STUDIES REGARDING CELLULOLYTIC ENZYMES PRODUCTION BY BACTERIAL STRAINS ISOLATED FROM NATURAL ENVIRONMENTS, GROWN IN LIQUID MEDIA WITH RAW AND PRETREATED SAWDUST

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Abstract:Lignocellulosic biomass is the most abundant renewable polymer on Earth, and represents the raw material for a large variety of products. It also has a high resistance at degradation, which has led to an increase in research focused on efficient hydrolysis of this material. For obtaining soluble sugars, one of the most valuable products, an enzymatic hydrolysis, a chemical one or both can be employed. In our study, we isolated a number of bacterial strains with cellulolytic abilities from different natural environments, which were then grown in liquid media containing sawdust. We show how the rates of enzymatic hydrolysis are influenced by a prior, acid pretreatment of the lignocellulosic material.

INTRODUCTION

Lignocellulosic materials are currently used as primary source for many commercially important products, among which the most notable is ethanol. The high sugar content of lignocellulosic materials and also their abundance makes them attractive for large-scale industrial processes. However, the complex structure of these materials raises a series of challenges in the process of obtaining sugars from them, limiting the yield and affecting the quality of the resulting products. The hydrolysis of cellulosic materials has been extensively studied, pioneering work in this area dating back to the first decades of the 20th century.

Under natural conditions, cellulosic materials are found as a complex made of lignin, hemicelluloses and cellulose, under various proportions, depending on the type of plant containing them. The three types of substances exhibit different chemical and physical properties, with lignin having a highly crystalline structure, while hemicelluloses are mainly amorphous. In order to recover as much sugars as possible from cellulose, lignin and hemicelluloses have to be removed by means of a pretreatment of the material. In nature, hydrolysis of lignocellulosic materials is performed by a series of cellulolytic microorganisms, mostly belonging to fungi and bacteria. However, enzymatic hydrolysis is rather slow, and it does not provide an extensive conversion on itself. Chemical hydrolysis, on the other hand, is performed using mainly acids (although there are many types of chemical hydrolysis), which allows a fast hydrolysis rate. The drawbacks of this type of processes include generation of unwanted byproducts and degradation of sugars. Acid hydrolysis can also be used as a pretreatment of lignocellulosic materials to remove lignin and hemicellulose, and it can be followed by an enzymatic hydrolysis of cellulose, to eliminate some of the disadvantages of the two methods. This configuration is currently used in many industrial processes, offering viable rates of conversion.

In our study, we apply an acid pretreatment to a lignocellulosic material, represented by sawdust. Several cellulolytic bacterial strains, isolated from different natural environments, are then grown in liquid medium containing raw and, respectively, pretreated sawdust to analyze the effect of the pretreatment upon the bacterial conversion of the material. The results show that the production of cellulolytic enzymes is increased, in most cases, for the strains cultivated in medium with pretreated sawdust.

MATERIALS AND METHODS

Isolation of cellulolytic bacterial strains

Since cellulolytic microorganisms play an important role in the circuit of carbon on the planet, they are likely to be found in places where cellulosic materials decompose. For our study, we chose three types of natural environments where this situation occurred, at locations near Aroneanu lake, close to the Iasi city. Samples were collected from moist soil at the margin of the lake, from degraded aerial shoots of Phragmites sp. and from leaf litter from the nearby forest.
Serial decimal dilutions were prepared out of each sample, up to dilution 10^{-5}. A culture medium for identifying aerobic cellulolytic microorganisms was then prepared, based on a recipe described in another work [1]. The medium contained Congo red for enhanced visualization. After preparation, the medium was poured into sterile Petri dishes. For inoculation, dilutions 10^{-3}, 10^{-4} and 10^{-5} from each sample were used. The inoculus was represented by 2 ml from each dilution, spread

onto separate plates. The plates were then allowed to incubate for 48h at 28°C. Meanwhile, culture tubes containing the same medium as above were prepared and stored at 4°C, in sterile conditions. After incubation, cellulolytic strains can be easily identified by observing lighter coloured areas around them. For selecting strains, a ratio between the diameter of the lysis area surrounding each colony and the diameter of the colony itself was calculated ($R=D_{la}/D_c$). Further selection was made using 1000x magnification with an optical microscope, to identify bacterial strains. Selected strains were transferred into culture tubes prepared before and stored at 4°C.

