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ORIGINAL PAPER

Petroleum Ferrofluid Influence on Cellulase Specific Activity in C*haetomium globosum*

AL. MANOLIU[*], Z. OLTEANU[*], L. OPRICA[*], M. M. ZAMFIRACHE[**], D. CREANGA[***]

[*]*Biological Research Institute, 20A Blvd. Carol I, 6600-IASI, ROMANIA,*
e-mail: a.manoliu@uaic.ro
[**]*Faculty of Biology, "Al. I. Cuza" University, 20A Blvd. Carol I, 6600-IASI, ROMANIA*
[***]*Faculty of Physics, "Al. I. Cuza" University, 11A Blvd. Carol I, 6600-IASI, ROMANIA*

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Abstract

The cellulase production (endoglucanase, exoglucanase, β-glucosidase) was investigated in the presence of five ferrofluid concentrations (20 μL/L, 40 μL/L, 60 μL/L, 80 μL/L and 100 μL/L) supplied in the liquid culture medium. The enzyme activity was spectrophotometrically assayed in the 7th and 11th day of growth.

The ferrofluid effect was found to be a stimulatory one in the case of relatively small concentrations (20 μL/L, 40μL/L and 60 μL/L), as observed in 7 th day for endoglucanase and exoglucanase and even for β-glucosidase (20 μL/L and 40 μL/L). In the 11th day, the stimulatory ferrofluid effect was maintained for endoglucanase (all concentrations) and β-glucosidase (40μL/L, 60 μL/L and 80 μL/L) but an inhibitory influence was noticed for exoglucanase. Relatively high ferrofluid concentrations (80 μL/L and 100 μL/L) gave inhibitory effects especially in older cultures (11 days).

Key words: β-glucosidase, cellobiohydrolase and endoglucanase biosynthesis, iron oleate

Introduction

As part of the complex biological and biophysical studies accomplished in our laboratories on the cellulosolyic fungus *Chaetomium globosum*, the main efforts of last years were directed towards the influence chemical and physical agents on some enzymes within this microorganism cell. So, we carried out experimental researche concerning the modifications determined on the level of the biosynthesis of the cellulase enzymes complex formed by endoglucanase, exoglucanase and β-glucosidase by the mineral nitrogen, aminoacids, trace elements, water-soluble vitamins, pH and temperature. Such investigation clearly showed that the enzymatic activity is individualized function of the enzyme type, culture age and external factors (Al. Manoliu & al, 1999a-e [1 - 5]). These data had confirmed the conclusions of other authors concerning the factors influencing cellulase activity in several fungi (Augustin & al, 1981 [6], Garg & Neelakanian, 1981 [7], Steward & Parry, 1981 [8], D'Souza & Volfova, 1982 [9], Reid, 1983 [10], Sandhu & Kalra, 1985 [11], Prakash & al, 1987 [12], Dwivedi & Sharma, 1989 [13], Uma Devi & Manohara Chary, 1992 [14], Magnelli & al, 1996 [15]).

The aim of this study was to determine the influence of a petroleum ferrofluid on the biology of *Chaetomium globosum* starting from the assumption that iron, though is the fourth most abundant element in the Earth crust, in aerobic conditions and neutral pH, it is unavailable to living organisms, being in the form of ferric hydroxides highly insoluble. However, microorganisms and some plants are able of iron internalization in the form of chemically specific iron ligands named siderophores (Winkelmann, 1985, [16]) .

Such compounds are released into the medium when the iron supply is limiting growth and chelates ferric ions. Metabolic energy is utilized in transporting the iron-siderophore complex across the plasma membrane into the cell. Siderophores are also involved in the storage of iron in the cell. In some fungi these siderophores have the same structure as those involved in transport, but in others they differ. Some fungi do not produce siderophores. These are transporting iron into the cell utilizing a ferric reductase at the cell surface. Fungi that do not themselves produce siderophores, may also utilize those produced by the other fungi if these are available in the environment (Carlile & Watkinson, 1994 [17]).

On the other hand, magnetic bacteria containing 'magnetosoms' were discovered in ocean water moulds (Blackmore & Frankel, 1981 [18]) - in this case iron being involved in the form of magnetite crystals (behaving as small natural magnets). In ferrofluid structure iron is present in the form of ferric and ferrous iron oxides, usually magnetite or ferrite. Iron oxide particles from ferrofluids, having sizes smaller than a magnetization domain, behave as small magnets only in the presence of a magnetic field - superparamagnetism. In a previous scientific paper (Manoliu & al., 1999 f [19]) we reported the petroleum ferrofluid influence on the growth rate as well as on the biomass accumulation dynamics in the fungus *Chaetomium globosum*.

