

Isolation and identification cellulolytic microorganism from bovine rumen at TH Truemilk dairy farm - Nghia Dan district - Nghe An province

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ABSTRACT: Using anaerobic techniques and the method of preparing media for roll tubes, we have isolated 18 bacteria strains from bovine rumen. The estimation of cellulose activity was carried out through the examination of glucose productivity and total percent of amount CMC was digested. Total cellulase activity has been at 0.022 U and over 80% amount of CMC was digested. The isolated strains named D1, D2, D4, D5, D6, D7, D13, D14, D15 were identified as high cellulase activity. The results indicate that these strains could be used for fermentation reaction to produce feeds for dairy cow. On the other hand, these strains could be used to treat pollution at TH truemilk dairy farm. This study is only initial, so it has to continue order to estimate potentiality of treatment pollution and fermentation feed for dairy cow.

Keywords: Rumen, cellulose, microorganisms, bovine rumen, glucose.

Introduction

TH Truemilk dairy farm at Nghia Dan district is the biggest dairy farm in Viet Nam. It plays important role for Nghia Dan economic. The feed to use for dairy cow has abundant of cellulose that is a material difficult to digest in natural.

Cellulose is a linear polysaccharide of glucose residues with β -1,4-glycosidic linkages. Abundant availability of cellulose makes it an attractive raw material for producing many industrially important commodity products. With the help of cellulolytic system, cellulose can be converted to glucose which is a multi-utility product, in a much cheaper and biologically favorable process.

Cellulolytic is a biological process which controlled and processed with cellulose system. Cellulase system consists of three classes of soluble extracellular enzymes, i.e 1,4- β -endoglucanases, 1,4- β -exoglucanases, and β -glucosidases (β -D- glucoside glucohydrolases or cellobiases) (Shewale, 1982). These enzymes hydrolyze cellulose to glucose (Ryu et al., 1980). One of the best source for cellulolytic system is microorganism in rumen fluid of dairy cow with cellulose as source of metabolizable sugar (glucose). Rumen fluid of dairy cow had microorganism which digests the cellulolytic feed (Saxena et al., 1993). Cellulase used in various industrial processes, including biofuels such as bioethanol, triphase biometha-

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nation (Chakraborty et al., 2000), plants and agriculture waste processing (Mswaka et al., 1998), chiral separation and ligand binding studies (Nutt et al., 1998). The rumen is a special digestive vessel, within which the digestion of cellulose and other plant polysaccharides occurs, through microbial activity. Fiber, especially cellulose and hemi-cellulose, is primarily broken down into the three volatile fatty acids, acetic acid, propionic acid and butyric acid in these chambers by microbes (bacteria, protozoa, and fungi). Even though the rumen and reticulum have different names, they represent the same functional space as digesta which can move back and forth between them. Microbes produced in the reticulo-rumen are also digested in the small intestine. Fermentation continues in the large intestine in the same way as in the reticulo-rumen. (Schwarz, 2001)

Almost all the glucose produced by the breaking down of cellulose and hemicellulose is used by microbes in the rumen, and as such dairy cow usually absorbs little glucose from the small intestine. Rather, dairy cow requirement for glucose (for brain function and lactation if appropriate) is made by the liver from propionate, one of the volatile fatty acids made in the rumen. (Hays, 2004) The bacteria *Fibrobacter succinogenes*, *Bacteroides succinogenes*, *Ruminococcus albus*, *Ruminococcus flavifaciens*, *Clostridium ochtheadii*, *Bacillus Licheniformis*, and *Streptococcus Anaerobius* are generally regarded as the predominate cellulolytic microbes in the rumen. (Findlay, 1998)

The objectives of this study are to isolate microorganisms from the rumen fluid of dairy cow and to test the ability of these organisms to hydrolyze cellulose.

Materials and methods

Materials

- Carboxy methylcellulose (CMC) media (Ulrich et al., 2008): 1 g $(\text{NH}_4)_2\text{SO}_4$, 1 g K_2HPO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, 0.001 g NaCl, 10 g CMC, 2 g Agar, Fill water to 1l and adjust pH = 7.