Pretreatment of sawdust

For pretreating sawdust, a dilute acid hydrolysis method was used. A quantity of 5 g of sawdust was placed into a recipient containing 50 ml of 1.5% H_2SO_4. The mixture was placed in a dry air sterilizer set at 195°C for 5 min. The acid was then neutralized using 100 ml of 3% NaOH, while continously monitoring the pH value of the mixture. Pretreated sawdust was then filtrated out of the solution and washed with distilled water thoroughly. The sawdust chosen for this experiment was beech sawdust, as beech is one of the woody species with the highest content of glucose in its wood [2]. Dilute acid hydrolysis was used because it has less requirements related to corrosion resistant materials, it necessitates smaller retention times and it can be performed at atmospheric pressure, as compared to other methods of pretreatment [3].

Preparation of liquid culture medium

For measuring the amount of cellulolytic enzymes produced by the selected bacterial strains, a liquid culture medium was prepared. The medium was a modified Weimer and Zeikuss medium, with the original recipe described in another work [4]. The cellulose in the medium was replaced with sawdust, either raw or pretreated. Following pretreatment, the sawdust was weighed again and it was equally distributed among flasks, for each of the selected bacterial strains. Similarly, 5 g of raw sawdust was equally distributed in a second set of flasks. From the prepared medium, 50 ml were placed into each flask, these having a capacity of 300 ml each, to allow proper oxygenation. The flasks were then capped and sterilized by autoclaving.

Cultivation of bacterial strains

After cooling the liquid culture medium, bacterial strains were transferred, from the culture tubes, which were allowed to warm to the room temperature, to the culture flasks, in sterile conditions. The flasks were then placed in a thermostated device, set at 28°C, and allowed to incubate for 5 days.

Measuring cellulolytic enzyme production

For determining the quantity of cellulolytic enzymes produced by each bacterial strain, samples were taken each 24 h over the incubation period. Samples were represented by 3 ml of liquid medium from each culture flask, which were then centrifuged to obtain the supernatant used for further determinations. The method we used relies on 3,5-dinitrosalicilic acid for determining the quantity of reducing sugars present in a solution. The acid changes its color from orange, proportional with the amount of reducing sugars in the solution [5]. The color shift is measured using a spectrophotometer calibrated at 640 nm. Cellulolytic enzymes are mainly divided into three classes, each acting upon different regions of the cellulosic polymer chain: glucanohydrolases, celobiohydrolases and β-glucosidase [6, 7]. The activity for each of these classes of enzymes was determined following the method described in another paper [5].

RESULTS AND DISCUSSIONS

Selection of strains

By using the Congo red solid medium mentioned above, identification of cellulolytic strains is facilitated, by observing lysis area around colonies. Congo red binds to the microcristalline cellulose in the medium, which is then used by cellulolytic microorganisms. Thus, the area around these colonies turns to a lighter colour (Photos 1, 2). Selection of the strains with the highest cellulolytic activity is then possible by comparing the diameter of the lysis area with the diameter of the colony (Tab. 1).

In our study, we considered that a significant cellulolytic activity is met at colonies which exhibit this ratio with a value of at least 3 (R>=3). Using these criteria, strains were selected only from plates belonging to samples taken from moist soil and degraded shoots. Colonies in plates corresponding to the leaf litter sample showed little or no cellulolytic activity. The colonies identified as having the highest cellulolytic activity were then tested for the type of microorganism present by observing at the microscope Gram stained slides prepared out of them (Photos 3, 4).

