

## The influence of shielded magnetic field on the cell viability and nitrogenase activity of *Azotobacter chroococcum*

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**Abstract.** This paper studies the magnetic field influence on the viability of *Azotobacter chroococcum* 2286<sup>T</sup> cells. The viability of the *Azotobacter chroococcum* cells incubated under shielded magnetic field was established according to the nitrogen accumulation rate in the culture medium, related to the control samples incubated in normal lab conditions. The inoculation was made from solid medium, and from liquid medium, at a bacterial density of  $1.5 \cdot 10^6$ . After incubation the  $\text{NH}_4^+$ ,  $\text{NO}_2^-$  and  $\text{NO}_3^-$  ions concentration in the medium was determined. The total nitrogen quantity was determined through Kjeldahl method. The nitrogenase activity was established by the reduction of  $\text{C}_2\text{H}_2$  to  $\text{C}_2\text{H}_4$  and by detecting it using a gas chromatograph.

**Keywords:** shielded magnetic field, viability, *Azotobacter chroococcum*.

**Résumé.** Cet étude suit l'influence du champ magnétique sur la viabilité des cellules d'*Azotobacter chroococcum* 2286<sup>T</sup>. La viabilité des cellules d'*Azotobacter chroococcum* a été établie en suivant les taux d'accumulation d'azote dans un milieu de culture liquide, dans la cage de Faraday, comparé avec le témoin incubé dans des conditions normales de laboratoire. L'inoculum a été effectué en deux variante, d'un milieu solide, avec une anse, et respectivement d'un milieu liquide, à une densité des bactéries de  $1,5 \cdot 10^6$ . Après l'incubation, on a déterminé la concentration dans l'environnement des ions  $\text{NH}_4^+$ ,  $\text{NO}_2^-$  et  $\text{NO}_3^-$ . Le montant total d'azote du milieu de culture a été déterminé par la méthode Kjeldahl. L'activité nitrogenasique a été déterminée par le test de réduction du  $\text{C}_2\text{H}_2$  à  $\text{C}_2\text{H}_4$  et sa détection avec l'aide du gaz chromatographe.

**Mots clés:** champ magnétique, viabilité, *Azotobacter chroococcum*.

**Rezumat.** Acest studiu urmăreşte influenţa câmpului magnetic ecranat asupra viabilităţii celulelor de *Azotobacter chroococcum* 2286<sup>T</sup>. Viabilitatea celulelor de *Azotobacter chroococcum* a fost stabilită prin urmărirea ratei acumulării azotului în mediul de cultură, în cutia ecranată, comparativ cu martorul incubat în condiţii normale de laborator. Inoculul s-a făcut în două variante, de pe mediul solid, respectiv din mediul lichid, la o densitate a bacteriilor de  $1,5 \cdot 10^6$ . După incubare s-a determinat concentraţia în mediu a ionilor  $\text{NH}_4^+$ ,  $\text{NO}_2^-$  şi  $\text{NO}_3^-$ . Cantitatea de azot total din mediul de cultură s-a determinat prin metoda Kjeldahl. Activitatea nitrogenazică s-a determinat prin testul de reducere a  $\text{C}_2\text{H}_2$  la  $\text{C}_2\text{H}_4$  şi detectarea acesteia cu ajutorul gaz-cromatografului.

**Cuvinte cheie:** câmp magnetic ecranat, viabilitate, *Azotobacter chroococcum*.

**Introduction.** The ecological distribution of *Azotobacter* species is affected by different environmental factors. It is proved that the distribution of these microorganisms is influenced by climatic conditions and soil characteristics but also by organic matter content, pH and the C/N ratio (Tilakk et al 2005). In order to study the growth characteristics and also the adaptability to different environmental conditions the influence of the shielded magnetic field on these parameters was recorded.

*Azotobacter* species are free, aerobic bacteria which may also grow at low oxygen concentrations (Tejera et al 2005). These are heterotrophic species, but related to their nitrogen nutrition they are autotrophic, introducing in biological cycle the anorganic form of nitrogen (Garritty et al 2004).

