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Research Article
Production Optimization of Fungal Cellulase and its Impact on Ruminal Degradability and Fermentation of Diet

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Abstract

Background and Objective: Cellulases enzymes are widely be interested due to its capability to degrade lignocellulosic materials. The current study was concerning on investigating different factors of cellulase production from fungal sources by using agricultural wastes and studying its impacts on ruminal digestion and fermentation. **Materials and Methods:** The study tested the cellulase enzyme production ability of fungal strains against different lignocellulosic. Simultaneously the effect of different fungal strains to choose according to the superiority of cellulase production, then environmental factors were studied such as carbon source concentrations, inoculum size, incubation period, initial pH and nitrogen source. Finally, produced cellulase was evaluated using *in vitro* batch culture technique. **Results:** *Penicillium chrysogenum* recorded the highest value for cellulase activity. Pea pods showed best carbon source with 17.5% concentration for cellulase production. About 4% inoculum size, 2 days of incubation and pH5 were recorded the highest value for cellulase production. *In vitro* fermentation results showed improvement of DM digestibility compared with control. **Conclusion:** The current findings showed potential possibilities to utilize agricultural wastes as a substrate for producing cellulase enzyme from *Penicillium chrysogenum* fugal strain which could be an effective additive to improve ruminant diet digestion and utilization.

Key words: Cellulases enzymes, agricultural by-products, rumen fermentation, gas production, digestibility, ruminal digestion

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cellulase is a family of enzymes capable of hydrolyzing β-1,4-glycosidic bonds of intact cellulose and other related cello-oligosaccharide derivatives. Complete cellulolytic enzymes are synergistically worked by action of 3 principal types of the enzymes, endoglucanase, exoglucanase and β-glucosidase which required to execute degradation of intact hydrogen bond ordered cellulose[1]. Many research being interested with utilizing agricultural as substrate for cellulases production[2-4].

Fungal cellulase is the main source of commercially produced enzymes for diet additives while, few bacteria and actinomycetes have also been investigated for the production of cellulolytic enzymes[5-9]. Mostly the cellulase enzymes production was reported from bacteria and fungi[1,2]. However, microbial cellulases varied according to the microbial strain, cultivation conditions (such as pH, incubation period and inoculums size) in addition to growth medium composition particularly carbon and nitrogen sources[3,4].

Agriculture sector produces a large amount of by-products and wastes that can be used for cellulase production including rice straw, wheat straw, bean straw, pea pods, corn stover and palm fronds[2,10]. Shahriarinour *et al.*[11] mentioned the great interest in utilizing cellulose wastes as feedstock through processes of fermentation by converting low cost starting materials into products of great value. There are few studies on cellulase production from raw biomass such as rice straw[4].

Improving feed utilization and nutritive values of feed stuffs through supplementing diets with cellulolytic enzymes were highly get interested during last dedicates. Many studies reported the role of fibrolytic enzymes in enhancing feed digestion in ruminants[12,13]. Moreover, cellulase enzyme can be an effective additive with agricultural by-product to produce simple glucose units which play a direct role in animals feeding by improved digestion in ruminants[14].

Therefore, using high yielding strains, optimal fermentation conditions and cheap production substrate is the way for maximum and economical cellulase production, especially with the increasing interest with utilizing cellulase in ruminant's diets.

So, the current study was carried out to investigate utilization of some agricultural by-products as substrate to produce fungal cellulase under the optimum fermentation conditions and the impacts of cellulase on ruminal *in vitro* degradability and fermentation.

MATERIALS AND METHODS

This study was carried out at laboratory of Dairy Science Department, National Research Centre, Giza, Egypt.

Waste materials used as substrates: Pea pods, rice straw, wheat straw, corn stover, bean straw and palm fronds were collected after harvesting from experimental station of National Research Centre. This material was air-dried then cut and dried at 70°C for 24 h in air-circulation oven and grounded at 5-10 mm electric grinder then stored in dry place at room temperature till use.