Another aspect observed was the purity of the colonies, selecting only pure ones. Thus, a total of 4 strains were selected and transferred onto fresh medium in culture tubes. Strains were designated as: T41, T51, T52 and S41, where T indicates degraded aerial shoots and S indicates soil, the first digit represents dilution and the second digit represents the number of the colony in the plate.

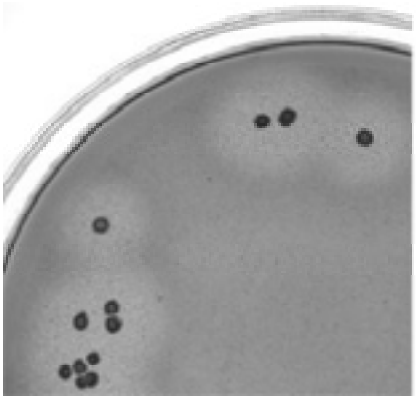

Photo 1. Cellulolysis areas at strain T52

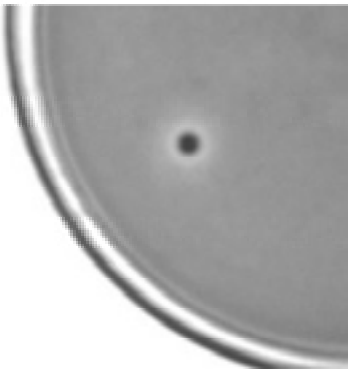

Photo 2. Cellulolysis areas at strain S41

Table 1. Ratio between diameters of lysis areas and diameters of colonies

Strain	Lysis area diameter (D_{la}) [mm]	Colony diameter (D_c) [mm]	$R=D_{la}/D_c$
T41	2.8	10	3.57
T51	3.4	11.5	3.38
T52	5	15.1	3
S41	4.8	15.4	3.2

Photo 3. Microscopic view of the strain T52 (1000x)

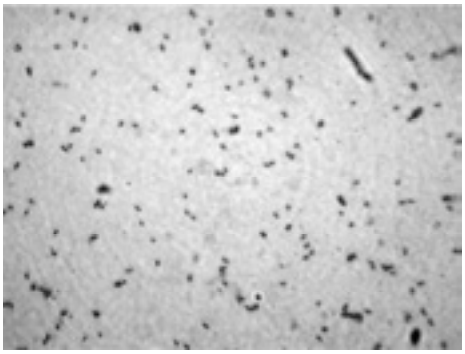

Photo 4. Microscopic view of the strain S41 (1000x)

Effect of pretreatment on the cellulolytic activity of selected strains

Following inoculation of liquid culture medium containing raw and pretreated sawdust with selected bacterial strains, cellulolytic activity for each strain was determined over the incubation period. Determinations were carried out each 24 h, using fresh biological material, for each of the three classes of cellulolytic enzymes. A peculiarity is observed as several values obtained for the enzymatic activity are negative. This phenomenon is explained in detail in a work dealing with the same method for determining the amount of reducing sugars as the one used here [8]. The 3,5-dinitrosalicilic acid used for determinations causes a decrease in the amount of reducing sugars by interacting with their aldehidic groups. The effect can be offset by adding known quantities of glucose to the solution, but this was not done in the present work.

For each of the three classes of cellulolytic enzymes, the activity of the selected bacterial strains is shown in figures 1-12. For both celobiohydrolases and β-glucosidase, higher values are recorded by the strains cultivated in medium containing pretreated sawdust. In the case of endoglucanases, two of the strains have a higher activity in medium with pretreated sawdust, while the other two present a higher activity in medium with raw sawdust. The latter situation might be explained by that the dilute acid used has a similar action with that of endoglucanases on the cellulosic chain, by generating reducing ends from the chain [9]. However, this hypothesis requires further testing.

Generally, the higher cellulolytic activity generated by the pretreatment is assumed to occur due to the release of cellulose from the complex formed with lignin and cellulose, to an increase in pore size and an increase in the contact surface between the enzymes and the cellulosic substrate [9].

