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Comparative Study on Microorganisms Used for the Bioethanol Production

Corina Amalia Macarie[1], Adina-Elena Segneanu[1], Ionel Balcu[1], Raluca Pop[1], Georgeta Burtica[2], Vasile-Daniel Gherman[2]

[1]National Institute for Research and Development in Electrochemistry and Condensed Matter, Timisoara, Romania; [2]The Faculty of Civil Engineering, Politehnica University of Timisoara, Timisoara, Romania.
Email: s-adinaelena@yahoo.com

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ABSTRACT

Two different methods, namely simultaneous saccharification and fermentation and two-stage hydrolysis and fermentation have been used for the conversion of the cellulose into bioethanol. Both aerobic and anaerobic conditions were employed in order to obtain two types of microorganisms—Trichoderma reesei and Zymomonas mobilis—that are used for the production of cellulases. The aim of the paper is to investigate also the efficiency of a microorganism's consortium in the fermentation stage of the two processes and the action of this microorganism's consortium on different concentrations of the cellulosic substrate, in order to determine the optimum parameters of the process. Good yields (45% - 70%) *of the cellulose degradation into fermentable sugars have been obtained.*

Keywords*: *Bioethanol, Lignocellulosic Biomass, Microorganism

1. Introduction

Due to the limitations of fossil energy, there is a continuous increasing interest in finding new alternatives for oil-based fuels. Renewable resources like sugarcane or agricultural crops have represented the major raw materials for the bioethanol production [1]. In the last years, lignocellulosic materials have gained much importance for the obtaining of bioethanol, mostly due to their large availability. The main disadvantage is represented by the higher costs caused by the small yields and by the difficulties encountered in the cellulose hydrolysis (deploymerization) in soluble sugars [2,3].

Chemical methods for the depolymerization of the cellulose are the acid, basic or enzymatic hydrolysis. Among them, enzymatic hydrolysis shows the advantages of mild reactions conditions and of avoiding the corrosion problems [4-7].

Cellulose transformation into soluble sugars during the enzymatic hydrolysis process occurs by the means of cellulase, an enzyme with a high cost of production. Lately, in the biofuels industry, cellulases obtained from the *Trichoderma reesei* fungus are used, with good results regarding the vegetal feedstock degradation [8,9]. This could enlarge the possible biomass resources. Another microorganism used for the bioethanol production is *Zymomonas mobilis* that may be used only for substrates that contain glucose, fructose and sucrose [10-12].

There are two main procedures used for the bioethanol production starting from cellulose. The first one implies two different stages, namely the hydrolysis of the cellulosic substrate, followed by the fermentation of the obtained sugars. The second method implies the simultaneous saccharification and fermentation [10,13,14].

The present paper presents the cellulose degradation under the action of *Trichoderma reesei* and *Zymomonas mobilis*, in order to determine the optimum conditions for the bioethanol production through the hydrolysis and fermentation of cellulose-based materials. The hydrolysis of the cellulosic substrate was performed using *Trichoderma reesei*, and for the separate fermentation of sugars there were used enzymes from *Zymomonas mobilis*. For the simultaneous saccharification and fermentation of a cellulosic substrate a consortium of the two microrganisms was employed. The results of the two fermentation methods (separate and simultaneous) have been quantified by means of the concentration of the unreacted fermentable sugars that were not transformed into ethanol.

2. Experimental Part

Lyophilized strains of *Trichoderma reesei* (*IHEM* 5652)

and *Zymomonas mobilis* (*ATCC* 29191) were used, that were reactivated at their transfer on the culture media.

2.1. The Obtaining of Cellulose and Hemicellulose Degrading Enzymes from Micro-Organism Cultures

Cellulases from *Trichoderma reesei* and *Zymomonas mobilis*.

The cultures were incubated for several days at a temperature of 30˚C, without shaking, both in anaerobic and aerobic conditions. At various time intervals samples were taken in order to analyze the cellulase activity on the substrate (carboxymethylcellulose standard) and also for the determination of the protein content.