Diazotrophs fix the molecular nitrogen in several steps, the first one consisting of reducing the triple bond between the two atoms of  $\text{N}_2$  molecule by bonding to H,

according to the reaction:  $N_2 + 3H_2 \rightarrow 2NH_3$  (Corbett et al 2006). The reaction is catalyzed by an enzymatic system known as nitrogenase, whose synthesis is controlled by genes clustered in the *nif* operon (nitrogen fixation), situated in the bacterial chromosome (Rees & Howard 2000; Einsele et al 2002). Therefore, the fixing  $N_2$  reaction product is  $NH_3$ , which immediately enters the synthesis cycles of nitrogen organic substances. The best known and most important pathway of nitrogen fixation is the so called Gogat pathway in which  $NH_3$  is incorporated into glutamate, through a two steps reaction: 1. L-glutamic acid accepts an  $NH_3$  molecule in a reaction catalyzed by glutamine-synthetase and a glutamine molecule is formed; 2. the amidic group from glutamine is reducibly transferred to  $\alpha$ -ketoglutarate resulting glutamate, reaction catalyzed by glutamate-synthetase (Berg et al 2002; Hu et al 2005).

Once entered in the composition of an organic substance, the nitrogen is still subjected to transformations common to all the living cells. Succeeding the death of bacterial cells, in the medium is released also  $NH_3$ , fixed by these from the air. This explains the detectable presence of  $NH_3$  in the culture medium, otherwise a substance toxic even to the bacteria (Giller 2001). It was expected not to detect the presence of  $NO_2^-$  and  $NO_3^-$  ions, because oxidation of reduced nitrogen compounds is carried out by bacteria belonging to another ecophysiological group – nitrifying bacteria (Christiansen et al 2000).

Researches concerning inorganic substrate transformation by microorganisms and organic substance synthesis through plant photosynthesis were also carried out by Dobrotă et al (2004).

The present study is part of a complex approach considering inorganic substances transformation by microorganisms, with the possibility of using them as a substrate by photoautotrophs, which produce organic substance. The aim of this research is to improve the premises of self supporting complex biological systems able to maintain themselves in successive life cycles.

**Materials and Methods.** In order to survey how magnetic fields affect the viability, the nitrogen accumulation in the culture media and the nitrogenase (an iron-sulphur enzyme) activity of *Azotobacter chroococcum*, the biological tests were incubated under shielded magnetic field and also in normal geomagnetic environment. The shielding level was about  $10^{-6}$  of the terrestrial magnetic field, provided by a double permalloy chamber.

The microorganism used in this study was *Azotobacter chroococcum* 2286<sup>T</sup> obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH).

The determination of nitrogen accumulation rate in the liquid culture medium, in the magnetic shielded chamber, relative to the control incubated in normal lab conditions, was accomplished by determining  $NH_4^+$ ,  $NO_2^-$  and  $NO_3^-$  ions concentration in the medium using Hanna C200 Multiparameter.

The total nitrogen quantity in the culture medium was determined by Kjeldahl method (Paulette 2007).

The nitrogenase activity was quantified by reduction of  $C_2H_2$  to  $C_2H_4$  and gas-chromatographic detection. Modified Burk (manitol B) liquid media were prepared (Atlas 2004) with three chelation agent concentrations (EDTA 0.008%, 0.01%, and 0.12%), and they were incubated for 12 hours (consonant with the growth curve) under normal geomagnetic conditions and also in shielded magnetic field.

**Results and Discussion.** The first step was to determine the nitrogen accumulation rate in the liquid culture medium, in the shielded chamber, related to the control incubated in normal lab conditions. The inoculation was made from solid medium, and from liquid medium, at a bacterial density of  $1.5 \cdot 10^6$ , determined through plate culture of successive dilutions method, the inoculation volume being 1 ml/100 ml fresh medium. During incubation the flasks were stirred manually, because an electric or magnetic stirrer would have modified the magnetic field conditions inside the shielded chamber. At the end of the incubation period  $NH_4^+$ ,  $NO_2^-$  and  $NO_3^-$  concentration in the medium was determined, using Hanna C200 Multiparameter.

By using Hanna C200 Multiparameter neither in control, incubated in normal lab conditions, nor in the samples incubated in the shielded chamber the presence of  $\text{NO}_2^-$  and  $\text{NO}_3^-$  ions was detected. Instead, the next quantities of ammonia nitrogen were distinguished (see Table 1).

Table 1

The nitrogen content in the bacterial culture medium

<i>Incubation</i>	<i>Inoculum</i>	<i>mg NH<sub>3</sub>-N/l culture medium</i>
Shielded magnetic field	solid	0.46
Shielded magnetic field	liquid	0.31
Control	solid	0.42
Control	liquid	0.38

As visible, at the control the values are very close. At the samples incubated in the shielded chamber, there are big differences between the two types of inoculation: the quantity of ammonia nitrogen in the flask with solid inoculum is much bigger than in the flask with liquid inoculum. Also, the quantity of ammonia nitrogen is bigger then in the media incubated in lab conditions, regardless of the inoculum type.

