyme was achieved by centrifugation of sample at 10,000 rpm for 15 minutes. Supernatant was used for determination of endoglucanase, exoglucanase and β glucosidase activities by Mendel's *et al.* method (1976) and Toyama and Ogawa method (1977) respectively.

2.7 Assay of cellulolytic enzymes

2.7.1 Substrates and reagents

a) Carboxymethyl cellulose (CMC) solution – 1% CMC solution
b) Cellobiose solution- 10mM solution
c) Filter paper strips- 6 x 1 cm sized whatman No. 1 strips
d) DNS solution
e) Sodium potassium tartrate solution (40%)
f) Citrate buffer – 0.1 M solution

2.7.2 Measurement of endoglucanase activity

Mandel *et al.* (1976) method was used for the measurement of endoglucanase activity. Aliquots (0.5 ml) of enzyme extract were taken in triplicate test tubes and 0.5 ml CMC solution was added to each tube. The mixture was heated in water bath at 50 °C for 30 minutes. Control was run simultaneously devoid of CMC substrate. For measurement of reducing sugar produced during reaction, 3ml DNS reagent was added to each tube and kept at boiling water for 15 minutes. While still hot, solution of sodium potassium tartrate (1ml) was added and the mixture was allowed to cool at room temperature followed by addition of 2 ml distilled water in each test tube. The % absorbance was measured at 575 nm using UV-VIS spectrophotometer 2800 model and thus concentration was read from standard curve enzyme activity was calculated using formula given below (in 2.7.5).

2.7.3 Measurement of exoglucanase activity

Mandel *et al.* (1976) method was used for the measurement of exoglucanase activity. Aliquots of 0.5 ml enzyme extract were taken in triplicate test tubes, 1 filter paper strip and 1 ml citrate buffer was added in each tube and were incubated at 50 °C for 10 minutes. Control of inactivated enzyme was run simultaneously. Reducing sugar produced during the reaction was measured according to DNS method by by Miller (1959) [9]. The % absorbance was measured at 575 nm using UV-VIS spectrophotometer 2800 model and thus concentration was read from standard curve enzyme activity was calculated using formula given in 2.7.5.

2.7.4 Measurement of β glucosidase activity

Toyama and Ogawa (1977) method was used for estimation of β glucosidase activity. Aliquots of 0.5 ml enzyme extract were taken in triplicate test tubes, 0.5 ml cellobiose solution was dispensed in each test tube and the tubes were incubated at 50 °C for 10 minutes. Control was run simultaneously as usual. Reducing sugar produced during the reaction was measured according to DNS method by Miller (1959). The % absorbance was measured at 575 nm using UV-VIS spectrophotometer 2800 model and thus concentration was read from standard curve enzyme activity was calculated using formula given below (in 2.7.5)

2.7.5 Enzyme units

The activity of cellulolytic enzymes were expressed in International units and international unit of cellulolytic enzyme may be defined as 1 micromole of reducing sugar released per minute per milliliter of enzyme extract, measured as glucose. Appropriate dilution factors were used as and when followed during estimation of enzyme activity

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

3. Results and Discussion

3.1 Proximate and chemical composition of poultry droppings and poultry dropping based biodigested slurry

The poultry droppings and the biodigested slurry coming out

of poultry dropping based biogas digester was analyzed for its proximate and chemical analysis. The results from table 1 indicated that both unautoclaved and autoclaved poultry droppings showed pH value of 5.8 and 6.7 thus poultry droppings are acidic in nature however poultry dropping based biodigested slurry were alkaline in nature showing a pH value of 7.93 and 8.26 under unautoclaved and autoclaved conditions respectively. Protein content in poultry droppings was high as compared to biodigested slurry. Under autoclaved conditions protein content in poultry droppings and biodigested slurry was 1.41mg/ml and 1.20 mg/ml respectively and under unautoclaved conditions protein content in poultry droppings and biodigested slurry was 1.31 mg/ml and 1.09 mg/ml respectively. This might be due to the fact that slurry was completely digested after biogas production and proteins could break down in simpler form after digestion.

