INFLUENCE OF MULTIPLE SHORT HIGHLY ENERGETIC X-RAY PULSES ON THE BIOSYNTHETIC AND PHYSIOLOGICAL ACTIVITY OF THE STRAIN Trichoderma reesei M7

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Abstract. In order to increase the biosynthetic capacity of the Trichoderma reesei-M7 strain – a notorious cellulase enzyme producer – the experimental biological object to be studied was exposed to the impact of the highly energetic X-ray pulses, produced by the dense plasma focus device (DPF). The X-ray pulses thus produced can have exceptionally high density of the flow of X-photons. The pulsed mode of irradiation fundamentally differs from the continuous when considering its effect on biological objects – they can rapidly absorb great amounts of radiation in a glimpse, leading to a qualitatively different interaction with their matter. In the experiments done the survivability was determined, as well as the vegetative biomass, four types of cellulase activity and the quantity of total protein after the treatment of the spores suspension (2.10^5 CFU/ml) of Trichoderma reesei M7 with this pulsed X-radiation. The absorbed doses were in the range of 7 to 45000 mSv. It was noticed that the survivability of the strain, if compared with the control samples, drops, reaching 57% of the initial value (of the untreated spores) for the sample with 45010 mSv of absorbed dose. From the data presented below it can be concluded that three of the samples with high absorbed doses, namely with 11754, 19439 and 42917 mSv, demonstrated increases their cellulase activities. In the case of 42917 mSv the increment of the endoglucanase activity, compared to the control, reaches 41,1%, FP arises with 30,3%, and the β-glucosidase activity nearly doubles – and increase of 92,7%.

1. Introduction
Hydrolysis of cellulose to glucose with the aim of biotechnological agents can be used in the synthesis of forage protein, of alcohol for energy uses, and the glucose produced can become an initial product for obtaining important chemical compounds for the industry. The degradation of cellulose using cellulolytic enzymes appears an economically profitable and perspective way to do the job.
The high energy X-radiation has the ability to change the structure of the macromolecules, thus altering the biophysical characteristics of the biologic objects. The pulsed nature of the radiation produced this way is due to the short (less than microseconds) duration of the discharge in the low-inductance circuitry of the machine. In order to increase the biosynthetic capacity of the Trichoderma reesei-M7 strain – a notorious cellulase enzyme producer – the experimental biological object to be studied was exposed to the impact of the highly energetic X-ray pulses, produced by the dense plasma focus device.

2. MATERIALS AND METHODS

2.1. Irradiation method, DPF and dose readings

The enzyme-based processes, used in the bioconversion of cellulose-containing substrates have taken their important positions in various industrial branches and the increased interest towards them is partially related to the intense search for nutrient sources, produced on the base of waste materials, as well as to the reveal of new, virtually unlimited chemical energy sources.

In ecologic perspective the cellulases are appropriate means for utilization of the constantly rising quantities of cellulose-containing wastes from the wide variety of human activities, accelerating in such way the natural transformation of the vegetative biomass.

It is proved for some plants and algae that not only the absorbed dose of radiation alters their cells biosynthetic potential, but also the time for which the same radiation dose was received by the test species [1,2].

The aim of this study was to establish the effect of the dose in a comparatively wide range on the organisms described. To achieve the high dose range a Dense Plasma Focus (DPF) device was chosen.

Dense Plasma Focus is a pulsed-operation gas discharge plasma device, using the effect of a current sheath, self-focussing due to interaction with its own magnetic field (so-called Z-pincho). It produces highly intense discharge in rarefied gas atmosphere and in a very compact space.

As an effect much accelerated particles are emitted in a short period of time (with the duration of the whole process being no more than a few microseconds and knowing that the plasma diode accelerating the particles to extreme velocities, takes no more than 100 – 300 ns of that time). Interaction of these particles, namely relativistic electrons and highly energetic ions takes place with the material of the metallic surfaces inside the chamber of the device. Electrons hit the anode cap, specially designed to have changeable metallic insert on its top and thus produce large amount of X-radiation, characteristic for the material of the insert. In our case it is tungsten and so tungsten Kα lines dominate the spectrum of the radiation bursts produced. The relativistic electron current, causing the X-radiation is quite high and reaches few tens of % of the full current through the discharge, although for a short period of time (few tens of ns). In our case this full current can be as high as about 200 kA.