In this study we present the next step in the investigations of ferrofluid influence in *Chaetomium globosum*, specifically the effects on the cellulase enzymes complex. The enzyme systems developed in the microorganisms that are able to degrade cellulose represent quite a variety of enzymes, characterized by different action mechanisms and specificity and acting cooperatively. The cellulasic enzyme system is formed by the next three enzymes:

-Endo-β-1,4-glucanase (1,4- β-D-glucan-4-glucan-hydrolase) has the role of randomly degrading β-1,4-glucosidic bonds from the middle of the cellulose molecule. It does not attack cellobiose but hydrolyzes cellodextrines and substituted celluloses, for instance carboxymethylcellulose. Its specificity is moderate, different sub-types having various affinities for different length oligosaccharides.

- Exo - β - 1,4-glucanase (1,4-β-D-glucan cellobiohydrolase)- cuts step by step cellobiose units from the non-reducing extremity of the cellulose cathene. It hydrolyses the cellodextrines but not the cellobiose. Its substrate specificity is rather high enabling it to degrade more than 80% from the crystalline cellulose while its degree of activity is different for different microorganisms strains.

- β-Glucosidase (β -D-glucoside glucohydrolase) - hydrolyzes both cellobiose and cellooligosaccharides in glucose. It is not able to degrade either cellobiose or cellodextrines with high molecular weigh but favorites this process by removing cellobiose and this way, diminishing the cellobiose accumulated in the medium - such accumulation could inhibit, by feed back, the activity of endo- and exoglucanase. The capacity of degrading the natural cellulose involves necessarily the biosynthesis of the whole enzyme system (Zarnea, 1994 [20]).

Materials and Methods

Chaetomium globosum, MO96 strain was cultivated in the presence of a magnetic fluid prepared from ferrous and ferric salts (Creanga & Cotae, 1994 [21]). The ferrophase was composed from iron oxides particles no larger than 100 Å, coated in oleic acid and dispersed in petroleum (**Figure 1**). The ferrofluid was characterized by 45% (wt) iron oxide particles (a Fe^{II}-Fe^{III} system in the form of γ-Fe_2O_3 and Fe_3O_4 (I.R. investigation, Creanga & Cotae, 1996 [22])). Electron microscopy showed an average physical diameter of 114.2 Å while the magnetic diameter (the difference between the physical diameter and the width of the non-magnetic iron oleate sheet, chemically formed around the magnetic particles) was of 97.6 Å (standard deviation of 0.186 - Creanga & Cotae, 1998 [23]). The volume concentration of magnetic particles was equal to 0.0895, the density was of 1177 kg/m^3 and the viscosity was equal to 4.2 10^{-3} Ns/m^2 (Cotae & al., 1997 [24]). The tested ferrofluid experimental variants in the culture medium were: 20 μL/L (V1), 40 μL/L (V2), 60 μL/L (V3), 80 μL/L (V4), 100 μL/L (V5). The fungal cultures were derived from an inoculum agar discs (diameter of 0.8 cm) transferred on 100 mL of liquid Haynes medium. Cultures were incubating at 28 ^{0}C without shaking.