- Mineral (M) media (Lee et al., 2002): 0.4 g KH_2PO_4 , 0.4 g K_2HPO_4 , 1 g NH_4Cl , 0.1 g MgCl_2 , 0.2 g yeast extract, 6 g NaHCO_3 , 0.5 g Cysteine-HCl, 0.25 g Na_2S , 0.001 g Resazurin, 10 ml master solution, 10 ml mixture solution of vitamins, fill distilled water to 1l, and adjust pH 7. Add 20 g Agar to prepare solid media

+ Master solution: nitrotriacetic 4.5 g, 0.4 g FeCl_2 , CoCl_2 0.12 g, $\text{ALK}(\text{SO}_4)_2$ 0.01 g, NaCl 1 g, CaCl_2 0.02 g, Na_2MoO_4 0.01 g, MnCl_2 0.1 g, ZnCl_2 0.1 g, H_3PO_3 0.01 g, CuSO_4 0.01 g, NiCl_2 0.02 g, fill distilled water to 1l.

+ Mixture solution of vitamins: Biotin 2 mg, Folic acid 2 mg, pyridoxine hydrochloride 10 mg, Thiamine 5 mg, Riboflavin 5 mg, 5 mg Nicotinic, Dl-calcium pantothenate 5 mg, B12 0.1 mg, D-amilobenzoic 5 mg, Lipoic 5 mg, fill distilled water to 1l.

- Hutchinson media: 2.5 g KNO_3 , 1 g K_2HPO_4 , 0.3 g MgSO_4 , 0.1 g CaCl_2 , 0.1 g NaCl, 0.01 g FeCl_3 , distilled water 1000 ml. Add 30 g Agar for preparation solid media.

Method

Sample collection and preparation

The sample was collected from the dairy farm - Nghia Dan district - Nghe An province. The samples were collected in sterile boxes and transferred to the laboratory. The sample was prepared by diluting 1 g rumen fluid into 50 ml distilled water, filtered by filter paper and col-

lected solution. Then, sample solution was serially diluted in 10 ml tube separately, like 10^{-1} to 10^{-5} . Take 5 μ l of each concentration by pipet was cultured on petri plates added CMC media and incubated at 37°C for 24 to 72 hours & colonies were observed.

Estimation of cellulase activity by determining the diameter of hydrolysis

The selected bacterial strains were cultured on CMC for 48hrs. at 30°C. The diameter of hydrolysis was measured by determining halo diameter of colonies after dying with 1% (w/v) Congo Red solution in 15 minutes. Follow that petri plates were washed with 1M NaCl solution. The possible formula of hydrolysis:

$$\text{(Halo diameter - colony diameter) / halo diameter} \times 100$$

Cellulase Assay

The selected bacterial strains were cultured on liquid Hutchison media for 48hrs. at 30°C. After that cultured solution was extracted by centrifuge at 5000 rpm for 20 mins. We took in the supernatant. Total cellulase activity was determined by incubating 0.5 mL supernatant with 1 mL of 0.05 M sodium citrate buffer (pH = 4.8) containing 50 mg of cellulose.

After incubation for 1 hour at 50°C, the reaction was terminated by adding 3 mL 3,5-dinitrosalicylic acid (DNS) reagent to 1 mL of reaction mixture. Reducing sugar was estimated spectrophotometrically using glucose as standards. [Miller, 1959] The enzymatic activity was defined in international units (IU). One unit of enzymatic activity defined as the amount of enzyme that releases 1 mol reducing sugar (measured as glucose) per mL per minute.

Results and Discussion

Isolation of cellulytic bacteria from bovine rumen fluid

From the sample of rumen fluid, we have isolated eighteen bacterial strains on CMC media. Eighteen isolated bacteria are named D1, D2, D3, D4, D5, D6, D7, D8, D9, D10, D11, D12, D13, D14, D15, D16, D17 and D18. We continued cultured these strains in liquid Hutchinson with copy paper (1 liter of liquid medium + 2 gam blotting paper), these strains are also live. Then, we have chosen nine bacterial strains as D1, D2, D4, D5, D6, D7, D13, D14, D15 which have digest ability of copy paper highest.

