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Full Length Research Paper

Efficient production of cellulase and xylanase by anaerobic rumen microbial flora grown on wheat straw

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Rumen bacteria, fungi and protozoa possess a wide range of enzymes which are capable of hydrolyzing most of the structural polysaccharides of the plant cell walls. In the study, the activities of cellulolytic and xylanolytic enzymes produced by anaerobic rumen flora were investigated. Mixed ruminal microorganisms were grown on wheat straw in anaerobic liquid medium that have been purged with oxygen-free CO_2 and allowed to ferment for 0, 24, 48, 72 and 120 h at 39°C. Then the supernatants of each culture were analyzed for cellulase and xylanase activities with dinitrosalicylic acid (DNS) method. Results showed that optimum time for maximum activity of both enzymes is 48 h incubation at 39°C and at each time pointed, xylanase activity was much higher than cellulase activity.

Key words: Rumen microbial flora, cellulase, xylanase.

INTRODUCTION

Plant fibers become available as a source of energy to the host animal through the activities of rumen microflora (Sait Ekinci et al., 2001). Rumen possesses major cellulolytic bacterial populations, highly fibrolytic anaerobic fungi and protozoa (Chen et al., 2008). Research has indicated an important role for anaerobic fungi in the degradation and subsequent fermentation of particulated substrates in the rumen (Lowe et al., 1987). The extracellular cellulases of rumen fungi have also been characterized and carboxymethyl cellulase (CMCase) activities have been demonstrated in supernatants from cultures grown on cellulose (Lowe et al., 1987; Mountfort and Asher, 1985). Furthermore, anaerobic fungi produce a large amount of xylanase constitutively together with cellulolytic enzymes even when cellulose is used as the carbon source (Srinivasan et al., 2001; Teunissen et al., 1991). Therefore the simultaneous production of cellulolytic and xylanolytic enzymes can be achieved by using cellulose xylanolytic enzymes (Srinivasan et al., 2001).

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Recently, the rumen cellulolytic bacteria have been studied extensively and have been shown to be the primary degraders of fiber (Chen et al., 2008). The major cellulose-degrading rumen bacteria are *Fibrobacter succinogenes, Ruminococcus flavefaciens* and *Ruminococcus albus* (Ekinci et al., 2001). As xylanase and cellulase have many applications in different industries, the objective of this study was to investigate the efficient production of both enzymes by using an inexpensive substrate (wheat straw and rumen fluid).

MATERIALS AND METHODS

Culture media

The culture was grown under strict anaerobiosis according to Hungate as modified by Balch and Wolfe (Mountfort and Asher, 1989).

The medium contained (per liter)

150 ml of salt solution 1 which contain 3.0 g dipotassium hydrogen phosphate (K_2HPO_4) in one liter of distilled water; 150 m l of salt

solution 2 which have KH_2PO_4, 3.0 g; $(NH_4)_2SO_4$, 6.0 g; NaCl, 6.0 g; $MgSO_4.7H_2O$, 0.6 g and $CaCl_2.2H_2O$, 0.6 g/l of distilled water; clarified rumen fluid (CRF), 150 ml; yeast extract, 2.5 g; tripticase pepton, 10.0 g; $NaHCO_3$, 6.0 g and L-cysteine hydrochloride, 1.0 g.

Clarified rumen fluid is added as a source of many growth factors including some that are not found in the usual bacteriological media. In order to prepare clarified rumen fluid, samples of rumen fluid were centrifuged (model 3 – 18 k, Sigma Co., Germany) at 10000 rpm for 5 min then collecting supernatants.

All above ingredients except for L-cysteine hydrochloride were mixed together and agitated for 2 h under a vigorous stream of O_2 free CO_2. Before dispensing in serum bottles, L-cysteine hydrochloride was added to culture media. In each serum bottle, 1% (W/V) wheat straw and 40 ml culture media were added and after CO_2 injection for removing headspace air, they were sealed with black rubber septum stoppers and aluminum serum-cap closures and then autoclaved at 121°C for 20 min.