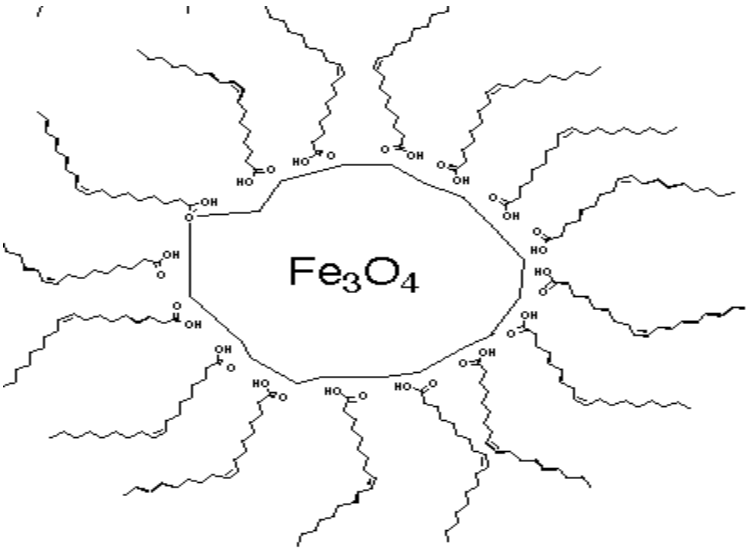

Figure 1. Magnetite particle (Fe_3O_4) stabilized with oleic acid

The cellulase activity, expressed as U/mL, was spectrophotometrically assayed (JASCO – V-530, UV-VIS spectrophotometer) in the culture supernatant using Petterson and Parth method modified by us with the aim of doubling the contact duration between enzyme and substrate (Manoliu et al, 1998f). One unit of cellulase activity, U, is defined as the amount of enzyme required to release reducing sugars equivalent to 1 mg of glucose /h (cellobiohydrolase) or 1mg of glucose /min (endo-β-1,4-glucanase, β-glucosidase)). Enzyme activity is expressed as U/mL. Standard curves with glucose were plotted in order to calculate enzymatic activities. Enzyme assays were accomplished after 7 and, respectively, 11 days from the inoculation.

Results and Discussions

Data concerning the influence of different concentrations of ferrofluid on the enzyme activity can be analyzed from the **Figures 2**, **3** and **4**. Results discussion need to take into account the fact that enzyme activity of the ferrofluid samples may be affected by two major

directions of ferofluid action: the influence upon the biochemical processes related to the enzyme biosynthesis and the influence of enzyme activity of the synthesized molecules. In the first case both chemical composition and physical properties of ferrofluid may be involved while in the second case the physical effect has a higher weight that we may suppose is capable of some destabilization of tertiary and quaternary enzyme structure. However we think that the influence of the iron as a chemical substance is dominant so in the next section we shall consider that the ferrofluid effect is focused on enzyme biosyntehsis. So, in **Figure 2** we can see that after 7 days the high ferrofluid concentrations appear as inhibitory for the biosynthesis of endoglucanase (only small concentrations seem to stimulate enzyme biosynthesis), but after 11 days all the samples present enzyme activity higher than the control. The control is characterized by approximately the same values in the 7th and 11th day - 0.147 U/mL and, respectively, 0.117 U/mL. Relatively small ferrofluid concentrations (V1, V2 and V3) determine the enzyme activity enhancing (from 0.216 U/mL to 0.307 U/mL) in the 7th day.

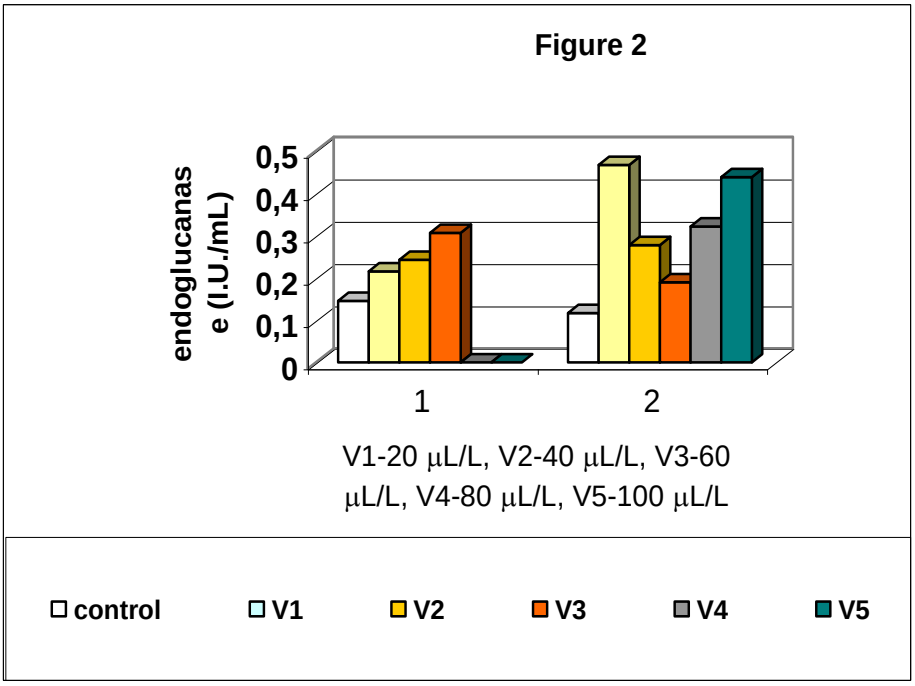

Figure 2. Endoglucanase activity at 7th (1) day and at the 11th day (2).

When assayed in the 11th day, the situation is reversed, the enzyme activity being diminished from 0.468 U/mL to 0.190 U/mL (more than twice). High ferrofluid concentrations (V4 and V5) correspond to an inhibition of enzyme biosynthesis until the 7th day, but a significant stimulation of this biosynthesis process can be seen in the 11th day of growth. Except the case of zero-biosynthesis all the other enzyme activity values are correlated with ferrofluid concentrations - values higher than that of the control. So, we may say that, except for the delay related to the higher ferrofluid concentrations, the endoglucanase biosynthesis was stimulated in samples in comparison to the control.