2.2. Cellulose Degradation by Enzymes Obtained from *Trichoderma reesei*

The *Trichoderma* strains were inoculated on a natural culture medium that contained 100 mL potato extract, 2 g glucose and 0.5 g, 0.75 g, 1.0 g, 1.5 g and 2.0 g Avicel, respectively. The cultures were sterilized in an autoclave, inoculated and thermostated for several days at 30˚C. Samples of a few milliliters were taken in order to determine the total sugar content.

2.3. Sugar Fermentation by Enzymes from *Zymomonas mobilis*

Cultures of *Zymomonas* in a synthetic medium were used. The synthetic culture medium was made by 0.5 g yeast extract, 0.1 g $(NH_4)_2SO_4$, 0.2 g K_2HPO_4, 0.1 g $MgSO_4$ and 2.0 g, 4.0 g, 6.0 g and 8.0 g glucose, respectively. The cultures were syntetized by the same way like in case of Trichoderma reesei strains.

2.4. Cellulose Degradation by Enzymes Obtained from *Trichoderma reesei* and *Zymomonas mobilis*

Cultures of *Trichoderma* and *Zymomonas* on natural medium were used. The natural culture medium contained 100 mL potato extract, 2 g glucose and 1.0 g, 1.5 g, 2.0 g, 3.0 g and 4.0 g Avicel, respectively. Sterylization procedure of cultures is identical with the previous method.

2.5. Determination of the Total Sugar Content

The total sugar content was calculated using the colorimetric method for the determination of the reducing sugars with the DNS (3,5-dinitrosalycilic acid) reagent. In order to calculate the total sugar content, Equation (1) was used.

$$\text{Total sugars} = \left[\left(A_{540} + 0.07447\right)/1.22612\right] \times \text{dilution factor} \quad (1)$$

where, A_{540}-solution absorbance (extinction) at 540 nm dilution factor-5.

The calibration curve used for the determination of the total sugar content (as glucose) is presented in **Figure 1**.

3. Results and Discussions

The total protein content obtained from cultures of *Trichoderma reesei* and *Zymomonas mobilis* both in anaerobic and aerobic is presented in **Figures 2-3** and **Tables 1-2**.

The protein content in aerobic and anaerobic conditions has been determined from the influence of the concentration of oxygen on the development of *Trichoderma reesei* and *Zymomonas mobilis*.

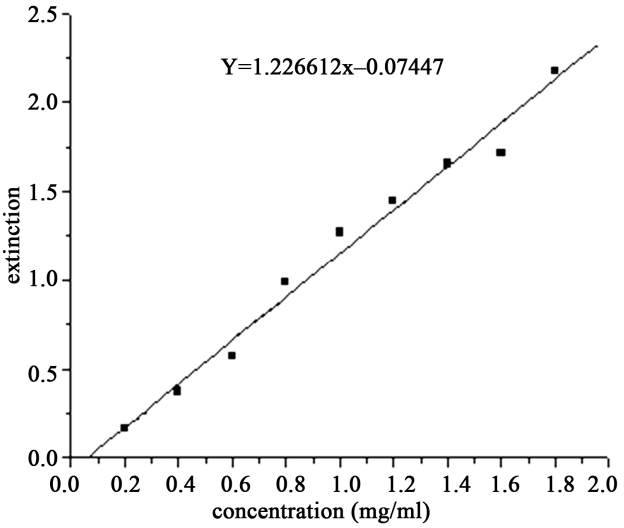

Figure 1. Glucose calibration curve using the colorimetric method (Correlation coefficient R(2) = 0.9908).

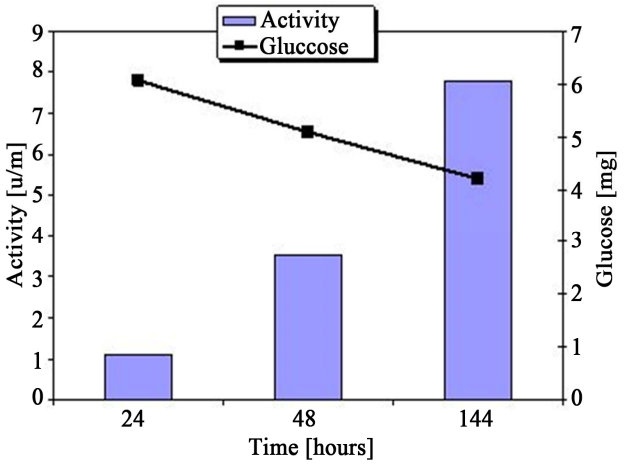

Figure 2. The evolution in time of the cellulase activity produced by *Trichoderma reesei* and of the amount of glucose obtained in anaerobic conditions.