The colonies of bacteria on CMC media were shown on **Figure 1**.

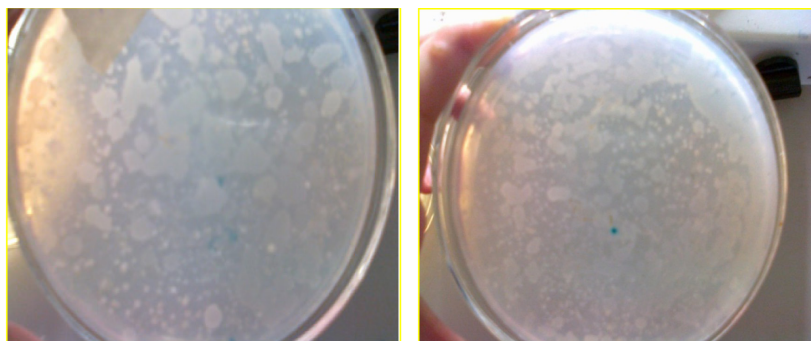


Figure 1 Bacterial colonies on CMC media from the rumen fluid

Determined of cellulase activity by diameter hydrolysis

Screening procedure was conducted after isolating. All bacterial cultures were isolated

from the rumen fluid were grown at 50°C on a screening media (Congo red-cellulose agar) to produce clear zone (**Figure 2**).

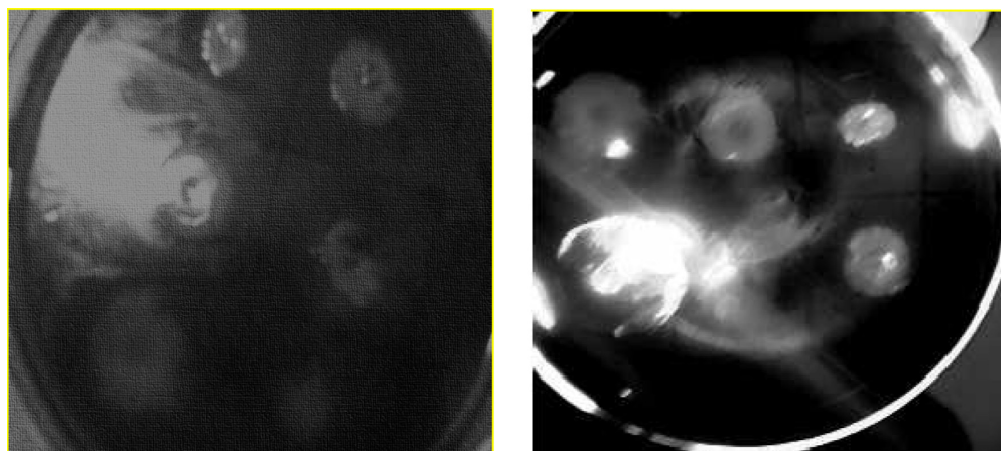


Figure 2 Clear zone on a screening media (Congo red-cellulose agar) after 48 hours incubation

CMC is a soluble pure cellulose or an amorphous cellulose which is more easily hydrolyzed than if taken from natural ones that is still bound to lignin and hemicellulose and still has a high crystalline structure (insoluble). Carboxymethylcellulose, measuring the activity of endo- β -1,4-glucanase, which is an important artificial

substrate for measuring cellulase activity due to the high solubility of these compounds in the water. CMC is used as a test medium to study the decomposition of cellulose in dairy rumen and other insects. Thus, isolates that produce the biggest diameter of clear zone is considered to have the highest cellulolytic activity.