In **Figure 3** the exoglucanase activity can be seen. The enzyme biosynthesis is spectacularly enhanced during the four days between the two assays both in control (10 times) and in the sample V1 (3 times). The apparently stimulatory effect of the ferrofluid in the V1, V2 and V3 samples is reversed by the inhibitory effect of the V4 and V5 samples where the enzyme activity in the 7th day is annulled.

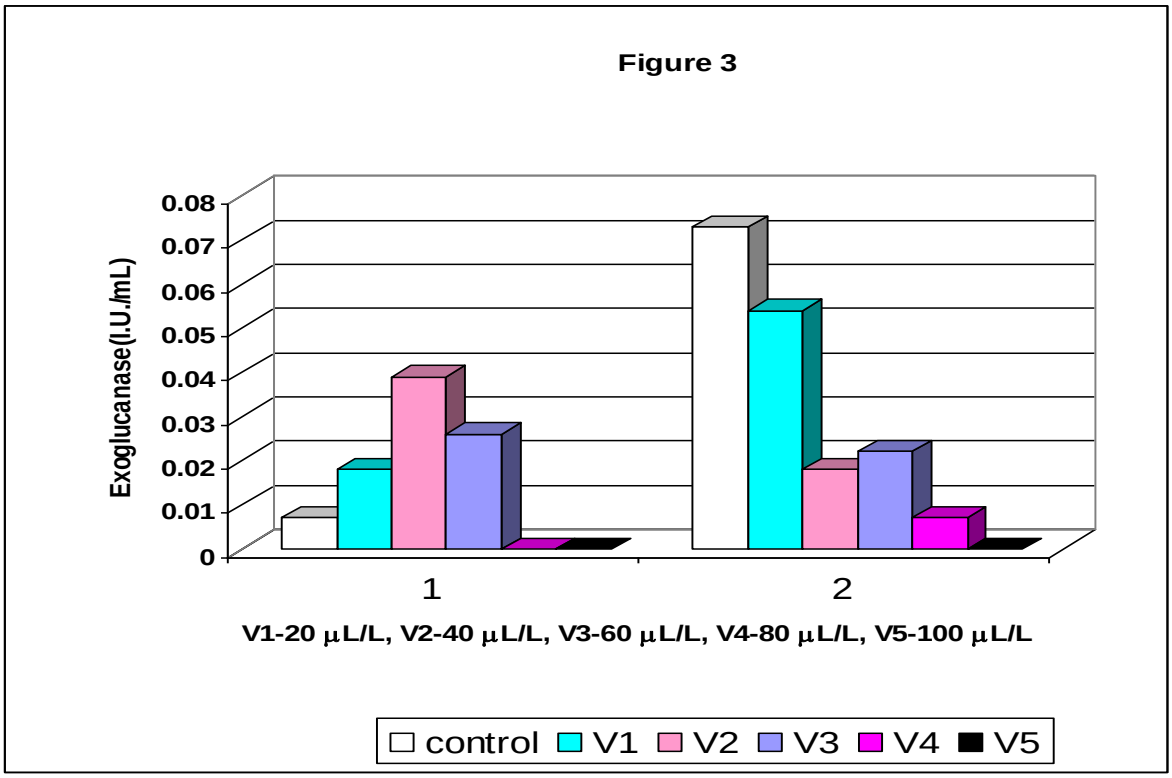

Figure 3. Exoglucanase activity at 7th (1) day and at the 11th day (2)

In the 11th growth day all the samples present smaller enzyme activity in comparison to the control, the V5 variant being characterized by null value. The ferrofluid influence on the β-glucosidase is showed in **Figure 4**.