The total nitrogen quantity in the culture medium was determined by Kjeldahl method (Paulette 2007). The method consists of digesting organic substances in order to convert nitrogen to ammonium and then calculating the amonium formed by distillation and titration. The total nitrogen quantity is calculated after the equation:

$$\frac{(a \times f_1 - b \times f_2) \times 0,0014}{m}$$

where: a = the volume of 0.1 N sulfuric acid solution in the colecting baloon (ml);

$f_1$  = the factor of 0.1 N sulfuric acid solution;

b = the volume of 0.1 N sodium hydroxide solution used at titration (ml);

$f_2$  = the factor of 0.1 N sodium hydroxide solution;

m = the mass of the analysed sample (g);

0.0014 = nitrogen quantity (g) corresponding to 1 ml solution of  $\text{H}_2\text{SO}_4$

0.1 N.

After the 14 days of incubation the following quantities of total nitrogen were determined:

Table 2

Total nitrogen assayed in the *Azotobacter chroococcum* culture

<i>Incubation</i>	<i>Inoculum</i>	<i>mg N/l culture medium</i>
Shielded magnetic field	solid	49.2
Shielded magnetic field	liquid	46.8
Control	solid	48.8
Control	liquid	49.3

The values of total nitrogen quantities, at the samples incubated in normal laboratory conditions, are very close to and higher than those recorded in the media incubated in the shielded chamber. It is possible that the bacterial development was slightly inhibited by the absence of the magnetic field in the shielded chamber. However the differences are quite small. In order to find an explanation for the bigger values of ammonia nitrogen quantities in the medium with solid inoculum incubated in the shielded chamber, at the end of the incubation period plate cultures were carried out from successive dilutions of

medium. Same time, the number of bacteria/ml culture medium was determined by microscopical examination (Table 3).

Table 3

Growth of bacterial cells in conditions of shielded magnetic field for 14 days

Incubation	Inoculum	<i>bacterial no./ml medium <math>\times 10^5</math> through the method:</i>	
		plates culture	microscopical
Shielded chamber	solid	16.4	17.1
Shielded chamber	liquid	15.6	15.8
Control	solid	17.3	17.5
Control	liquid	16.2	16.4

As a fact in all the culture media, regardless of the inoculum and the incubation conditions, the number of bacteria/ml culture medium is at  $10^6$  level, with small differences, which features the better developement of bacteria in normal laboratory conditions. The bacteria number counted by plate culture method was just slightly smaller than the one determined by microscopical examination.

A relevant difference was found only at the medium with solid inoculum incubated in the shielded chamber. That means that a bigger percentage of the bacteria which appear on the microscope are not viable. This observation also explains the bigger ammonia nitrogen recorded in this case. In other words a bigger number of bacteria entered a decaying state, freeing in the medium bigger quantities of amonium, the primary form of fixation for the molecular nitrogen.