Table 1 Hydrolytic capacity measurement of bacterial isolates

Colony	Colony diameter (mm)	Clear zone diameter (mm)	Hydrolytic capacity
1	5.1	26.7	80.90
2	4.7	23.6	80.08
4	4.9	25.3	80.63
5	3.9	22.3	82.51
6	4.3	24.6	82.52
7	5.3	27.1	80.44
13	4.1	26.8	84.70
14	5.3	27.7	80.87
15	4.3	25.4	83.07
Average			81.75

The nine isolate has the ability to degrade CMC substrate. It can be seen from the clear zone around the colony. The formation of clear zone indicates that the CMC substrate agar is hydrolyzed by cellulase. The ability to form clear zones on CMC substrate showed the enzyme endo - β - 1,4 - glucanase can break the bonds of 1.4 glycosides on the cellulase fibers randomly and the number of amorphous regions on a substrate can hydrolyze CMC more efficiently (Goto et al., 1992).

All bacteria were able to grow on CMC media at temperature of 37^oC and pH=7.1. The addition of congo red dye can clarify the diameter of clear zone. Formation of a clear zone around the colony explains the secretion of extracellular cellulase. Nine isolates D1, D2, D3, D4, D5, D6, D7, D8 and D9 (Table 1) that grown at 50^oC were then selected for further study. The measurement of isolates hydrolytic ability showed that the highest clear zone was on D13 84.7 followed by

D15 87.07, respectively.

The hydrolytic capacity value (Table 2) obtained is from 80.08 to 84.7 percent. We found that the hydrolytic ability average value of nine isolated bacteria is 81.75 percent. This indicates that the bacterial isolate had ability to degradate the cellulose so highly, so it can digest cellulose to produce glucose for carbon sources.

Cellulase assay

Cellulase system consists of 1,4 - β - endoglucanase, 1,4 - β - exoglucanase, and β - glucosidase (β - D -glucoside glucohydrolase or cellobiase). (Shewale, 1982) The synergy of the third enzymes do complete hydrolysis of cellulose to glucose. (Ryu et al., 1980) In this study, glucose used as standard and DNS reagent used to stop the enzymatic reaction, so the reaction product can be measured. The reaction between glucose and the DNS reagent gave maximum absorption at 450 nm.

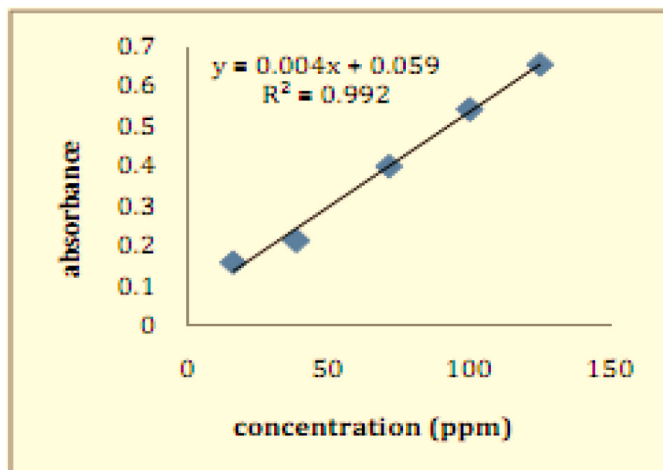


Figure 3 Glucose calibration curve

Incubation of cellulase and CMC gave absorbance of 0.547 for total cellulase activity. Hence, we found that the glucose concentration was 122.0 ppm. So, bacterial isolates had total cellulase activity at 0.022 U, respectively.

Conclusion and Suggestion

The cellulose utilizing organism isolated and studied for the cellulolytic activity, the observation and result shows that the isolates have potent cellulose degrader. We found that the isolates bacteria from rumen fluid has digestion ability of cellulose highly, specially bacterial strains are named D1, D2, D4, D5, D6, D7, D13, D14, D15. A survey generated by cellulase assay, we found that the total cellulase activity at 0,022 U. The highest activity strains are D5, D6, D13, and D15. Suggested applications of this strain into reality as produce feed that makes increase cellulose digestion ability of dairy cow, resolution of straw and other cellulose waste products to help the environment or handling of organic products. The further identification is needed from the national laboratories for confirmation of genus and species.

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