To be precise in the assessment of the absorbed dose, we have used thermo-luminescent dosimeters, placed in the same conditions as the test tubes, containing the spore material and also in the same type of a test tube, packed in groups in order to carefully take an average and exclude accidental errors during the reading process. These dosimeters are packed in a 10 μm aluminium foil to preserve them from light, contamination with components of the environment such as dust and moisture (as they are highly sensitive to such contamination) and are faced down so as to point towards the radiation source. That is how the X-radiation dose is measured, having the X-rays passing through a window in the chamber of the DPF. The dose rate is calculated based on the oscillography images of the signal of the scintillation detector, monitoring this type of radiation in time. It can be as high as 1 to 5 mSv/ns, depending on the charge of the capacitors, pressure of the working gas, atomic number of the anode cap insert and some other less important conditions.

TLDs are read in a special dark oven, with the temperature inside linearly increasing and constantly measuring the radiation from the decaying excited states in the material with a photomultiplier. So a curve is achieved, showing the quantity of radiation produced by each tablet during its heat-reading.
The integral of this curve is proportional to the absorbed dose and is carefully calibrated to respond the numerical value of the real dose absorbed by the material. More about this PF device and the details of the irradiation procedure can be found in the previous paper [2].

2.2. Microorganism and fermentation conditions
The fungal strain Trichoderma reesei M7 was obtained from the strain collection of the Department of Biotechnology, Faculty of Biology, Sofia University St. Kliment Ohridski (Bulgaria) and used in the present study. The M7 strain is obtained from Trichoderma sp.914 by screening technique and successful mutagenic treatment [3,4].

The cultures were maintained on potato dextrose agar at 28±1°C for four days. Inoculums were obtained in 500 cm3 flasks which contained 100 ml Mandels mineral salt medium [5] with added 2% glucose and 1% maize extract at 28°C with constant shaking (250 rpm) for 24 hours. Fermentation mixture (50 ml) was composed of Mandels mineral salt medium, 2% microcrystalline cellulose Avicel® and 1% wheat bran.

2.3. Determination of enzyme activity
The methods we have adopted and used in our studies of the enzyme activities, taking place in the composition of the cellulase complex, are in accordance with the ones, recommended by the International Union of Pure and Applied Chemistry (IUPAC). Here they are:

2.3.1. Endo-1,4-β-glucanase activity. Endo-1,4-β-glucanase activity was detected on sodium carboxymethyl cellulose (Na-CMC) as substrate according to Wood and Bhat [6]. Reaction mixtures containing 0.5 ml 1% solution of Na-CMC in 0.05 M Sodium-acetate buffer (pH 4.8) and 0.5 ml enzyme solution, were incubated at 50°C for 30 min.

2.3.2. Determination of FPA. Whatman № 1 filter paper (special 1 x 6 cm stripes), is used as a substrate for determination of the overall cellulase activity [5]. The reaction mixture so obtained is incubated for 60 min at 50°C, then the quantity of reducing groups is defined according to the Shomogy-Nelson procedure [7]. For a unit of activity this enzyme quantity is assumed, which leads to the release of 1 µmol of glucose for 1 min, according to the experimental conditions.

2.3.3. β-glucosidase activity. Activity is determined using p(4)-nitrophenyl-β-D-glucopyranoside (pNPG) (Acros organic) as a substrate for the enzyme to act on. The reaction mixture contains 0,2 ml of suitably rarified enzyme solution (cultivation liquid), 0,2 ml substrate (14 mM pNPG) and 0,4 ml 0,1М acetate buffer (pH 4,5). The mixture is incubated for 60 min at 40°C. After addition of 20 ml sodium carbonate (Na2CO3) the reaction is terminated [8]. The quantity of p-nitro phenol is measured spectrophotometrically at a 400 nm wavelength. Then activity of β-glucosidase is defined as the enzyme quantity, leading to catalytic release of 1 µmol p-nitro phenol for 1 min, according to the experimental conditions.

2.3.4. Endoxylanase activity. Endoxylanase activity was determined by measuring the amount of reducing sugars released by hydrolysis of birchwood xylan (Sigma) through a colorimetric assay [9–11], based on the Somogyi–Nelson method with xylose as a standard [7]. One unit of xylanase activity was defined as the amount of enzyme required to liberate 1 µ mol of xylose per min at 40°C, pH – 4.0.