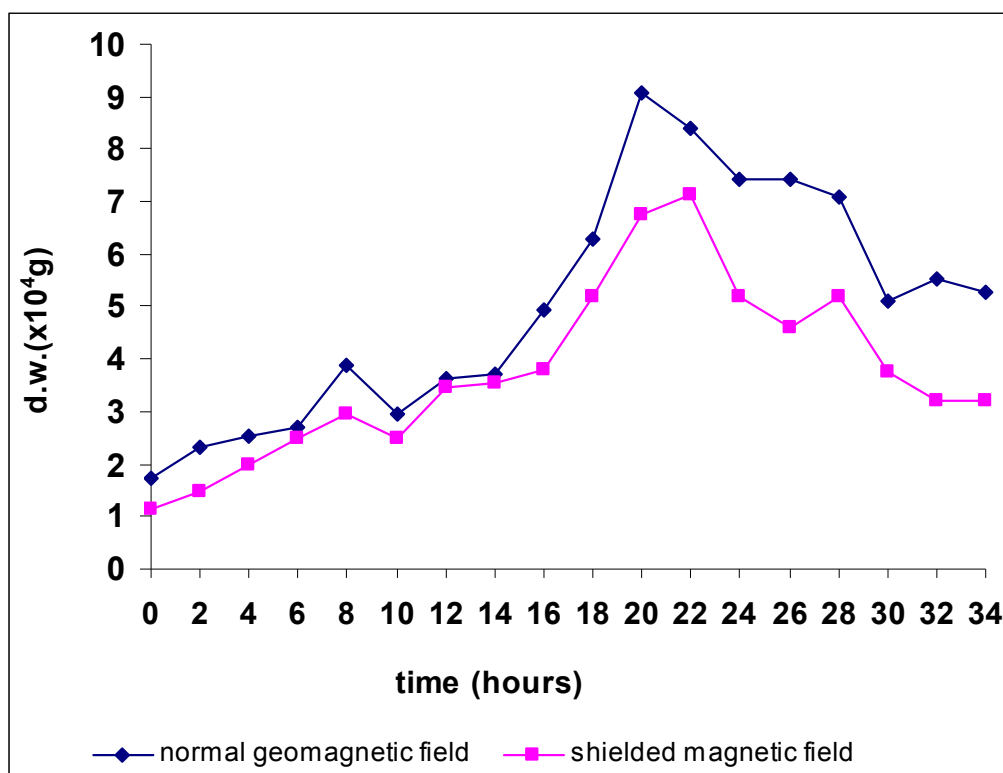


Figure 1. Growth curves of *Azotobacter chroococcum*

To assess *in vivo* nitrogenase activity it is necessary to use a bacterial culture in the middle of *log* state. For this, growth curves were previously attained, both for the control and for experimental variants. The indirect method was used, measuring the bacterial culture turbidity at 600 nm, by a spectrophotometer.

In order to represent the growth curves, the dry weight corresponding to 5 ml of bacterial culture was assessed, by correlating the optical density with the dry substance contained in the given volume. The drying process took place in the drying chamber until the weight of the sample attained the same value at three consecutive measurements.

The reduction of  $C_2H_2$  to  $C_2H_4$  and detection by gas-chromatography were performed in order to determine the nitrogenase activity. Previously standards were tested for acetylene and ethylene, finding the time of appearance on the chromatogram for the drops of the two hydrocarbons.

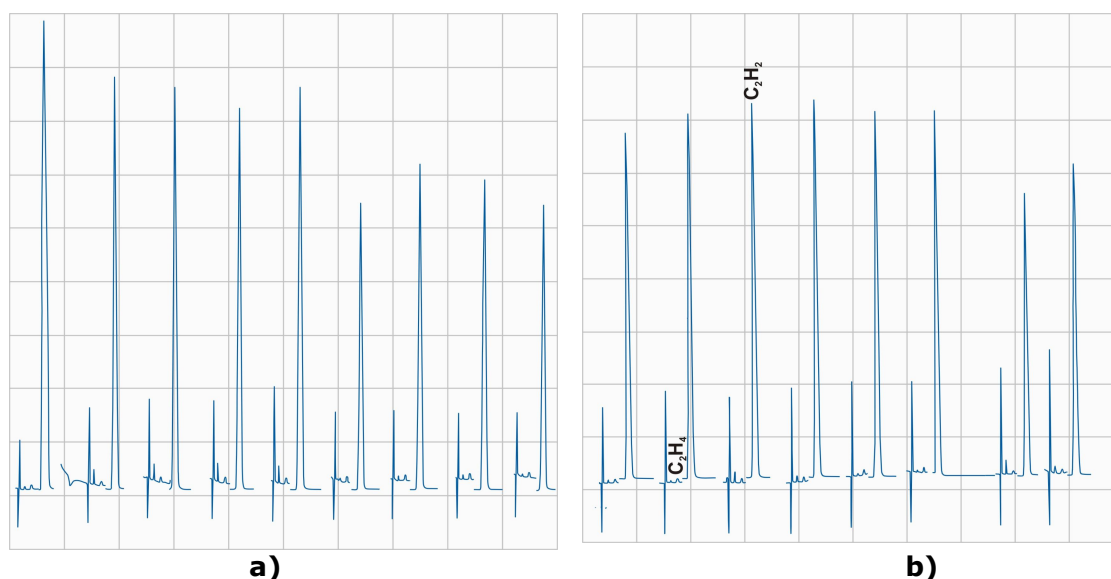


Figure 2. Chromatograms in normal geomagnetic field (a) and in shielded magnetic field (b), concentration 0.008% EDTA

The simple normalisation of drops areas method was used to calculate the concentrations of the consumed substrate ( $C_2H_2$ ) and of the formed product ( $C_2H_4$ ). In this case the percentage composition is calculated as follows: the drops areas of the chromatogram are found; the sum of all the areas is computed; the normal area of each drop is calculated and expressed as percentages of the sum of all the areas  $\%Ax = (Ax/\sum ai) \times 100$ . The area of one drop was computed as follows: the height of the drop was multiplied with its width at half the height.

For an exact interpretation of the drops areas calculated on the chromatogram the attenuation used to bring the detector signal in the field of the recorder was taken into account. In order to obtain a correct chromatogram the attenuation was chosen properly, so that all the drops are distinct and do not go beyond the scale of the recorder. So, for  $C_2H_4$  the attenuation was 2 and for  $C_2H_2$  it was 512.