Fungal cultures, media and inoculum preparation: Seven fungal cultures were used for investigating its ability to produce cellulase enzymes; *Penicillium chrysogenum*, *Aspergillus niger*, *Fusarium oxysporum*, *Fusarium avenaceum*, *Aspergillus fumigatus*, *Trichoderma viride* and *Cephalosporium acremonium* were obtained from Laboratory of Plant Pathology, National Research Centre, Cairo, Egypt and *Aspergillus flavus* NRRL 5521 (non-aflatoxin producer strain) which obtained from National Center for Agriculture Utilization Research, Microbial Genomics and Bioprocessing Research Unit, Department of Agriculture, Peoria, Illinois, USA.

Culture conditions for cellulase production: Static cultures were used for studying fungal cellulase production under variable condition including fungal cultures effect (*Penicillium chrysogenum*, *Aspergillus niger*, *Fusarium oxysporum*, *Fusarium avenaceum*, *Aspergillus fumigatus*, *Cephalosporium acremonium*, *Trichoderma viride* and *Aspergillus flavus* NRRL 5521). The effect of fungal cultures was studied through inoculation of 3 flasks each with one of the 8 mentioned fungal cultures incubated for 3 days at 29+1°C and the levels of cellulase activities were determined in the cultures filtrate[2].

Effect of substrate carbon source was investigated through replacing the cellulose powder in CPM by different cellulolytic waste materials including pea pods, rice straw, wheat straw corn stover, bean straw and palm fronds.

Effect of carbon source concentrations (2.5, 5, 7.5, 10, 12.5, 15, 17.5 and 20%), inoculum size (1, 2, 3, 4, 5, 6, 7 and 8% v/v), incubation period (1, 2, 3, 4, 5, 6 and 7 days), initial pH (3, 4, 5, 6, 7 and 8) and nitrogen source (ammonium sulphate, ammonium chloride, sodium nitrate, meat extract, yeast

extract and peptone) were investigated at an equivalent concentration of 0.33 g N/l media, these nitrogen sources replaced the original nitrogen present in the CPM[2]. The level of a parameter optimized in an experiment was maintained in the subsequent studies.

Cellulase enzyme extraction and activity assay: The fermented substrate for each flask was mixed with 25 mL of 0.02 M acetate buffer (pH 5.0) to extract the enzyme. The carboxymethyl-cellulase activity (CMC) for resultant enzyme was determined as described by Khattab *et al*.[1].

Enzymes source for *in vitro* batch culture experiment: Pan-Zyme: a commercial enzyme source produced by Baytara for pharmaceuticals technology, Sadat Industrial City under license of VTR Guangdong VTR Bio-Tech Co., Ltd-China each (g) of it contains 600 cellulase units.

Ruminal *in vitro* batch culture experiment procedures: *In vitro* incubation procedures were carried as described by Khattab *et al*.[15], rumen fluid was collected from 3 ruminal cannulated Holstein dairy cows (mean weight 680±30 kg). The rumen fluid was collected before morning feeding, mixed and squeezed through a 4-layers cheese cloth under continuous flushing with CO_2 and immediately transported to laboratory at 39°C (used as a source of inoculum). Treatments were: 50% concentrates feed mixture, 25% Egyptian clover and 25% wheat straw. The experimental ration was supplemented separately with locally produced cellulase (Pan-Zyme) and commercial cellulase source Pan-Zyme at 4 levels (21.08, 42.16, 63.24 and 84.32 IU kg^{-1} DM) compared with the control. Each treatment was tested in 8 replicates accompanied by blank bottles (no substrate). The experiment run was replicated in different weeks. Substrate (400 mg) was added to the incubation bottles of 100 mL capacity. Then the bottles were incubated at 39°C for 48 h. After 48 h of incubation, gas production (GP) was recorded using the pressure reading technique then bottles were uncapped, pH was measured using a pH meter and the contents of each bottle were filtered to obtain the non-fermented residue for determination of degraded substrate.

Statistical analysis: Data obtained from this study were statistically analyzed by SPSS software, the Duncan's multiple range test was used to test the significance among means.