Table 1: Proximate and chemical analysis of poultry droppings and poultry dropping based biodigested slurry.

Parameters	Poultry droppings		Poultry dropping based biodigested slurry	
	Unautoclaved	Autoclaved	Unautoclaved	Autoclaved
pH	5.8 ± 0.05	6.7 ± 0.08	7.93 ± 0.1	8.26 ± 0.21
Total solids (TS% dry weight basis)	41 ± 0.015	38.53 ± 0.23	22.53 ± 0.03	18.21 ± 0.01
Volatile solids (VS % dry weight basis)	66.16 ± 0.31	62.86 ± 0.93	22.54 ± 0.02	50.44 ± 0.01
Total organic carbon (TOC% dry weight basis)	36.75 ± 1.37	34.92 ± 0.31	12.49 ± 0.04	28.04 ± 0.86
Ash(% dry weight basis)	36.63 ± 0.21	37.96 ± 0.03	77.7 ± 0.19	49.54 ± 0.02
Cellulose (%)	15.94 ± 0.42	9.58 ± 0.36	8.47 ± 0.03	5.96 ± 0.05
Hemicellulose (%)	23.62 ± 0.25	18.66 ± 0.15	11.29 ± 0.02	13.65 ± 0.14
Lignin (%)	41.87 ± 0.0.5	27.98 ± 1.28	22.61 ± 0.17	18.33 ± 0.24
Silica (%)	3.14 ± 0.16	1.86 ± 0.06	2.24 ± 0.03	2.81 ± 0.06
Nitrogen (%)	15.77 ± 0.11	14.69 ± 0.33	13.53 ± 0.03	15.39 ± 0.18
Protein (mg/ml)	1.31 ± 0.02	1.41 ± 0.37	1.09± 0.02	1.20 ± 0.02

*Values in ±indicates the standard error of data in triplicate

Cellulose content in autoclaved poultry droppings and biodigested slurry was 9.58% and 5.96% respectively however cellulose content in unautoclaved poultry droppings and biodigested slurry was 15.94% and 8.47% respectively. This suggested that poultry droppings and biodigested slurry were sufficient source of cellulosic biomass hence can be used as efficient substrates for cellulolytic enzyme production using microorganisms

Oyewole (2010) [10] reported 23.6% total solids, 55.3% volatile solids, 44.8% ash% in biodigested slurry while 86.5% total solids, 64.3%, volatile solids, 35.7% ash% in poultry droppings was reported. pH of biodigested slurry was 6.9 and pH of poultry droppings was 6.7. Shahariar *et al.* (2013) [11] reported that the pH of poultry droppings was 7.61 and that pH of biodigested slurry was 7.69 Owen *et al.* (2008) [12] reported 20% crude protein was present in poultry droppings. Adegbola *et al.* (1990) [13] and Lamidi (1995) [14] investigated protein content in poultry droppings was 16.5% and 25 % respectively.

3.2 Cellulolytic activity of poultry droppings based biodigested slurry.

Cellulolytic enzyme activities and protein content was determined in unautoclaved and autoclaved poultry dropping based biodigested slurry. Results from table 2 shows a comparison between enzymatic activities and protein content of unautoclaved and autoclaved poultry dropping based biodigested slurry. Unautoclaved biodigested slurry showed relatively more enzyme units as compared to autoclaved biodigested slurry. Among all, endoglucanase activity was highest in unautoclaved and autoclaved biodigested slurry i.e. 60.05 U/ml and 43.81 U/ml of BDS respectively. Exoglucanase activities were found to be 21.15 U/ml and 9.06 U/ml of BDS in unautoclaved and autoclaved respectively. Among all the three enzymes, activities of β glucosidase were found to be 4.81 U/ml and 2.13 U/ml of BDS in unautoclaved and autoclaved slurry respectively. Protein content is also high in unautoclaved (4.53 mg/ml) than autoclaved (2.13 mg/ml) biodigested slurry. As biodigested slurry is rich in organic matter along with consortium of microorganisms of diverse groups, thus contributing towards higher activities of endoglucanase, exoglucanase and β glucosidase. However after autoclaving, reduction in enzyme activities was reported which might be due to the fact that high temperature (121°C) would degrade and denature some of the proteins and thus affecting catalytic activities of enzyme. These results also suggest that use of unautoclaved slurry for enzyme production will save the cost of autoclaving which is energy consuming process.