The cellulosic substrate degradation with the enzymes obtained from *T. reesei* was quantified by the determination of the sugar content of the culture medium at different times. The *Zymomonas* strains present less efficiently in aerobic conditions (**Table 2**) then in anaerobic conditions (**Table 1**), but in this case also, no cellulase was produced. We can conclude that it cannot be used to produce cellulase in the given, studied, conditions.

The results are presented in **Table 2** and, in a graphic form, in **Figure 3**. As a comparison, the theoretical quantities that could be obtained from the cellulose degradation with a 100% yield were calculated (**Table 3**). Data from the **Tables 3** and **4** were used for the calculation of the yield of the cellulose enzymatic hydrolysis to sugars, results that are presented in **Table 4** and **Figure 4**.

As it may be observed, best results are obtained for the culture media with a low content of cellulose (higher values are obtained for the methods where an initial con-

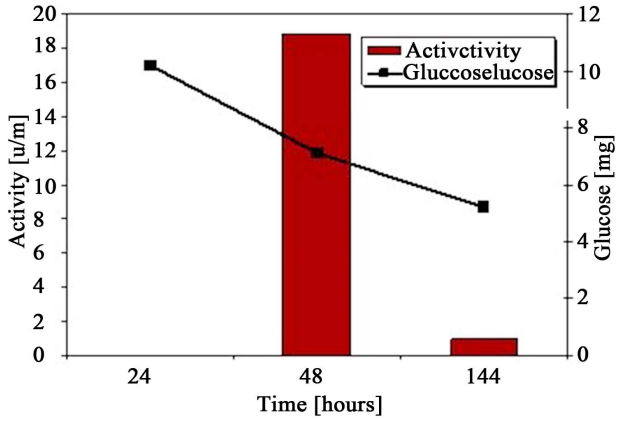

Figure 3. The evolution in time of the cellulase produced by *Trichoderma reesei* and of the amount of glucose obtained in aerobic conditions.

Table 1. The total protein content in *Zymomonas mobilis* cultures in anaerobic conditions.

Time [h]	Carbon source	Protein [mg/ml]
24	Cellulose 5 g/L	0.971
48		0.771
144		0.918

Table 2. The total protein content in *Zymomonas mobilis* cultures in aerobic conditions.

Time [h]	Carbon source	Protein [mg/ml]
24	Cellulose 5 g/L	0.482
48		0.438
144		0.524

Table 3. Maximum theoretical concentrations of total sugars and ethanol, considering maximum efficiency of the hydrolysis and fermentation processes.

No. of culture	Initial cellulose concentration (g/L)	Theoretical sugars concentration (g/L)	Theoretical ethanol concentration (g/L)
1	5	43.21	22.08
2	7.5	45.41	23.21
3	10	47.61	24.33
4	15	52.01	26.58
5	20	56.41	28.83

Table 4. Results of the cellulose hydrolysis into fermentable sugars by means of enzymes obtained from *Trichoderma reesei.*