RESULTS

Effect of Fungal culture on cellulase production: Different fungal strains were investigated for production of cellulase enzyme (Fig. 1). Results shows significant superiority ($p<0.05$) of *Penicillium chrysogenum* for cellulase activity by recording 0.84 μmole mL^{-1} min^{-1}, followed by *Fusarium oxysporum*, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Fusarium avenaceum* and *Trichoderma viride* (0.69, 0.64, 0.60, 0.55, 0.41 and 0.33 μmole mL^{-1} min^{-1}, respectively). On the light of these results, *Penicillium chrysogenum* was chosen for further studies for cellulase production on CPM.

Effect of carbon source on cellulase production: Different agricultural by-products were tested as a carbon source in growth culture for producing cellulase enzyme from *Penicillium chrysogenum* (Fig. 2). Pea pods media recorded the highest value of cellulase activity ($p<0.05$) as 0.98 μmole mL^{-1} min^{-1}. Corn stover, bean straw, palm fronds and wheat straw cultures lowered cellulase activity (0.85, 0.62, 0.46 and 0.43 μmole mL^{-1} min^{-1}, respectively) as compared with pea pods media. From the current results, pea pods were chosen as carbon source for carrying the further experiments of cellulase production by *Penicillium chrysogenum*.

Effect of carbon source concentration on cellulase production: Gradual concentrations of pea pods as carbon source in culture of cellulase production from *Penicillium chrysogenum* were used (Fig. 3).

Data illustrated in Fig. 3 showed effect of different concentration of pea pods powder ranged from 2.5-20% (w/v) on cellulase production by *Penicillium chrysogenum*. Maximum cellulase activity reached (5.68 μmole mL^{-1} min^{-1}) was obtained at 17.5% (w/v) pea pods concentration ($p<0.05$), while the minimum activity (1.8 μmole mL^{-1} min^{-1}) was obtained at 2.5% of pea pods concentration of modified cellulose powder medium (CPM). Higher moisture levels as in case of 2.5% pea pods concentration of modified CPM may negatively affect oxygen transfer which in turn may reduce the level of fungal growth. Based on these data, pea pods concentration at 17.5% (w/v) was chosen for further studies on the modified CPM.

Effect of nitrogen source on cellulase production: Data of Fig. 4 showed that among 5 nitrogen sources tested for screening their effect on cellulase activity. Ammonium