Table 2: Cellulolytic activity and protein content of poultry dropping based biodigested slurry

Parameters	Biodigested slurry	
	Autoclaved	Unautoclaved
Endoglucanase (U/ml of BDS)	43.81±0.14	60.05 ± 0.10
Exoglucanase (U/ml of BDS)	9.06 ± 0.12	21.15 ± 0.09
β glucosidase(U/ml of BDS)	2.13 ± 0.07	4.81 ± 0.05
Protein Content (mg/ml of BDS)	2.13 ± 0.07	4.53 ± 0.014

*****Cultural conditions: Incubation temperature – 30± 2° C; Slurry concentration – 100 ml; pH – 8.26 (unautoclaved), 7.93 (autoclaved); Autoclaving temperature - 121°C; pressure 15psi; The data represents the mean of three determinations each; ± values indicate standard error, BDS: Biodigested Slurry

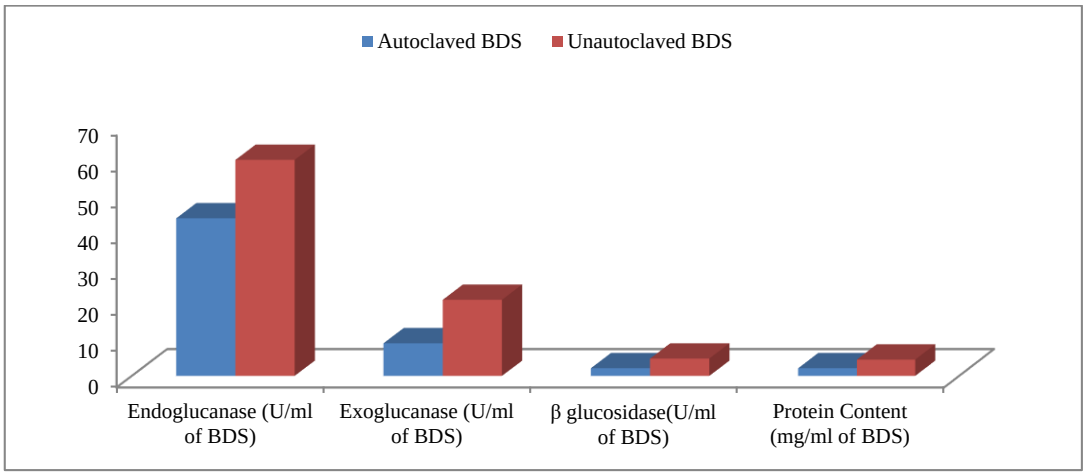

Fig 1: Cellulolytic activity and protein content of poultry dropping based biodigested slurry

3.3 Cellulolytic activity of poultry droppings based biodigested slurry inoculated with *P. roqueforti* NCIM 712

The autoclaved and unautoclaved poultry dropping biodigested slurry was inoculated with *P. roqueforti* NCIM 712 using spore concentration of 10^6 spores/ml and was incubated for 7 days. After incubation cellulase activities were observed. Maximum yield of endoglucanase was observed i.e. 145.76 U/ml of BDS in unautoclaved biodigested slurry and 62.91U/ml of BDS in autoclaved biodigested slurry. The activities for exoglucanse were 46.46 U/ml and 41.31U/ml of BDS in unautoclaved and autoclaved biodigested slurry. The activity of β glucosidase was low in both conditions i.e 11.61 U/ml of BDS in unautoclaved and 9.98 U/ml of BDS in autoclaved biodigested slurry.