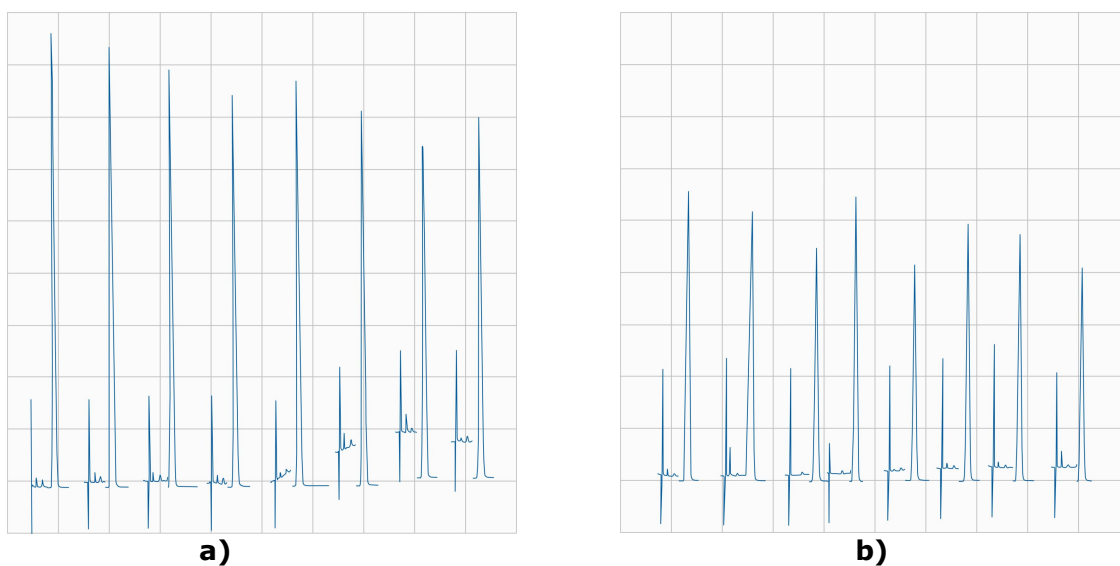


Figure 3. Chromatograms in normal geomagnetic field (a) and in shielded magnetic field (b), concentration 0.01% EDTA