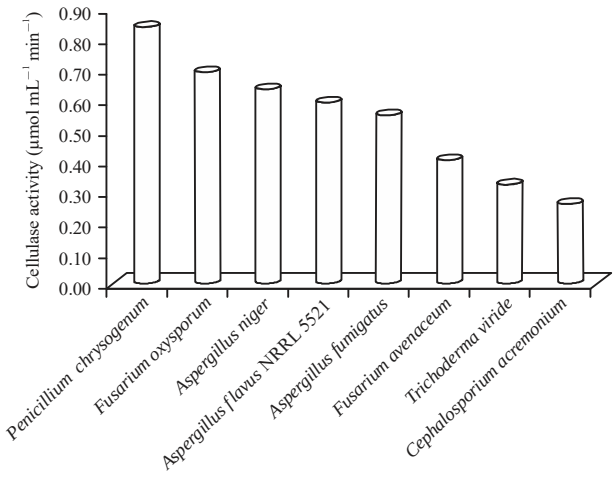

Fig. 1: Effect of fungal culture on cellulase production

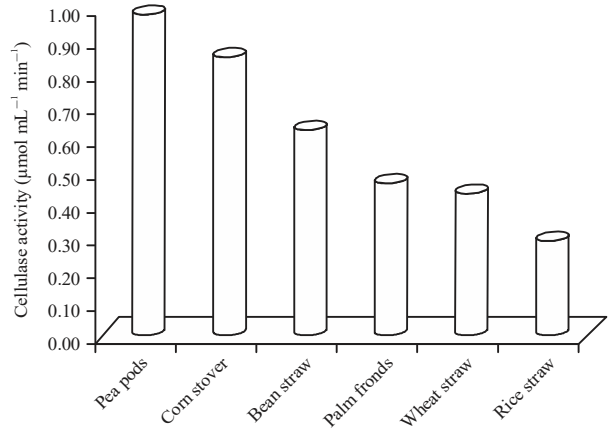

Fig. 2: Effect of carbon source on cellulase production

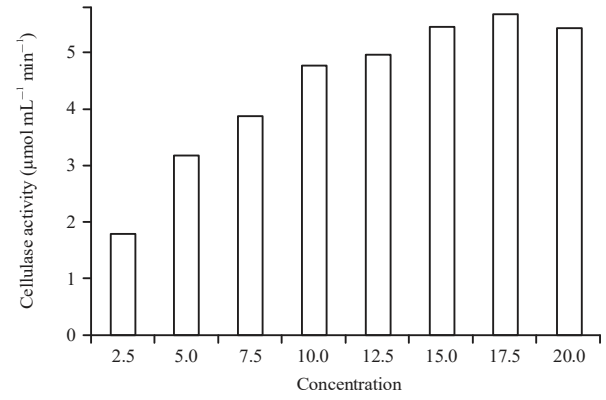

Fig. 3: Effect of carbon source concentration on cellulase production

chloride was reported as optimum nitrogen source for producing the highest level ($p<0.05$) of cellulase activity reached 5.40 µmole mL^{-1} min^{-1} by *Penicillium chrysogenum*.

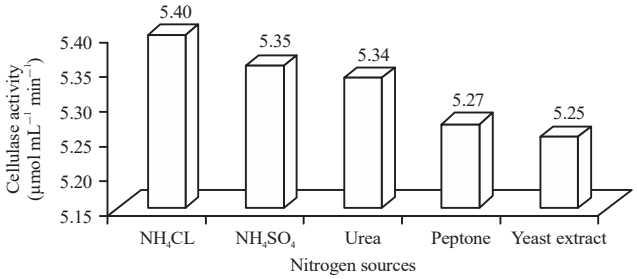

Fig. 4: Effect of nitrogen source on cellulase production

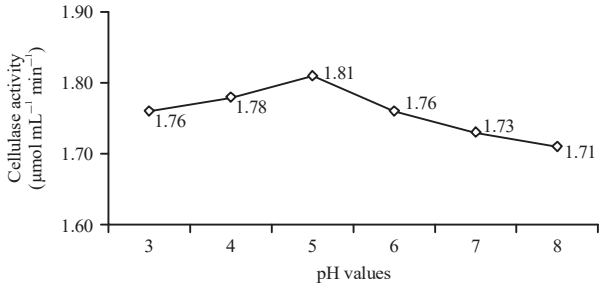

Fig. 5: Effect of culture pH on cellulase production

These data indicating that the source of nitrogen should be inorganic for better cellulase production.

Effect of culture pH on cellulase production: As shown in Fig. 5 primary pH of the fungal growth medium has clear effect on cellulase production. Cellulase enzyme production by *Penicillium chrysogenum* in different pH of modified CPM produced the highest value of cellulase activity ($p<0.05$) at pH 5.0 (1.81 µmole mL^{-1} min^{-1}), further more increasing pH level, the decrease of enzyme production was noticed. Based on current results, pH 5 was adjusted in the subsequent experiments on the modified CPM.

Effect of culture inoculation size on cellulase production: Penicillium chrysogenum has exhibited different responses to variations in inoculum size from 1-8% (v/v). The results of (Fig. 6) showed that maximum cellulase activity ($p<0.05$) was detected at 4% inoculum size (6.87 µmole mL^{-1} min^{-1}) and further increase in the inoculum size led to decrease in cellulase activity. In the light of these results, 4% inoculum size was selected for conducting further studies on modified CPM.