No. of culture	Initial cellulose conc. (g/L)	Time (h)	Total sugars concentration (g/L)	Sugars yield (%)
1	5	24	22.19	51.3
		48	24.84	57.5
		72	30.19	**69.9**
		96	28.17	65.2
2*	5	24	28.05	64.9
		48	29.60	68.5
		72	30.23	70.0
		96	28.20	65.3
3	7.5	24	25.91	57.1
		48	28.08	61.8
		72	31.36	69.1
		96	29.87	65.8
4*	7.5	24	27.55	60.7
		48	26.12	57.5
		72	29.44	64.8
		96	29.11	64.1
5	10	24	29.70	62.4
		48	28.08	**59.0**
		72	28.64	**60.2**
		96	31.01	65.1
6*	10	24	28.08	**59.0**
		48	23.35	49.0
		72	32.32	67.9
		96	28.89	60.7
7	15	24	20.11	38.7
		48	19.83	38.1
		72	26.40	50.8
		96	23.74	45.6
8*	15	24	25.15	48.4
		48	23.53	45.2
		72	31.04	59.7
		96	30.49	58.6
9	20	24	34.18	60.6
		48	30.85	54.7
		72	30.0	53.2
		96	31.13	55.2
10*	20	24	25.05	44.4
		48	25.18	44.6
		72	28.16	49.9
		96	29.54	52.4

*There were used five cultures (in duplicate) of *Trichoderma reesei*.

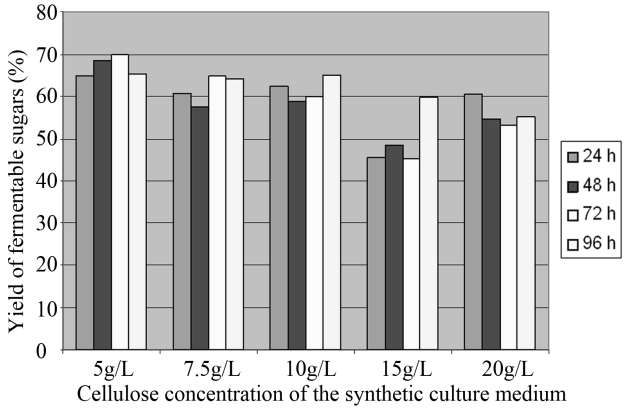

Figure 4. Yields of total sugars obtained from cellulose (best results of each duplicate culture are presented).

centration of 5 g/L, 7.5 g/L and 10 g/L cellulose was used). The maximum yield of the cellulose in glucose transformation is 70% and it is obtained for a concentration of the cellulosic substrate of 5 g/L and a reaction time of 72 h. An increase of the reaction time leads (except the case of the substrate with the greatest cellulose content) to better results in concentrations of the fermentable sugars.

The fermentation process of the glucose using *Zymomonas mobilis* resulted in small amounts of unfermented sugars. Results presented in **Table 5** and **Figure 5** show that only small concentrations of unfermented sugars were obtained. It may also be observed that the best results are obtained during a longer reaction time (48 h - 96 h) and when shaking is applied.

Table 5 shows the untransformed sugar contents after the glucose fermentation in the presence of *Z. mobilis* and, starting from these experimental values, theoretical ethanol yield was calculated. The results presented in Figure 5 show that, independent from the substrate concentration, the best results are obtained after an enzyme action time of 72 - 96 h.

A series of cellulosic substrates (**Table 6**) were subjected to the simultaneous action of *Trichoderma reesei* and *Zymomonas mobilis* for a 30 days period. The results are presented in **Table 6** and **Figure 6**.

The results show no significant differences among the content of untransformed total sugars, independent of the initial cellulose concentrations. It appears that, in this case, higher initial concentrations of cellulose have positive influence on the total amounts of fermented sugars.

4. Conclusions

Trichoderma reesei cultures showed a lower growth of the microorganism under aerobic conditions than under anaerobic conditions. Cellulase was not detected after the first day, but after 48 hours it was formed in sufficiently

Table 5. The total sugar content experimentally resulted and the calculated theoretical ethanol concentration at sugars fermentation with *Zymomonas.*

No. of culture	Initial glucose conc. (g/L)	Time (h)	Total sugars conc. (g/L)	Theoretical ethanol conc. (g/L)
1*	20	24	2.21	9.09
		48	1.07	9.67
		72	0.45	9.99
		96	0.29	10.02
2	20	24	0.23	10.10
		48	0.42	10.00
		72	0.46	10.00
		96	0.28	10.02
3*	40	24	2.17	19.33
		48	0.79	20.04
		72	0.72	20.08
		96	0.74	20.06
4	40	24	0.35	20.26
		48	0.58	20.15
		72	0.43	20.22
		96	0.36	20.26
5*	60	24	0.89	30.21
		48	1.03	30.14
		72	1.03	30.14
		96	0.94	30.19
6	60	24	2.29	29.50
		48	0.65	30.33
		72	0.62	30.35
		96	0.52	30.40
7*	80	24	2.80	39.50
		48	1.09	40.33
		72	1.47	40.14
		96	1.30	40.22
8	80	24	2.17	39.78
		48	0.49	40.64
		72	0.46	40.65
		96	0.45	40.65

*shaken at 150 rot/min.