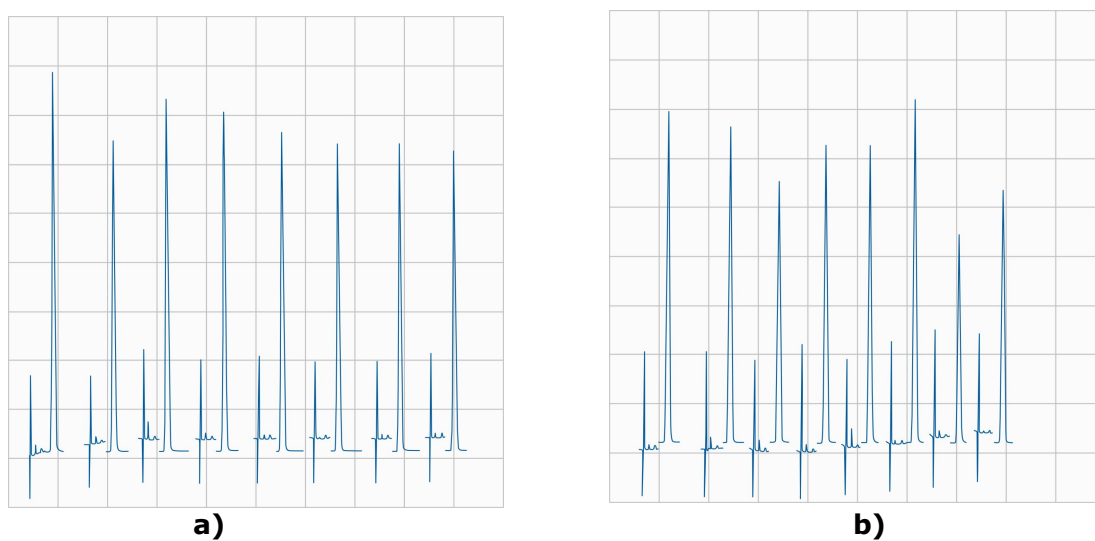


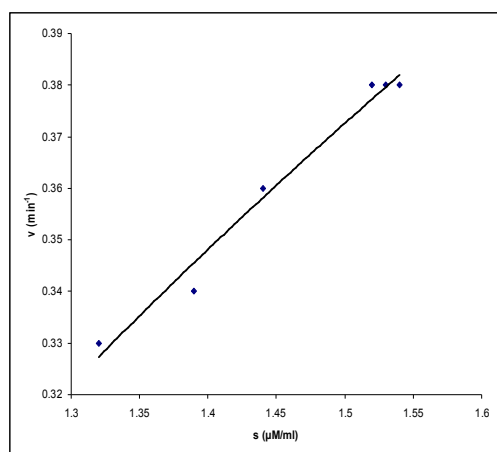
Figure 4. Chromatograms in normal geomagnetic field (a) and in shielded magnetic field (b), concentration 0.012% EDTA

The enzymatic kinetics is Michaelian and the activity of the enzyme was evaluated by examining the Lineweaver-Burk representation of kinetic data.  $K_m$  and  $V_{max}$  derived from interpreting the data of the hyperbolic function  $v = V_{max} [S]/K_m + S$ .

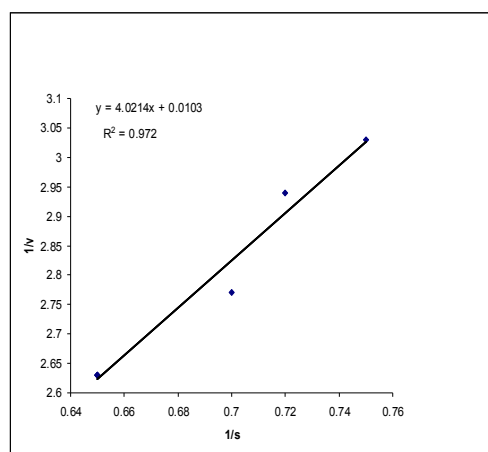
Table 4

Finding the parameters of Michaelis-Menten equation by Lineweaver-Burk linearization for EDTA 0.008% culture from shielded magnetic field (a) and for EDTA 0.008% culture from normal geomagnetic field (b)

$C_2H_4$ (ppm)	$S$ ( $\mu M/ml$ )	$V$ ( $min^{-1}$ )	$1/s$	$1/v$	$C_2H_4$ (ppm)	$S$ ( $\mu M/ml$ )	$V$ ( $min^{-1}$ )	$1/s$	$1/v$
34.432	1.32	0.33	0.75	3.13	34	1.13	0.32	0.77	3.07
36.23	1.39	0.34	0.72	2.94	35.61	1.35	0.33	0.74	2.96
39.582	1.52	0.48	0.65	2.63	36.279	1.14	0.35	0.71	2.85
39.901	1.53	0.38	0.55	2.63	39.907	1.53	0.38	0.65	2.61
44.824	1.81	0.47	0.63	2.17	49.339	1.9	0.48	0.52	2.1
44.784	1.86	0.43	0.53	2.17	51.674	2.10	0.5	0.5	2.14
40.243	1.53	0.28	0.65	2.63	58.737	2.26	0.57	0.44	1.77
37.559	1.44	0.26	0.7	2.67	40.688	1.56	0.39	0.64	2.56

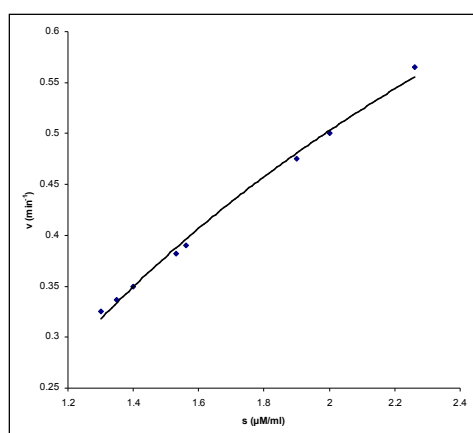


a)

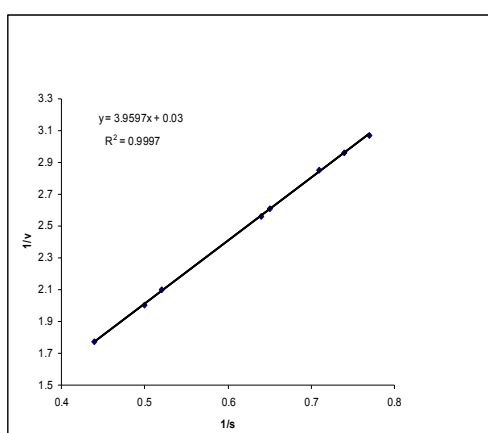


b)