Effect of culture incubation period on cellulase production: Production of cellulase on modified CPM was monitored for a period of seven days (Fig. 7). The highest

Table 1: Effect of treatments on *in vitro* ruminal fermentation and digestibility

Items	Control	Cell-pro-1	Cell-pro-2	Cell-pro-3	Cell-pro-4	Pan-Zyme-1	Pan-Zyme-2	Pan-Zyme-3	Pan-Zyme-4
pH	6.40	6.36	6.48	6.42	6.51	6.35	6.42	6.49	6.52
NH3	43.90^{a}	31.00^{b}	25.20^{c}	28.60^{bc}	33.10^{b}	35.90^{b}	32.40^{b}	37.70^{b}	36.10^{b}
TVFA	5.50	5.25	5.45	5.65	5.25	5.05	5.50	5.60	5.30
Nutrients digestibilities									
DM	57.50^{c}	60.10^{b}	64.07^{ab}	63.18^{ab}	62.24^{ab}	61.51^{ab}	65.09^{a}	63.68^{ab}	63.23^{ab}
NDF	38.17	38.85	46.43	38.88	39.43	42.08	45.58	41.81	37.40
ADF	35.11	35.14	38.24	34.88	38.74	37.73	45.80	37.61	34.40
cellulose	32.92	38.58	40.34	37.83	46.96	41.68	50.04	41.58	37.05
Hemicellulose	42.34	43.91	57.58	44.31	40.36	48.01	45.28	47.53	41.48
Gas production									
Total GP	155	155	156	159	156	151	160	152	147
GP/ gm DM	416	410	417	418	413	407	423	408	402
GP/ gm NDF	932	918	934	935	924	911	948	913	901
GP/ gm ADF	1617	1593	1621	1622	1603	1581	1644	1584	1563

Different superscripts in the same row are significantly different at (p<0.05), cell-pro-1, 2, 3 and 4: Experimental ration supplemented with locally produced cellulase (Pan-Zyme) at 4 levels (21.08, 42.16, 63.24 and 84.32 IU kg^{-1} DM), Pan-Zyme-1, 2, 3 and 4: Experimental ration supplemented with locally produced cellulase (Pan-Zyme) at 4 levels (21.08, 42.16, 63.24 and 84.32 IU kg^{-1} DM)

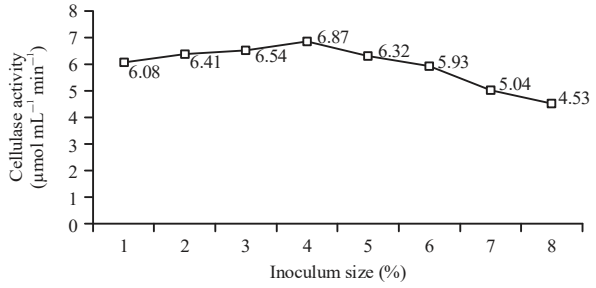

Fig. 6: Effect of culture inoculation size on cellulase production

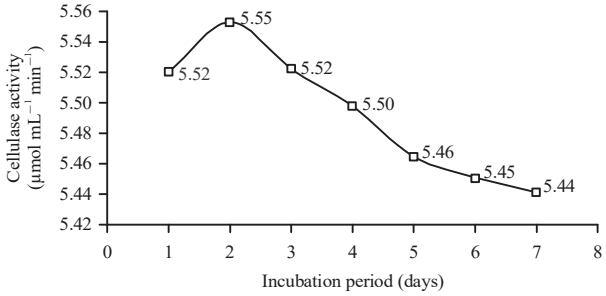

Fig. 7: Effect of culture incubation period on cellulase production

cellulase activity (5.55 µmole mL^{-1} min^{-1}) (p<0.05) was recorded after two days of incubation with *Penicillium chrysogenum*. The reduction of cellulase activity returns to the consumption of cellulolytic substrate during the 1st days[2]. From the previous data, 48 h incubation period was selected for conducting further studies on modified CPM.

Effect of different cellulase supplementing levels on ruminal *in vitro* parameters: Results of *in vitro* evaluation of different supplementing level of both commercial and produced cellulase enzyme on some ruminal parameters were introduced in Table 1.

Ruminal fermentation parameters showed no significant differences in pH values and TVFA concentrations among treatments (p>0.05). While, ammonia concentrations showed a reduction in treatments compared with control (p>0.05).

Digestibility parameters showed that adding cellulase enzymes significantly (p<0.05) improved DM, while there was a slight improvement (p>0.05) but not significant in NDF, ADF and cellulose digestibilities in treatments compared with control.

No significant differences were recorded between all treatments for gas production.