Growth condition

Rumen fluid (containing different kinds of microorganism) was sampled from the rumen of cows fed with a diet of hay and one milliliter of it was inoculated in each serum bottle under sterile condition then incubated at 39°C for 24 h. After incubation time, 1 ml of culture fluid of each bottle was sampled by syringe and injected to new serum bottles. Then cultures had incubation time of 24 h. Experimental cultures (new serum bottles) were inoculated by transferring 0.2 ml of each 24 h old cultures grown on wheat straw. After separating the blank samples (time 0), other experimental cultures were incubated at 39°C for 24, 48, 72 and 120 h.

Enzyme assay

The supernatants from cultures grown on wheat straw in each serum bottle were separated by centrifugation at 10000 rpm for 10 min and were used for xylanase and cellulase activities. Culture supernatant was analyzed for reducing sugar with dinitrosalicylic acid (DNS) (Miller, 1959).

Xylanase activity was measured by incubating 0.1 ml of supernatant with 0.5 ml xylan solution (0.5 g xylan was dissolved in 10 ml of 1.0 N sodium hydroxide and pH was adjusted to between 5 - 5.2 by HCl. Then by addition of 0.1 M sodium acetate buffer, the volume of solution was reached to 25 ml) in 0.4 ml of 0.1 M sodium acetate buffer (pH 4.8) at 50°C for 30 min in a water bath. The reaction was terminated by placing tubes in a boiling water bath for 10 min. The supernatant was analyzed for reducing sugar with DNS reagent and absorbance was read at 550 nm by Uv-Visible spectrophotometer (T80, PG Instruments Limited, England). A standard curve of D-xylose was used as reference. Each unit of xylanase activity (U) is that amount of activity which released 1 µmol D-xylose min-1 (Mountfort and Asher, 1989; Rezaeian et al., 2005).

Cellulose activity was determined by measuring glucose released from CMC. The reaction mixture consisted of 0.5 ml CMC solution (0.75 g CMC in 50 ml distilled water) in 0.4 ml citrate-phosphate buffer (pH 6.5). Culture supernatant (0.1 ml) was added to substrate and the reaction mixture was incubated at 50°C for 30 min. The reaction was terminated by placing tubes in a boiling water bath for 10 min. Glucose released was determined by DNS reagent and absorbance was read at 550 nm by Uv-Visible spectrophotometer (T80, PG Instruments Limited, England). A standard curve of D-glucose was used as reference. Cellulose activity was expressed in terms of micromoles of D-glucose equivalents released per min per ml of the culture (Miller et al., 1960; Rezaeian et al., 2005).

Statistical analysis

An analysis of variance (ANOVA) for the obtained results was investigated by SAS. Three replicates were conducted with five tubes at each time period.

RESULTS AND DISCUSSION

Enzyme activities

The activity of extracellular enzymes that were produced by incubation of cultures of rumen anaerobic flora on wheat straw under strict anaerobic condition was determined. Most studies on the location of fiber-degrading enzymes produced by rumen fungi indicate that they are extracellular, being free in the culture fluid (Akin and Borneman, 1990; Mountfort and Asher, 1989).

Extracellular activity on CMC by accumulation of reducing sugars and increasing the absorbance at 550 nm, are shown in Figure 1. As shown in Figure 1 cellulase activity was measured after 0, 24, 48, 72 and 120 h incubation. The mean values of cellulase activity at these time courses were 0.017, 0.040, 0.070, 0.047 and 0.060 IU ml^{-1}, respectively. According to Tukey's (HSD) mean analysis (alpha 0.05), incubation of culture media until 48 h had significant effect on increasing cellulase activity (0.070 IU ml^{-1}) compared to blank sample (time 0) and 24 h incubation and the maximum activity was determined at this time course.

