COMPARATIVE STUDY ON ENZYMATIC HYDROLYSIS OF CELLULOSE

A.E. SEGNEANU[a], C. MACARIE[a], M. UNGUREANU[b], I. BALCU[a],
V. GHERMAN[b], I. GROZESCU[a*]
[a]National Institute for Research and Development in Electrochemistry and
Condensed Matter, Timisoara, 144 A.P. Paunescu, 300569, Timisoara, Romania,
[b]Politehnica University of Timisoara, Timisoara, Romania

Identification and optimization of strains with high enzyme activity able to overcome constrains imposed by the cellulosic structure represents an important step in the development of new biotechnologies for bioethanol. this paper aims to reveal the advantages and disadvantages of the cellulase enzymes derived from two complet different microorganisms: *Trichoderma ressei,* a very known cellulase producers and *Butyrivibrio fibrisolvens,* a ruminal bacteria. Both organisms were inoculated under the same conditions (strict anaerobic, Sabouraud dextrose agar media, pH and temperature). The cellulose degradation was investigated by the time evolution of cellulase activity and the amount of reducing sugar released (glucose as standard) from carboxymethyl cellulose.

1. Introduction

The enzymatic hydrolisis of cellulose still represents an important step of the bioethanol production's cost. The identification of a highly efficiency cellulolytic enzymes (cellulases) with a high specific activity, will have a major impact on the availability of cost competitive biofuels market.[1-8] Althrough it is well known that physical properties of cellulose affects the rate of the enzimatic degradation process, recent studies aims to develop an innovative biotechnology based on suitable cellulolytic microorganisms capable to produce a complex and efficient enzyme system, being able to ensure a coordinate and a very efficient hydrolysis of hemicellulosis and cellulose, from different lignocellulosic substrates.[2-12]

Under the enzymatic attack occurs the depolymerisation of cellulose in easily fermentable saccharides. Usually, the microorganisms which degrade cellulose, also degrade hemicellulose.[8-20]

There are a great diversity of microorganisms for lignocellulose degradation: *Trichoderma, Aspergillus, Clostridium, Humicola, Talaromyces, Acrophialophora, Thermoascus, Bacillus* and *Penicillium* species.[12-25]

Comparative studies on the efficiency of aerobic and anaerobic microorganisms had proved that not only 5-10 % of cellulose is degraded in nature under anaerobic conditions, but there are many differences between these two types of cellulolytic microorganisms.[1,23-30] The best known strict anaerob microorganism is *Clostridium thermocellum*, but the main disadvantage consists in that it is necessary high temperature for cellulose degradation.[2,3]

Researchers have paid special attention on anaerobic and, facultative, on anaerobic cellulolytic microorganisms isolated from a rumen microbial environment, because it is well known that cellulolytic rumen microorganisms (bacteria, fungi, etc) can convert the carbohydrates, from cellulose and other several types of biomasses, in carbon and energy sources. Even the

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* Corresponding author: ioangrozescu@gmail.com

complex mechanism of cellulolysis system is not yet complete elucidated, has been studied intensively the competitive and synergic interaction between rumen microbes.[4-6, 23, 25-29]

Most of the rumen cellulolytic microbes are: bacteria species (*Pseudomonas aeruginosa*, *Bacillus*, *Micrococcus*, *Streptococcus*, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *Ruminococcus albus)*, while the fungi isolated were species of *Fusarium, Penicillium, Aspergillus and Mucor*.[5,6]

Studies on biodegradation of lignocellulose biomass under the action of rumen bacteria can represent an promising step for the development of an innovating biotechnology for bioethanol production.[23, 25-29]

This paper investigate the cellulolysis efficiency of enzymes isolated from an rumen bacteria *Butyrivibria fibrisolvens* in comparison with an industrially important cellulolytic filamentous fungus, *Trichoderma reesei.*

2. Experimental

Materials and instruments

All the reagents are analytical grade. Folin-Ciocalteu reagent, 3,5-dinitrosalicylic acid (DNS), sodium hydroxide, potassium sodium tartrate, copper sulphate, glacial acetic acid, glucose, 3,5- dinitrosalicylic acid (DNS), phenol, sodium sulphite and sodium carbonate were obtained from Merck. The bovine serum albumin BSA, carboxymethyl cellulase (CMC) are from Fluka.

The microorganism used for inoculation of culture media were: *Butyrivibrio fibrisolvens*. The culture inoculation was carried out in sterile conditions in Laminar Flow Advanced Bio Safety Cabinet, BIOQUELL Medical Limited, England.