Pericin (2008) [15] evaluated the use of pumpkin oil cake as a as substrate for the cellulase production by *P. roqueforti* in solid state fermentation. Pumpkin oil cake and mixed substrate of pumpkin oil cake and wheat bran were used as sole nutrient source and thus produced sufficient quantities of enzyme, which were 48.49 and 37.07 Units per gram dried solid respectively.

Table 3: Cellulolytic activity and protein content of poultry dropping based biodigested slurry inoculated with *P. roqueforti* NCIM 712.

Parameters	Biodigested slurry	
	Autoclaved	**Unautoclaved**
Endoglucanase (U/ml of BDS)	62.91 ± 0.33	145.76 ± 0.19
Exoglucanase (U/ml of BDS)	41.31 ± 0.23	46.46 ± 0.16
β glucosidase(U/ml of BDS)	9.98 ± 0.34	11.61 ± 0..19
Protein Content (mg/ml of BDS)	5.46 ± 0.99	4.80 ± 0.98

*****Cultural conditions: Incubation temperature – 30± 2° C; Slurry concentration – 100 ml; Spore concentration - 10^6 spores/ml; pH – 8.26 (unautoclaved), 7.93 (autoclaved); Autoclaving temperature - 121°C; pressure 15psi; The data represents the mean of three determinations each; ± values indicate standard error, BDS: Biodigested Slurry

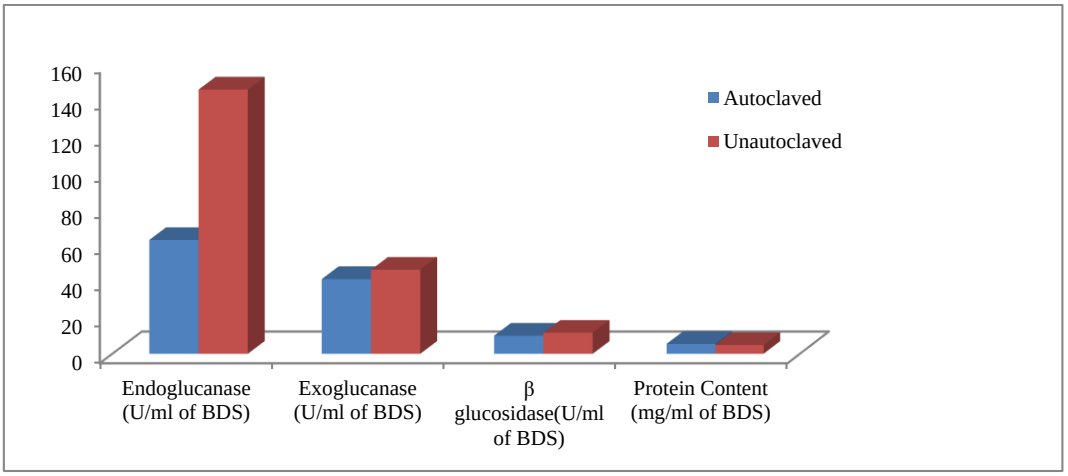

Fig 2: Cellulolytic activity and protein content of poultry dropping based biodigested slurry inoculated with *P. roqueforti* NCIM 712.

Conclusion

Chemical and proximate analysis of autoclaved and unautoclaved poulry droppings and poultry dropping based biodigested slurry showed the presence of organic matter rich in volatile solids, cellulose, hemicelluloses, lignin and silica, protein, nitrogen content and cellulolytic enzymes. Thus this highly nutritive substrate can be used for cellulolytic enzyme production using fungal cultures and hence, commercial market can be benefitted by further scaling up of process technology using it as a low cost, economical substrate for commercial cellulase production.

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