The same result was seen for xylanase activity. In Figure 2, the effect of incubation time (0, 24, 48, 72 and 120 h) on xylanase activity is shown. As it is obvious, at 48 h after incubation, the xylanase activity was significantly ($p < 0.05$) increased (1.974 IU ml^{-1}) compared with that of the control treatment (0.127 IU ml^{-1}). At other incubation time (24, 72 and 120 h) on wheat straw, the mean values of xylanase activity were 1.043, 1.442 and 1.266 IU ml^{-1}), respectively.

In the study, production of both enzymes (cellulase and xylanase) was detected during the growth of mixed ruminal microorganisms in culture medium on wheat straw. Xylanase activity was generally higher than the cellulase activity. Cellulase and xylanase activity increased sharply within the first two days of inoculation (Figures 1 and 2). Cellulase activity to the end of the experimental period increasing from 0.017 IU ml^{-1} (time 0) to 0.060 IU ml^{-1} of culture supernatant at 120 h incubation. No significant change in the activity of this enzyme was observed in cultures after 120 h with 48 h incubation. However, the xylanase activity of the culture supernatant was significantly ($p < 0.05$) higher than that of the control treatment (time 0) beyond 48 h incubation and the difference between the level of activity of this enzyme between the 48 and 120 h was significant ($p < 0.05$).

One of the mechanisms of the improved fiber break down in the rumen is considered to be the high fibrolytic enzyme activity (Lee et al., 2004). Bauchop (1979)

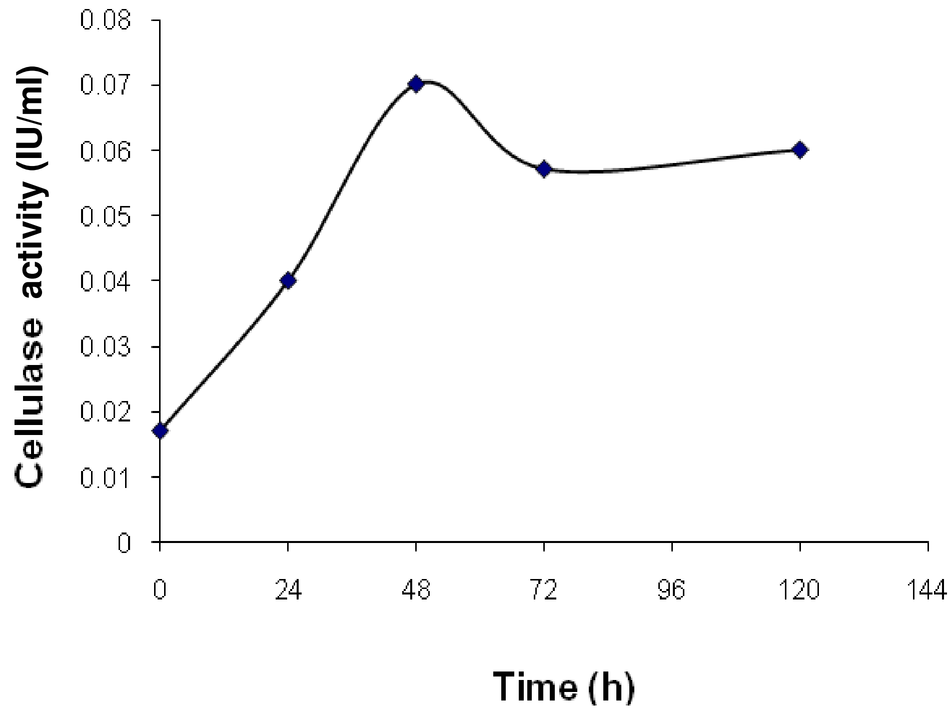

Figure 1. Cellulase activity from culture supernatant, after different incubation time courses.