2.4. Determination of the total soluble protein
The soluble protein concentration was determined according to Lowry et al. [12], using bovin serum as standard.
2.5. **Biomass Dry Weight (BDW) measurement**

The biomass dry weight (BDW) was estimated by centrifugation of 30 ml aliquots of culture at 4000 x g for 15 min. Each pellet was washed twice and dried at 105° C until the weight was invariant by analytical hygrometric balance (KERN).

2.6. **DPF device**

To obtain highly energetic pulsed X-radiation, which was used to treat the strain, a dense plasma focus device of the Madder type was used. The plasma inside is formed in argon gas with the charging voltage of 20 kV and pressure of 0.8÷0.9 Torr in the chamber of the DPF. The plasma, being superheated to extreme temperatures by the current and shrunk by its magnetic field, starts to emit highly energetic charged particles, including electrons. These electrons hit the tungsten top surface of the anode and so release X-radiation with a characteristic spectrum.

2.7. **Irradiation of the spore material**

The dose absorbed by the producer and the solution is represented in miliSievert units (mSv). Spore material with concentration of 2.10^5 CFU/ml was preliminarily suspended in 0.9% solution of NaCl before irradiation. Absorbed doses of X-radiation were adjusted in a wide range, extending from 7 to 45 000 mSv and measured with TLD.

**3. RESULTS AND DISCUSSION**

In this study the survivability, the biomass, four hydrolase activities and total protein were measured after treatment of spores suspension (2.10^5 CFU/ml) of the producer *Trichoderma reesei* M7 with highly energetic pulsed X-radiation.

Biomass has been measured after turning it to Biomass Dry Weight (BDW). Measurements were carried out after 24 hours of submerged cultivation process of the samples in a nutritious medium. Despite the decrement of survivability of the spores material and the decreased quantity of BDW in review, it must be underlined the overall resistibility of the strain, sustaining vitality even after being subjected to extreme radiation loads. The quantity of biomass accumulated, as a consequence of the lower survivability of the strain mentioned, drops. Above doses of the order of 10 000 mSv the biomass drops from 42.8% to 52.3% (for 45 010 mSv).

Within the 230-700 mSv range of absorbed doses the accumulation of biomass decreases not proportionally to their values. The extracellular protein, measured at 120-th hour of the fermentation process, is in excess, which comes to show the inverse dependence between the biomass and the protein in this range.

The vegetative material obtained is used for sowing in fermentation nutritious medium (2% crystal cellulose) and for fruition of depth synthesis hydrolytic enzymes. In table 2 the values of 4 enzyme activities measured are shown – that of the endoglucanase, FPA, β-glucosidase and endoxylanase are shown, as well as the total protein at 120-th hour of the cultivation process.

The viability of the spores’ material is measured relatively to a control sample with the same concentration, about 2.10^5 CFU/ml in this study (Table 1) after sewing the spores on PDA (Potato Dextrose Agar), followed by cultivation at 28°C for 7 days.

With concentration of the spores suspension of 2.105 CFU/ml the survivability of the strain, compared to the control samples, drops and in the case of the material, irradiated with 45 010 mSv this survivability is about 57% of the corresponding value for the untreated ones.

The experiment was continued with the consequent cultivation stage – the obtaining of vegetative biomass (Table 1).

It can be seen from the data present that in three of the cases, where the material has high doses absorbed, namely 11754, 19439 and 42917 mSv – that the cellulase activities rise.
Table 1  Effect of absorbed radiation dose on the viability of a spore material (2.105 CFU/ml) of the T. reesei M7 strain and on the accumulation of producer biomass.