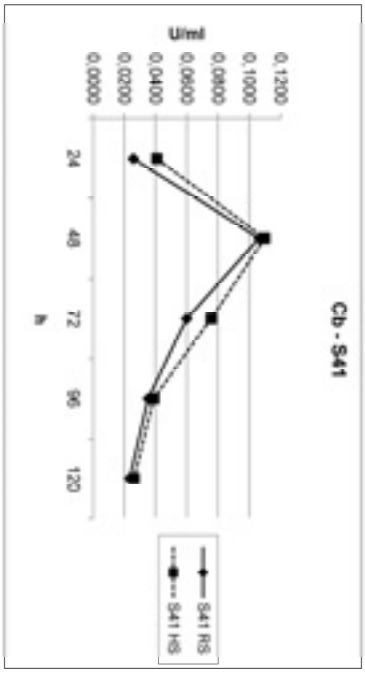

Figure 1. Dynamics of β-glucosidase biosynthesis for strain S41

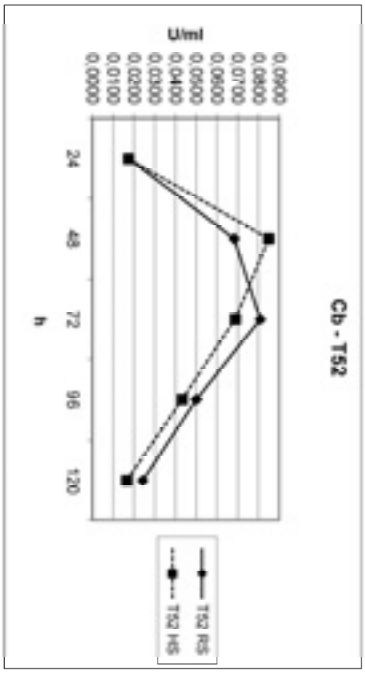

Figure 2. Dynamics of β-glucosidase biosynthesis for strain T52

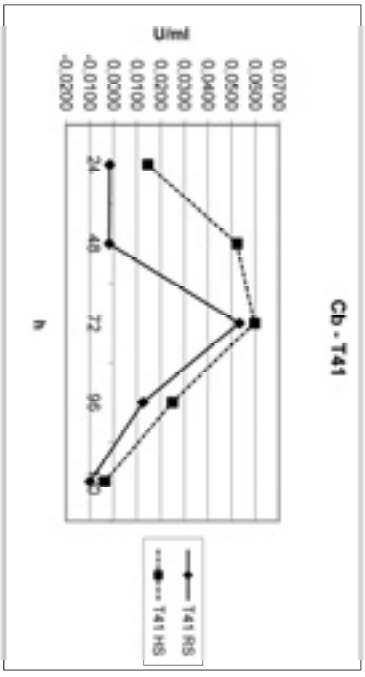

Figure 3. Dynamics of β-glucosidase biosynthesis for strain T41

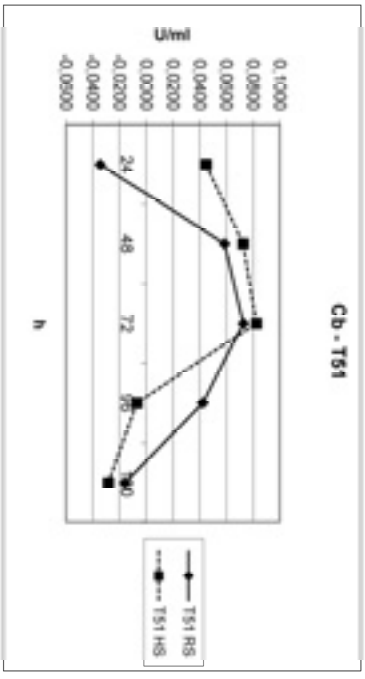

Figure 4. Dynamics of β-glucosidase biosynthesis for strain T51

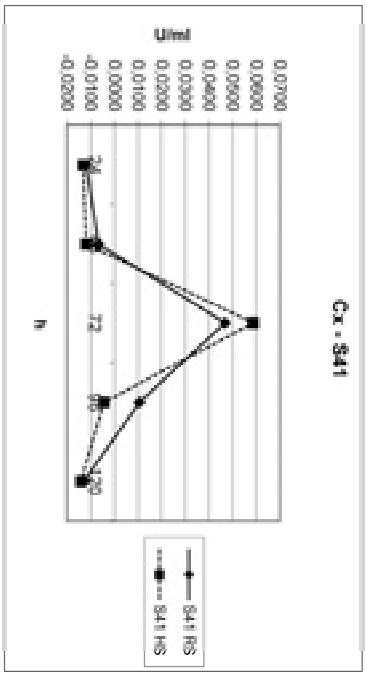

Figure 5. Dynamics of cellobiohydrolases biosynthesis for strain S41

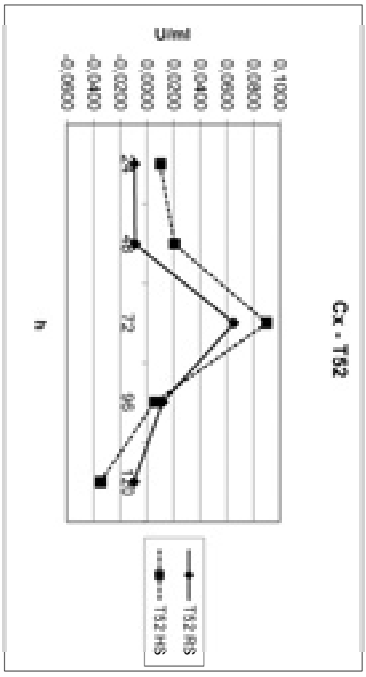

Figure 6. Dynamics of cellobiohydrolases biosynthesis for strain T52

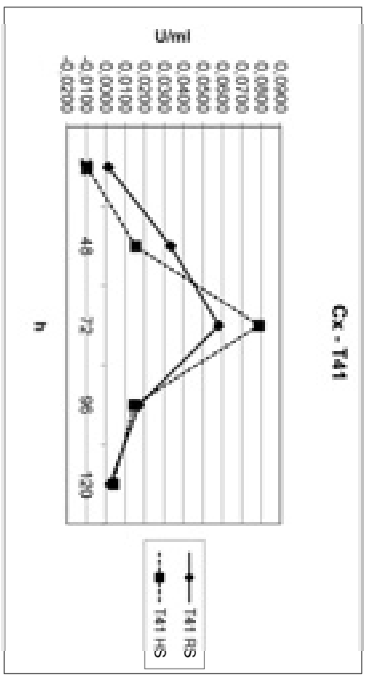