3. Experimental procedure

Estimation of protein concentration was determinated by Lowry method using bovine serum albumin (BSA) as the standard. This chromogenic procedure is inexpensive, easy to perform, very sensitive and highly reproducible, but the major disadvantage is because its accuracy depends on the pH of the solution.[31-32]

A) Different dilutions of BSA solutions are prepared by mixing stock BSA solution (1 mg/ mL) and water in the test tube as given in the table 1. The final volume in each of the test tubes is 2 mL. The BSA range volume is 0.02 to 0.2 mL.

B) The alkaline cooper reagent was prepared by mixing 0.5 mL of 0.5% cupric sulfate with 0.5 mL of 2% sodium potassium tartrate, followed by the addition of 50 mL of 2% sodium carbonate in 0.1 N NaOH. The mixture was then allowed to incubate at room temperature for 10-15 minutes.

C) There were taken portions from the standard solution of Bovine serum albumin (BSA) and placed into 10 tubes by filling with distilled water to 0.2 mL, 5mL of alkaline copper reagent and 0.5mL Folin-Ciocalteu reagent were added in this order.

D) Calibration solution of albumin bovine serum (BSA) in distilled water (1 mg/mL).

The samples were mixed and the color was allowed to develop for 30 minutes at room temperature and the absorbance measured at 660 nm against a blank obtained under the same condition, by replacing the BSA solution with distilled water. For safety determination two samples have been made in parallel. The results are being presented in the table 1.

Table 1. BSA solution calibration with Lowry method.

Nr. crt.	BSA solution (mL)	Distilled water (mL)	Extinction	Protein quantity (mg)
1. (blank)	-	0,20	-	0,00
2.	0,02	0,18	0,0496	0,02
3.	0,04	0,16	0,0957	0,04
4.	0,06	0,14	0,1442	0,06
5.	0,08	0,12	0,2022	0,08
6.	0,10	0,10	0,2538	0,10
7.	0,12	0,08	0,2838	0,12
8.	0,14	0,06	0,3342	0,14
9.	0,16	0,04	0,3562	0,16
10.	0,18	0,02	0,4349	0,18
11.	0,20	-	0,4169	0,20

The plot of the extinction depending on the protein amount shows a straight form.This dependence may be expressed by the next equation:

$$E = b \cdot C_P$$

where:

E = the extinction
b = the slope
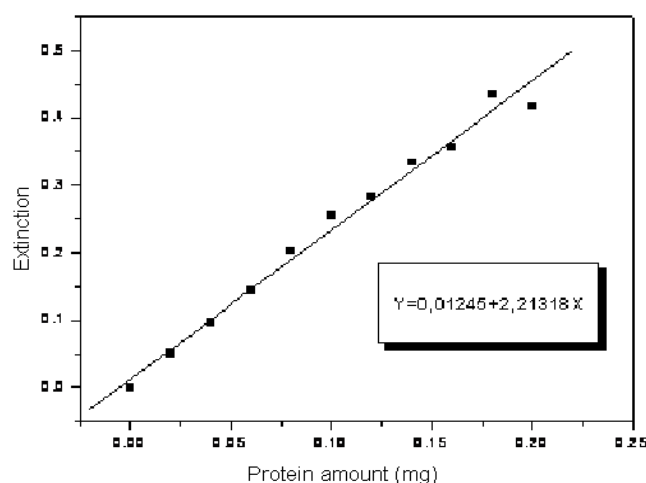C_P = the protein amount, (mg)

Fig. 1. Calibration curve of BSA solution by Lowry method

Simultaneously have performed an estimate of a protein concentration from an unknown solution

A volume of 2 mL sample, 0,2 mL distilled water, 5 mL copper alkaline reagent and 0,5 mL Folin-Ciocâlteu reagent were added into a test tube and left ageing for 30 minutes at the room temperature. After this, the extinction was determinated at 660 nm against a blank obtained in the same condition but replacing the sample with distilled water. For a safety determination two samples have been made in parallel.

The protein content of the sample was determined with the relationship:

$$C = \frac{1}{2,21318} \cdot E \cdot 5 \cdot F \text{ (mg/ml)}$$

Where: 5 = sample dilution at 1 mL
F = the dilution factor of enzymatic solution

Evaluation of Cellulase Activity usig the carboxymethylcellulase as substrat was performed by Miller method with 3,5-dinitro-salicylic acid (DNS).

D-glucose standards were prepared in 0,05 M sodium acetate buffer pH 4.8 at concentation between 0.25 and 1.5 mg/mL (Table 2) and the reaction was carry out with a reagent solution composed from: 1,0 g DNS, 200 mg phenol, 50 mg of sodium sulphite and adjust to volume with 1% NaOH.

Table 2. Preparation of standard glucose solutions.