DISCUSSION

The most benefits of cellulolytic microorganisms mostly degrade carbohydrates to simple units such as glucose and generally not depended on lipids and proteins as energy source for metabolism and growth[16]. Among them, in ruminant nutrition and feeding the most important microorganisms are anaerobic cellulolytic ruminal bacteria[17,18]. While, anaerobic microbial species have specific cellulolytic activity restricted to cellulose and its hydrolytic products[19,20]. Cellulolytic fungal strains are widely studied and have the ability to convert desired as well as native cellulose to glucose. Different fungal species like *Trichoderma*, *Penicillium* and *Aspergillus* are widely studied with notable high cellulolytic activity. Also, some other bacterial species include; *Pseudomonas*, *Bacilli*, *Actinomycetes*, *streptomycetes*, *Cellulomonas*, *Streptomyces* and *Actinomucor* were investigated[21,22]. Certain species of fungi were practically

used for cellulose hydrolysis especially species of *Aspergillus*, *Penicillium* and *Trichoderma* which appeared practical implementation to produce high yields of cellulases[23]. *Penicillium chrysogenum* was the best organism for cellulase production using pure cellulose as carbon source. The findings of the present study were coincided with other studies which concluded that *P. chrysogenum* superiority than other fungal strain for cellulase production[23], in contrast, these finding do not match with those obtained by other studies who found that *A. niger* recorded the highest cellulase production value then followed by *Penicillium chrysogenum*[3,24]. *Penicillium chrysogenum* showed a good command for cellulase production by cultivating on pea pods than other carbon sources, which might due to the superiority of pea pods in chemical composition compared with other investigated agricultural by-products especially in crude protein and less contents of lignin[25].

In the concern of inoculum size, it was well stated that impact of inoculant amount on cellulase production was noted as lower inoculum size makes substrate utilization and cellulase formulation takes a long time, while the highest one can ensure rapid fungal biomass synthesis to a certain limit, after it the enzyme production could decrease[26]. The current results showed a reduction in cellulase production with an increase of inoculum size might be due to ensure rapid fungal biomass synthesis to a certain limit, after it the enzyme production could decrease, also, because of nutrients depletion due to biomass enhancement[27].

Many of *in vitro* studies reported the positive effects of fibrolytic enzymes supplementation on the nutrients digestibility[2,3,10,28]. The current results of DM degradability in agreement with previous studies investigated supplementing diets with cellulase[2,3,10,28]. The improving effect of supplementing diet with cellulase are interaction period give the chance for creating a stable enzyme-feed complex which protects the supplemented enzymes from proteolysis by the rumen microorganisms[29]. Increasing ability of the ruminal microorganisms to attachment and/or access to feed particles and subsequently accelerate the rate of digestion[30] and enhancing the hydrolytic activity of the ruminal microbes due to added enzyme activities and/or synergy with rumen microbial enzymes[31,32].

It is well known that there is a negative correlation between gas production and cell wall contents (NDF and ADF) which tend to reduce the microbial activity[33]. Different studies stated that addition of fibrolytic enzymes reduced methane production[34]. Addition of fibrolytic enzymes stimulated the reductive acetogens in the rumen that alters hydrogen (H_2) metabolism and its utilization by methanogens in a manner that reduces CH_4 formation and emissions[35].

Overall, supplementing ruminant diets with cellulase is recommended for improving the utilization of diets and enhancing animal performance especially regarded to the gas emission from ruminant.

CONCLUSION

Results showed that *Penicillium chrysogenum* has a potential cellulase production characterizes to utilize agricultural wastes as a substrate for producing cellulase enzyme which could be an effective additive to improve ruminant diet digestion and utilization.

SIGNIFICANCE STATEMENT

This study was carried out to utilize of some agricultural by-products as substrate for producing cellulase and determining the optimum production conditions, then evaluate the effects of produced cellulase enzyme on ruminal digestion and fermentation. This study founded that pea pods can be beneficial substrate for producing cellulase enzyme from *Penicillium chrysogenum.* Also, the produced cellulase enzyme showed a potential improvement in ruminal digestion and fermentation, especially for DM degradability.

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