| Absorbed dose [mSv] | Viability of spores [%] | BDW* [mg/ml] |
|---------------------|------------------------|--------------|
| Control             | 100                    | 3.90         |
| 7                   | 100                    | 3.48         |
| 28                  | 96                     | 3.29         |
| 37                  | 96                     | 3.27         |
| 89                  | 94                     | 3.21         |
| 134                 | 95                     | 3.22         |
| 178                 | 95                     | 3.19         |
| 206                 | 94                     | 3.20         |
| 232                 | 95                     | 2.76         |
| 475                 | 92                     | 2.61         |
| 515                 | 91                     | 2.53         |
| 541                 | 91                     | 2.56         |
| 618                 | 88                     | 2.68         |
| 669                 | 89                     | 2.52         |
| 1174                | 85                     | 2.81         |
| 2360                | 83                     | 2.78         |
| 4216                | 74                     | 2.75         |
| 5601                | 74                     | 2.61         |
| 6265                | 73                     | 2.58         |
| 7318                | 74                     | 2.51         |
| 7491                | 71                     | 2.54         |
| 9542                | 70                     | 2.29         |
| 10346               | 69                     | 2.23         |
| 11754               | 67                     | 2.28         |
| 12662               | 66                     | 2.21         |
| 19439               | 64                     | 2.13         |
| 31893               | 61                     | 1.96         |
| 42413               | 59                     | 1.87         |
| 42917               | 60                     | 1.89         |
| 45010               | 57                     | 1.86         |

*BDW: Biomass Dry Weight

For the leading endoglucanase activity the increment, compared to the control sample, is as follows: 11754 mSv – 16.1%; 19439 mSv – 22.4%; 42917 mSv - 41.1%. Rise of the FPA can be seen with 42917 mSv – 44.4% higher than normal values with 11754 mSv absorbed and peaks with 42917 mSv, where the activity of the enzyme is 92.7% higher than the control. Also, some overall rise of the enzymes biosynthesis in this last case takes place, which can be noticed not only for the cellulases, but for the endoxylanase activity as well.

The negative effect of the ionizing radiation is most visible for the experimental spore material with absorbed doses between 12662 mSv and 45010 mSv – here an overall drop of the biosynthetic potential of the strain is established.

Results for the 6265 mSv sample are quite interesting – here we see higher FP-activity (4.13 FPU/ml) than the control, but lower endoxylanase activity (4.92 IU/ml) as well. Or the sample with 7491 mSv, where it’s practically the opposite – lower FPA compared to the control (3.41 FPU/ml), but higher for the endoxylanase (6.12 IU/ml). This finding needs further study.

The data, related to the extracellular protein released, in short, show the absence of considerable change in the extracellular quantities, synthesized by the treated material under consideration despite the different intensities of irradiation. The minimal such quantity measured is 1.69 mg/ml for 5601 mSv and the maximal - 2.31 mg/ml for 541 mSv (having the control sample with 2.11 mg/ml).
Table 2 Biosynthesis of hydrolytic enzymes after irradiation of spores material (2.105 CFU/ml) of the strain M7 with a DPF X-radiation source