Glucose solution (mL)	Sodium acetate buffer solution 0,05 M, pH 4,8 (mL)	Dilution	Concentration (mg/mL)
1,0	2,3	1 :3,3	1,5
1,0	3,0	1 :4	1,25
1,0	4,0	1:5	1,0
1,0	5,7	1:6,7	0,75
1,0	9,0	1:10	0,5
1,0	19,0	1:20	0,25

Glucose standard curve

Determination of the calibration curve for glucose solution required the next procedure:

a) the blank sample contains: 0.4 mL acetate buffer solution (0,05 M, pH 4.8), 0.4 mL distilled water and 1.2 mL DNS solution;

b) glucose sample was prepared from 0.4 mL standard glucose solution dissolved in 0.4 mL distilled water and was added 1.2 mL DNS solution;

The both obtained samples were boiled for 15 minutes and then cooled at room temperature and absorbance at 540 nm was determinated.

The standard curve for glucose standards (Figure 2) has a linear form described by the following equation:

$$E = a + b \cdot C_G$$

where:

E= absorbance
C_G = concentration of glucose, mg/mL
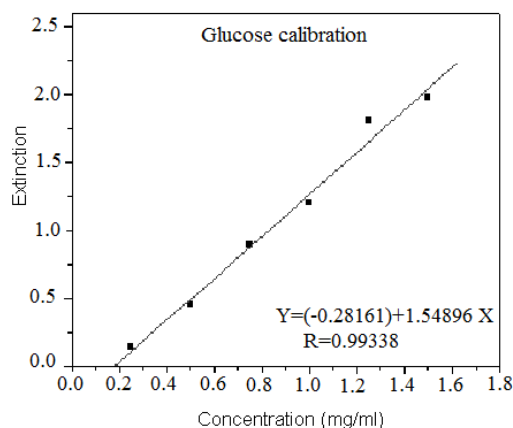a = origin ordinate
b = slope

Figure 2. D-glucose calibration curve

The amount of glucose expressed as mg glucose from the sample was determinated from the calibration curve using the following relation:

$$C_G = \frac{(E_P - E_M)}{1,54896} \cdot sample\ dilution \cdot V\,[\text{mg glucose}]$$

where: V- total volume (1,5 mL).

Cellulase preparation from *Trichoderma reesei*

The *Trichoderma reesei* was innoculated on a Sabouraud dextrose agar media prepared from 200 mL of potato extract and 2 g cellulose/l , in serum bottle sealed with rubber stoppers. The serum bottles were autoclaved (20 min, 120°C, 1 atm pressure) and then flushed with nitrogen. The culture was grown in anaerobic condition, ten days at 30°C. Then was prelevated samples for enzymatic activity assay and for protein assay.

Cellulase preparation from *Butyrivibrio fibrisolvens*

The *Butyrivibrio fibrisolvens* was innoculated on a Sabouraud dextrose agar media prepared from 200 mL of potato extract and 2 g cellulose/l , in serum bottle sealed with rubber stoppers. The serum bottles were autoclaved (20 min, 120°C, 1 atm pressure) and then flushed with nitrogen. The culture was grown in anaerobic conditions, ten days at 30°C.

Determination of cellulase activity

The 1 mL sample was added to 0.5 mL of 2% carboxymethyl cellulose (CMC). The mixture was incubated in a water bath at 50ºC for 30 minutes. Comparative was prepared a blank sample consiting from 1.0 mL cellulase solution and 0.5 mL substrate solution CMC 2%. The evaluation of the enzymatic activity was determinated in basis of the remaining unreacted glucose and is expressed in units. A unit is the amount of enzyme from 1mL enzyme solution which releases 1 mol of reducing sugars (glucose as standard) in a minute at 30°C.

The calculation formula of the activity is:

$$A_{CMC} = \frac{\text{mg glucose} \cdot 1000}{180 \cdot 1,0 \cdot 30} \quad [U/ml]$$

4. Results and discussions

According to the literature, *Butyrivibrio fibrisolvens* is an anaerobic, butyric acid forming bacteria which produce an multi-enzyme complex with predominantly xylanase activity.[1-6,23,25-29] This study is important in terms to investigate the enzymatic activity of the pure *Butyrivibrio* species isolated and particularly, their cellulolytic enzyme activity by comparison with a well known *cellulolytic* microorganism, *Trichoderma reesei*. Was chosen this widely used fungus *Trichoderma reesei,* for cellulase production, because of its ability to produce significant quantities of enzymes.

The specific activity of pure cellulase-producing strains was carried out using traditional technique in strict aerobic condition by determination of the amount of reducing sugar released (glucose as standard) from carboxymethyl cellulose.

The results of the enzyme activity per time are presented in the Tables 3 and 4 and figures 3 and 4.