Figure 7. Dynamics of cellobiohydrolases biosynthesis for strain T41

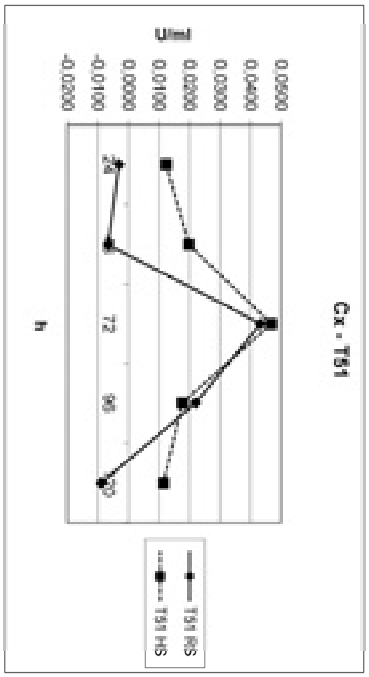

Figure 8. Dynamics of cellobiohydrolases biosynthesis for strain T51

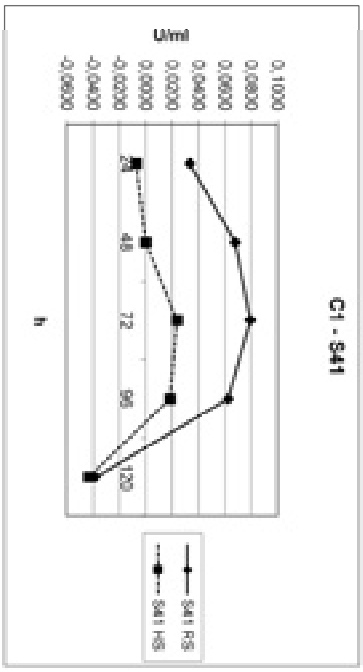

Figure 9. Dynamics of endoglucanases biosynthesis for strain S41

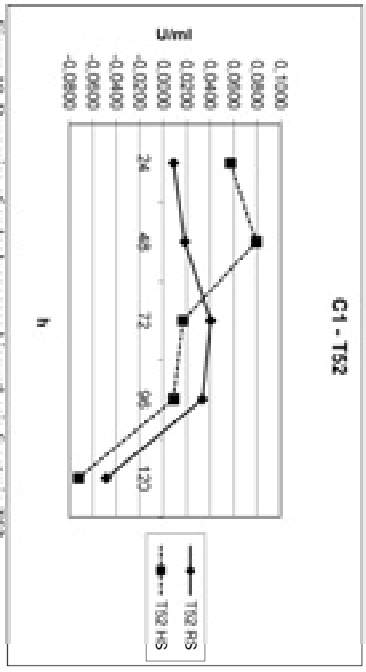

Figure 10. Dynamics of endoglucanases biosynthesis for strain T52

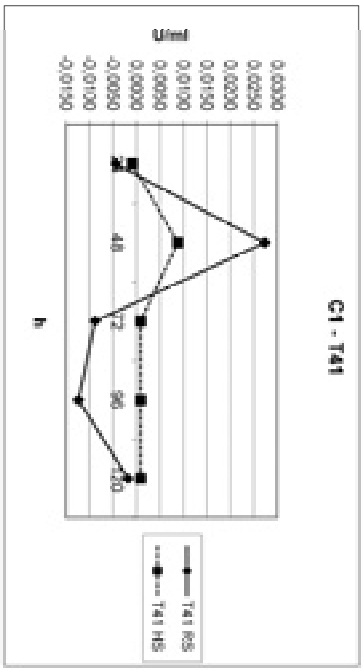

Figure 11. Dynamics of endoglucanases biosynthesis for strain T41

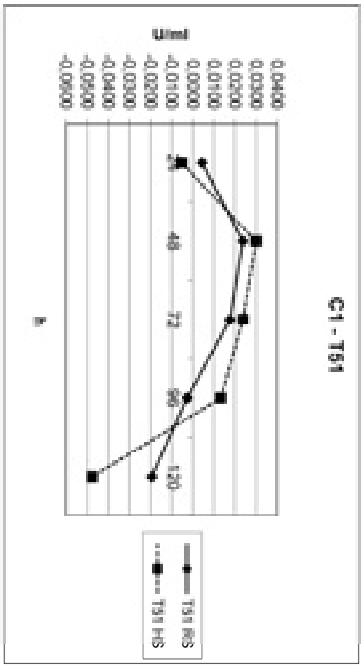

Figure 12. Dynamics of endoglucanases biosynthesis for strain T51

CONCLUSIONS

Conversion of lignocellulosic materials to valuable products is a subject of continuous interest among researchers. Effectively combining chemical and enzymatic hydrolysis seems to be the key to economic success, however improvements are to be made. Recent advancements in chemical treatment of these materials offers higher percentages of recovered sugars, while genetically engineered strains have the ability to convert cellulose faster and even use the resulted sugars [10, 11]. Acid hydrolysis remains one of the preferred methods of converting and pretreating materials, and cellulolytic microorganisms will continue to play an important role in this area of research.

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