Figure 5 (a). Speed variation of enzymatic reaction depending on substrate concentration for the culture with 0.008% EDTA from shielded magnetic field; (b). Lineweaver-Burk representation for the 0.008% EDTA containing culture from shielded magnetic field



a)



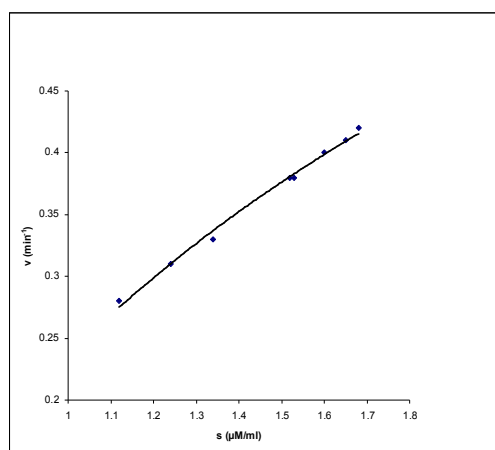
b)

Figure 6 (a). Speed variation of enzymatic reaction depending on substrate concentration for the control culture containing 0.008% EDTA; (b). Lineweaver-Burk representation for the 0.008% EDTA containing control culture

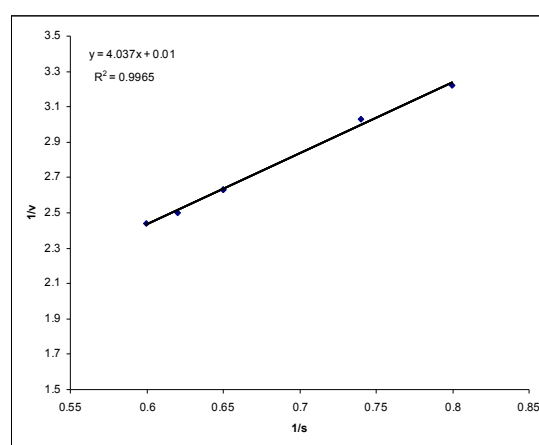
Table 5

Finding the parameters of Michaelis-Menten equation by Lineweaver-Burk linearization for EDTA 0.01% culture from shielded magnetic field (a) and for EDTA 0.01% culture from normal geomagnetic field (b).

$C_2H_4$ (ppm)	$S$ ( $\mu M/ml$ )	$V$ ( $min^{-1}$ )	$1/s$	$1/v$	$C_2H_4$ (ppm)	$S$ ( $\mu M/ml$ )	$V$ ( $min^{-1}$ )	$1/s$	$1/v$
29.179	1.12	0.28			39.858	1.53	0.38	0.65	2.63
32.461	1.24	0.31	0.8	3.22	42.178	1.62	0.4	0.61	2.5
34.86	1.34	0.33	0.74	3.03	42.178	1.62	0.4	0.61	2.5
39.699	1.52	0.38	0.65	2.63	43.059	1.65	0.41	0.6	2.44
43.816	1.68	0.42	0.59	2.38	45.719	1.75	0.43	0.57	2.32
41.668	1.6	0.4	0.62	2.5	43.042	1.65	0.41	0.6	2.44
40.005	1.53	0.38	0.65	2.63	46.24	1.77	0.44	0.56	2.27
42.904	1.65	0.41	0.6	2.44	44.514	1.71	0.42	0.58	2.38

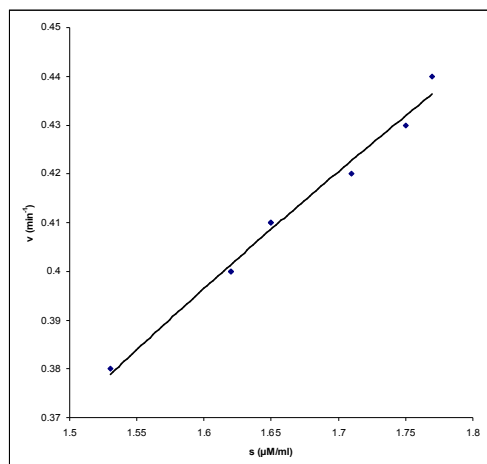


a)