Tabelul 3. Cellulasic activity and total protein content in the Trichoderma reesei culture

Time [h]	Carbon source	Glucose (mg)	Activity [U/mL]	Protein [mg/mL]
24	Cellulose 5 [g/L]	6,06	1,122	0,964
48		5,90	3,520	0,998
144		4,19	7,760	1,039

Table 4. Cellulasic activity and total protein content in the Butyrivibrio fibrisolvens culture

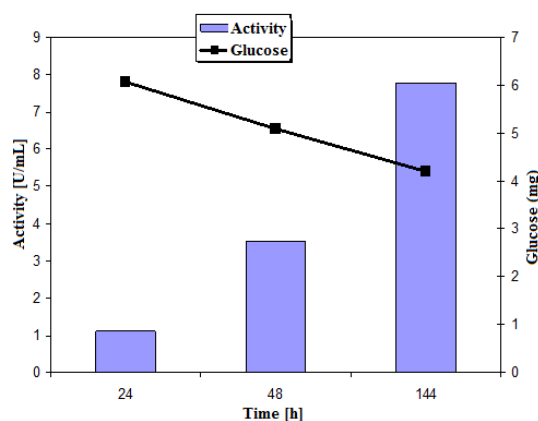| Time [h]	Carbon source	Glucose (mg)	Activity [U/mL]	Protein [mg/mL]
24	Cellulose 2 [g/L]	7,972	9,885	0,495
48		5,338	30,90	0,529
144		3,477	0,644	0,664

Fig. 3. The evolution in time of the cellulase produced by Trichoderma
reesei and of the glucose quantity.

Analysis of experimental showed that the both microorganisms growth occurs fast on the Sabouraud dextrose agar media. The evolution of the cellulasic activity present several features for each microorganism investigated. For instance, *Trichoderma reseei* stains showed a continuous increase of the cellulasic activity throughout the monitored period, yielding a value of 7.76 U/mL after 144 hours (Figure 3). These results are comparable to those reported in the literature[30-33], which give higher values for cellulase activity of *Trichoderma reesei* cultures after 7 days, and the can be difference can be attributed to the type of the culture media which ensure the mantainance of the pH value at a specific value. The total protein concentration exhibit only a mild variation. Evolution of the amount of glucose indicate a slight decrease until at value of 4.19 mg after 144 hours, which shows that there is still required condition for further enzymes production.

The rumen cellulolytic bacteria, *Butyrivibrio fibrisolvens,*acted in different way. It was found that the maximum enzyme production was 30,90 U/mL and was optimum at 48 h, then have showed a dramatic decrease in enzyme production level was detected at day six (0,644 U/mL) (Figure 4). In contrast, the protein concentration recorded present a steady growth, which shown that the culture media used was appropriate from the point of view of ensuring the required nutritional factors. After six days, was retrieved aproximative half o the glucose amout from day one (24 hour), which demonstrates that there were growth resources of biomass, however this fact is not important in given condition because it was of interest only in the production of cellulase.
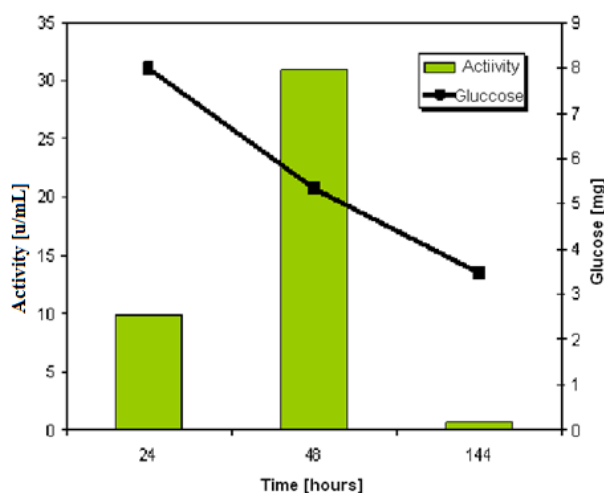

Fig. 4. The evolution in time of the cellulase produced by Butyrivibrio
fibrisolvens and of the glucose quantity

The results obtained has been suggested that enzymatic activity of the cellulolytic bacteria start present an early rate exponential (until the second day), unlike the industrial fungus, whose enzyme production increases slowly. Further studies are neccesary to optimise the culture conditions (pH, temperature, carbon sources and nitrogen sources) to achieve the maximum yield of the cellulase activity.

5. Conclusions

The purpose of this study was a comparative analysis of enzymatic activity of two type of microorganisms, innoculated on the same culture condition. *Butyrivibrio* species isolated in this experiment can be considered as a higher source of cellulolytic enzyme. The results of this investigation have suggested that this cellulolytic ruminal bacteria endowed with the capacity to digest native insoluble cellulose and relatively high specific growth rates, the must be regarded as a very promising solution for industrial enzymatic hydrolysis step.

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