| Absorbed dose [mSv] | Endoglucanase activity [IU/ml] | FPA* [FPU/ml] | β-glucosidase activity [IU/ml] | Endoxylanase activity [IU/ml] | Total soluble protein [mg/ml] |
|---------------------|-------------------------------|----------------|-----------------------------|-----------------------------|-----------------------------|
| control             | 30.4                          | 3.79           | 1.51                        | 5.62                        | 2.11                        |
| 7                   | 29.2                          | 3.81           | 1.54                        | 5.98                        | 2.15                        |
| 28                  | 28.6                          | 3.78           | 1.39                        | 5.89                        | 2.09                        |
| 37                  | 29.2                          | 3.51           | 1.54                        | 5.91                        | 2.13                        |
| 89                  | 28.4                          | 3.73           | 1.47                        | 5.77                        | 2.01                        |
| 134                 | 28.3                          | 3.81           | 1.61                        | 6.18                        | 1.97                        |
| 178                 | 29.9                          | 3.49           | 1.58                        | 5.83                        | 2.21                        |
| 206                 | 30.8                          | 3.81           | 1.47                        | 5.57                        | 2.08                        |
| 232                 | 32.1                          | 3.89           | 1.61                        | 5.98                        | 2.20                        |
| 475                 | 33.8                          | 3.77           | 1.47                        | 6.19                        | 2.19                        |
| 515                 | 35.2                          | 4.35           | 1.75                        | 6.43                        | 2.29                        |
| 541                 | 35.8                          | 4.21           | 1.89                        | 7.08                        | 2.31                        |
| 618                 | 31.3                          | 3.91           | 1.66                        | 7.14                        | 2.25                        |
| 669                 | 30.6                          | 3.82           | 1.54                        | 6.32                        | 2.17                        |
| 1174                | 28.1                          | 3.50           | 1.31                        | 5.87                        | 1.97                        |
| 2360                | 28.7                          | 3.63           | 1.49                        | 5.69                        | 2.06                        |
| 4216                | 29.4                          | 2.49           | 1.38                        | 5.46                        | 1.84                        |
| 5601                | 26.8                          | 3.41           | 1.57                        | 5.11                        | 1.69                        |
| 6265                | 29.9                          | 4.13           | 1.61                        | 4.92                        | 1.87                        |
| 7318                | 28.3                          | 3.66           | 1.35                        | 5.87                        | 1.98                        |
| 7491                | 31.5                          | 3.41           | 1.62                        | 6.12                        | 1.86                        |
| 9542                | 30.2                          | 3.66           | 1.34                        | 5.68                        | 1.94                        |
| 10346               | 28.3                          | 3.18           | 1.80                        | 6.42                        | 2.08                        |
| 11754               | 35.3                          | 3.83           | 2.18                        | 5.93                        | 2.24                        |
| 12662               | 25.4                          | 3.45           | 1.41                        | 4.76                        | 2.01                        |
| 19439               | 37.2                          | 3.86           | 1.36                        | 6.79                        | 2.17                        |
| 31893               | 30.6                          | 3.77           | 1.62                        | 5.74                        | 1.99                        |
| 42413               | 29.8                          | 3.94           | 1.43                        | 5.67                        | 2.09                        |
| 42917               | 42.9                          | 4.94           | 2.91                        | 7.65                        | 2.18                        |
| 45010               | 23.7                          | 2.81           | 1.23                        | 4.41                        | 1.74                        |

FPA*: Filter Paper Activity

It is characteristic for the material, irradiated in the dose range of 230 – 700 mSv that it shows rise of the enzyme synthesis and also increased quantities of full protein released. Maximal rise is observed in the 515 and 541 mSv cases, where for 541 mSv the endoglucanase activity is increased by 17.8%, FPA with 11.1% and β-glucosidase activity with some 25.2%. Considerable increment of the synthesized endoxylanase – 26% - is also demonstrated.

On the basis of the quantity of the extracellular protein and the corresponding hydrolase activity, the specific enzyme activities at 120-th hour of the fermentation process are calculated and shown on Figure 1.

The results, present at Figure 1, show that the dose of 42917 mSv has the greatest effect in terms of increasing the specific enzyme activity. The observed increment in the specific activities suggests that the major part of the released extracellular protein consists of cellulase protein. This is especially evident for the specific activity of β-glucosidase, which is about 84.7% higher in the case.

High specific activities of the β-glucosidase and endoglucanase are observed at doses of 11754 and 19439 mSv as well.

The material irradiated with a dose of 6265 mSv shows a rise in the specific FPA and this irradiated with a dose of 5601 mSv of X-radiation shows a high specific β-glucosidase activity. The higher
specific activities in those cases could be due to the relatively low extracellular protein, established in
the respective case.

Figure 1 Specific hydrolase activities after irradiation of T. reesei M7 with a 4 kJ DPF device: A) Endoglucanase, B) FPA, C) β-glucosidase, D) Endo xylanase.

4. CONCLUSION
Having all the results considered, it can be said that the DPF device with the radiation generated by it alters the physiological and biochemical indices of the spores material treated. With the exception of the 230 – 700 mSv dose range (where specific repeated dependence takes place, namely reduced biomass – increased biosynthetic potential, including extracellular protein) in the general case the change of the metabolite expression of the strain is not proportionally related to the absorbed dose.

It again deserves pointing out the high vitality, kept by the producer despite the relatively large amounts of radiation accepted. Moreover, not only the vitality appears high, but also the activity of the metabolite expression measured not only remains the same, but in some cases considerably exceeds the control values.

Considering the wide variety of treated specimens and their specific hydrolase activities, the supposition appears ever more justified that despite the extremely wide absorbed dose range in consideration (between 7 and 45010 mSv), the quantitative range of metabolite expression remains much narrower. In other words, despite the variability of treatment the micromycete retains its biosynthetic expression nearly constant.

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