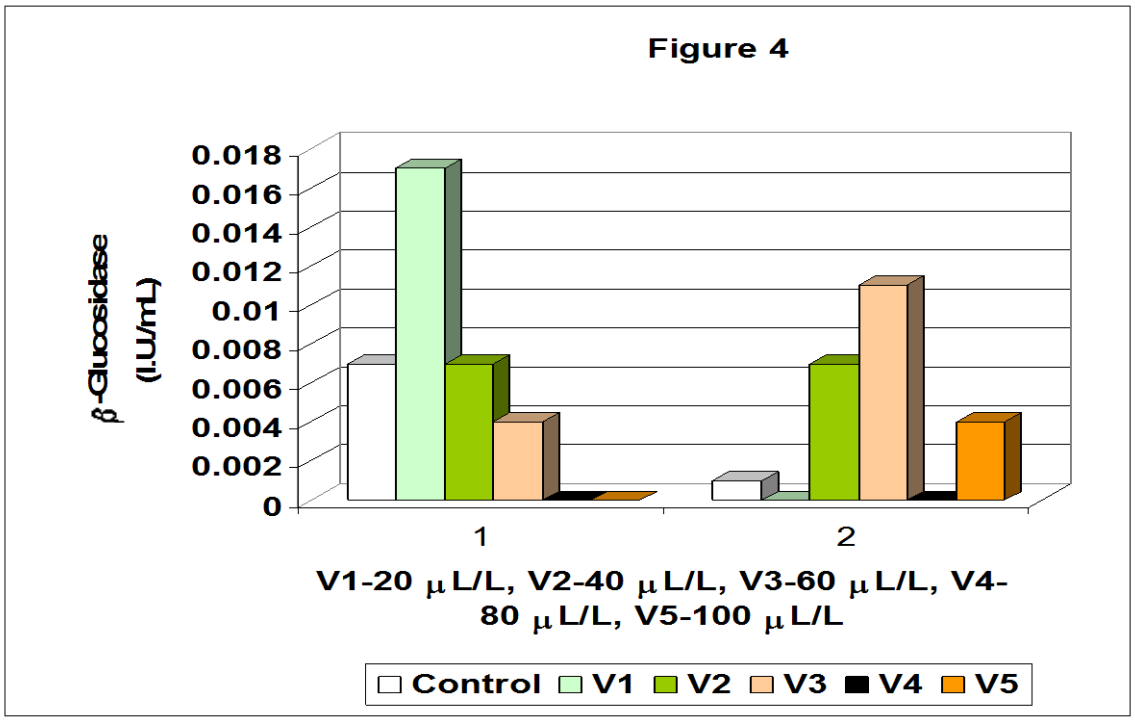

Figure 4. β-Glucosidase activity at 7th (1) day and at the 11th day (2)

For the first 7 days of growth the smallest ferrofluid concentration (20 μL/L) gave an enzyme activity (0.017 U/mL) more than twice higher than in the control (0.007U/mL). But for a ferrofluid concentration of 40 mL/L the enzyme biosynthesis is kept at the same level than in the control (0.007 U/mL), and further, it is reduced to 0.004U/mL for a ferrofluid concentration of 60 μL/L. Finally it is annulled for the ferrofluid concentrations of 80 and 100 μL/L.

In the 11th observation day the control presents the enzyme biosynthesis annulling, as well as the 20 μL/L ferrofluid variant. Higher ferrofluid concentrations have a neutral effect (40 μL/L and 80 μL/L variants let the enzyme activity at the same levels as in the control) or a stimulatory one (60 μL/L variant enhances more than twice - from 0.004 U/mL to 0.011 U/mL the enzyme activity while the 100 μL/L variant enhanced from zero to 0.004 U/L the enzyme activity).

Taking into account all of the above discussion, the question that issued is: how could the ferrofluid actually influence the enzyme biosynthesis? Is there only a chemical effect, or is a magnetic one, or both? The answers must be given considering the ferrofluid properties (Manoliu et al., 2001a-b [25-26]). First, the ferrofluid is a colloidal suspension. This colloidal medium means not only the formation of colloidal particles of magnetite in the petroleum carrier fluid, but also the appearance of a sheet of iron oleate at the magnetite particle surface. In this compound, oleic acid is chemically bond to the iron particles reducing the physical diameter of ferrophase particles to the magnetic diameter (which is smaller than the physical diameter with an aliquot equal to the double of the size of the elementary crystallization cell of magnetite).

So, a significant quantity of iron oleate is delivered to the fungus culture medium besides the iron in the form of magnetite. It is possible that this way the microorganism can easier internalize the iron atoms within its body cell, siderophores based on iron complexes being indispensable to their metabolism. The magnetic effect is negligible since the cultures developed in liquid media where the putative elementary magnets of the ferrophase particles move freely and the resultant magnetic field is statistically zeroed.

Conclusion

The main issue of the present study is that the utilization of this fungus in some cellulose biotechnological projects could be monitored by means of the ferrofluid addition to the culture medium, due to the iron content of such material. We noticed that ferrofluid concentration range is important as well as the culture age: small concentration values led to stimulatory effects in younger cultures while higher concentration values had mainly inhibitory effects, especially in older cultures.

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