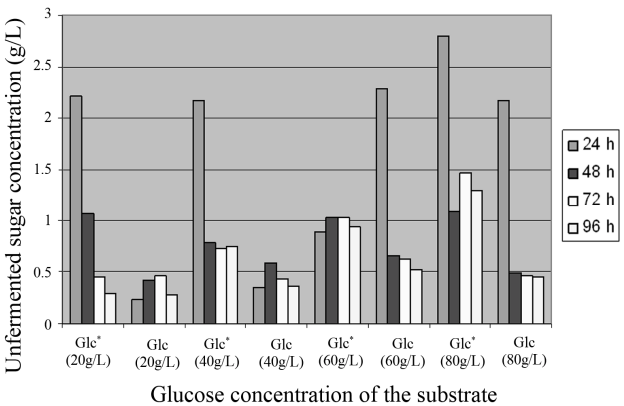

Figure 5. Total sugar content after glucose fermentation with *Zymomonas mobilis.*

Table 6. The total sugar content at the action of a mixture of *T. reesei* and *Z. mobilis* on cellulosic substrates.

No. of culture	Initial cellulose conc. (g/L)	Time (days)	Total sugar content (g/L)	Theoreticalethanol conc. (g/L)
1	10.0	30	1.077	23.78
2	15.0	30	1.091	26.03
3	20.0	30	1.110	28.77
4	30.0	30	1.112	32.76
5	40.0	30	1.065	37.28

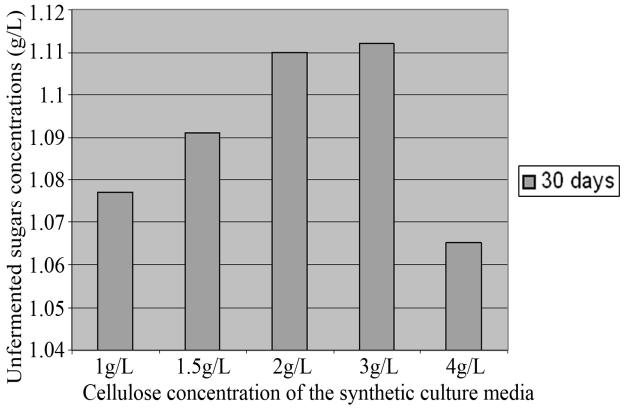

Figure 6. Total content of untransformed sugars after the action of a mixture of *T. reesei* and *Z. mobilis.*

large amounts. after 6 days, it disappeared almost completely from the medium. Cellulase can be also biosynthesised under aerobic conditions from Trichoderma reesei cultures, but it is very important to know the optimum time for stopping the culture if the cellulase activity is elevated. *Zymomonas* strain presented a lower growth under aerobic conditions than under the anaerobic ones, but cellulase was not produced in none of the cases. It may be said that the studied *Zymomonas* strain cannot produce cellulase in the culture media that were used in our experiments.

The results of the study show that *T. reesei* action on cellulose led to the obtaining of sugars (determined as glucose) in 45% - 70% yield. Higher content of sugars are obtained for an initial cellulose concentration of 5 g/L - 10 g/L. Cellulose concentrations of 15 g/L - 20 g/L show an inhibitory effect on the cellulose degradation.

The experimental values of the total sugar content after the *Z. mobilis* action on glucose show that only small sugar amounts had not been transformed. Similar values of untransformed sugars are obtained after the action of a mixture of *T. reesei* and *Z. mobilis* on cellulosic substrates. As results from the values presented in **Table 6** and **Figure 6**, higher cellulose concentration of the synthetic culture medium does not have negative consequences on the cellulose degradation process.

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