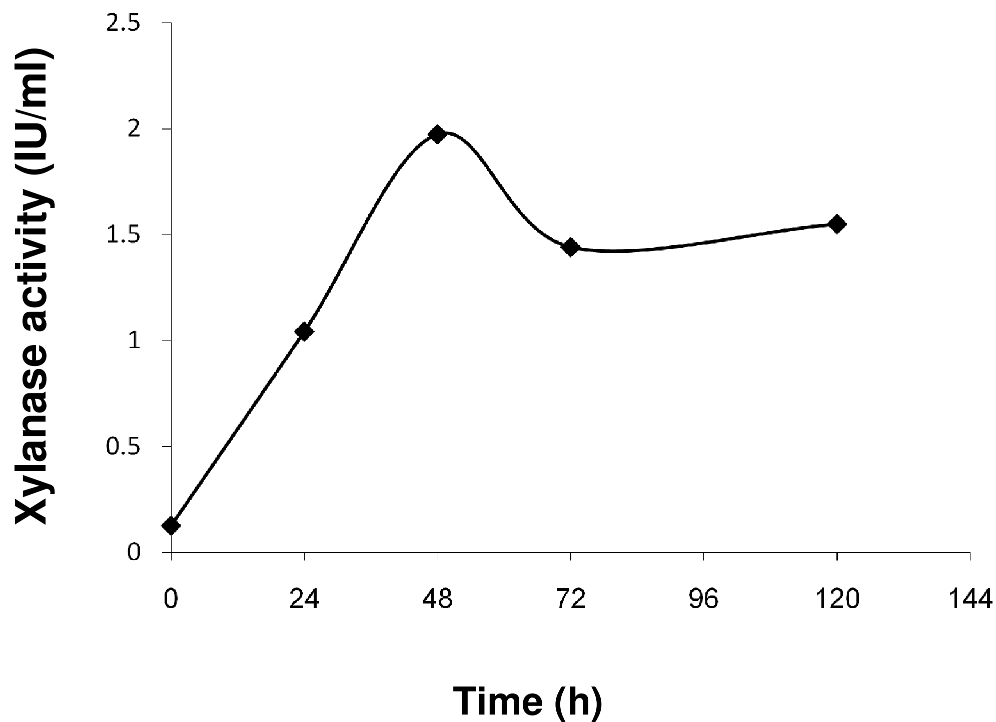

Figure 2. Xylanase activity from culture supernatant, after different incubation time courses.

showed that fungi were more prevalent in ruminants fed high fiber diets than in those fed less fibrous ones. Rumen fungi obviously must interact with a variety of protozoal and bacterial species in their ecosystem (Akin and Borneman, 1990). They produce a wide range of polysaccharide degrading enzymes (eg. cellulose and

xylanase) during growth on various soluble and insoluble carbon sources (Rezaeian et al., 2005). The activities of ruminal fungal polysaccharolytic enzymes and xylanolysis by the fungi are significantly increased by cocultivation with methanogenic bacteria. As methanogens are often coisolated with fungi from ruminal contents, it is probable that such interactions also occur in the ruminal ecosystem (Joblin et al., 1990). So it appears that the production of fungal fibrolytic enzymes in our experiment could best be achieved because fungus was in co-cultivation with methanogenic bacteria. The decrease in the rate of both enzyme productions after 2 days of incubation (Figures 1 and 2) may be explained by reduced substrate availability and the consequence of repressive monomers released during digestion (Rezaeian et al., 2005). Glucose has been shown to repress cellulase production (Rezaeian et al., 2005; Akin and Borneman, 1990). Xylanase production was influenced by both soluble sugars and xylan concentration and was higher with crude wheat straw hemicelluloses compared with xylan from different sources (Akin and Borneman, 1990). However, the much higher xylanase activity compared with cellulase activity is in agreement with previous studies (Rezaeian et al., 2005).

Microbial xylanases have attracted considerable research interest in recent years because of their potential application in the food, animal feed, paper and pulp industries (Yan et al., 2009). Because of the easy accessibility of both enzyme (mainly releasing into the culture fluid), there seem to be some justification for considering their possible application in many industries.

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