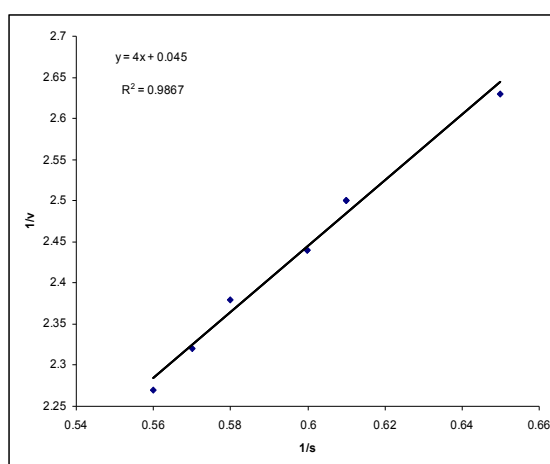


b)

Figure 7 (a). Speed variation of enzymatic reaction depending on substrate concentration for the shielded magnetic field culture containing 0.01% EDTA; (b). Lineweaver-Burk representation for the 0.01% EDTA containing shielded magnetic field culture



a)



b)

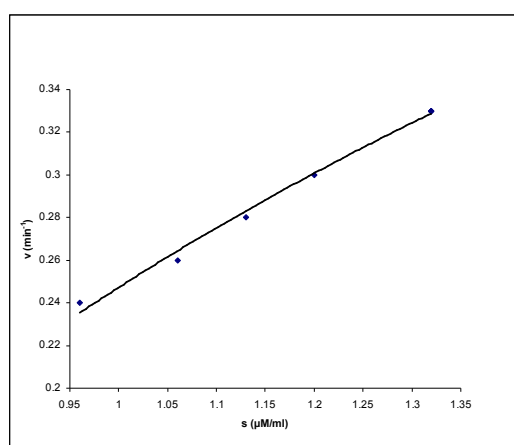
Fig. 8 (a). Speed variation of enzymatic reaction depending on substrate concentration for the control culture containing 0.01% EDTA; (b). Lineweaver-Burk representation for the 0.01% EDTA containing control culture



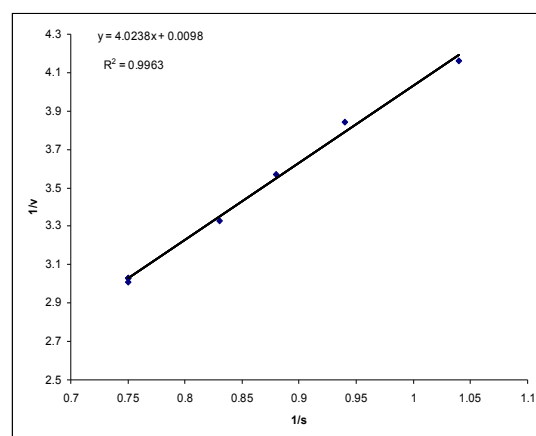
Table 6

Finding the parameters of Michaelis-Menten equation by Lineweaver-Burk linearization for EDTA 0.012% culture for shielded magnetic field (a) and for EDTA 0.012% culture for normal geomagnetic field (b)

$C_2H_4$ (ppm)	$S$ ( $\mu M/ml$ )	$V$ ( $min^{-1}$ )	$1/s$	$1/v$	$C_2H_4$ (ppm)	$S$ ( $\mu M/ml$ )	$V$ ( $min^{-1}$ )	$1/s$	$1/v$
25.012	0.96	0.24	1.04	4.16	24.187	0.93	0.23	1.07	4.34
27.654	1.16	0.26	0.94	3.84	26.671	1.02	0.25	0.98	4
29.48	1.13	0.28	0.88	3.57	29.369	1.13	0.28	0.88	3.57
34.393	1.32	0.33	0.75	3.13	33.613	1.3	0.32	0.77	3.12
35.281	1.35	0.34	0.84	2.94	38.126	1.46	0.36	0.68	2.77
32.256	1.3	0.33	0.77	3.13	38.953	1.5	0.37	0.66	2.7
34.176	1.32	0.23	0.75	3.23	40.369	1.55	0.38	0.64	2.63
31.249	1.2	0.23	0.83	3.33	39.976	1.53	0.38	0.64	2.63

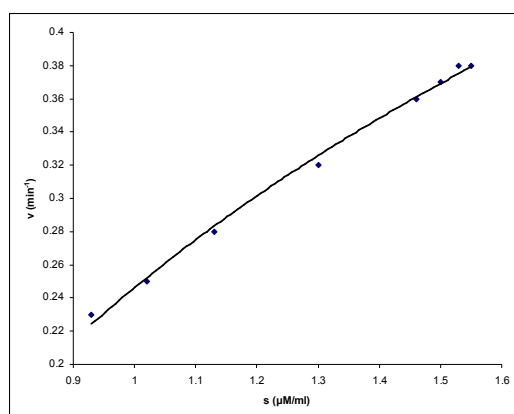


a)

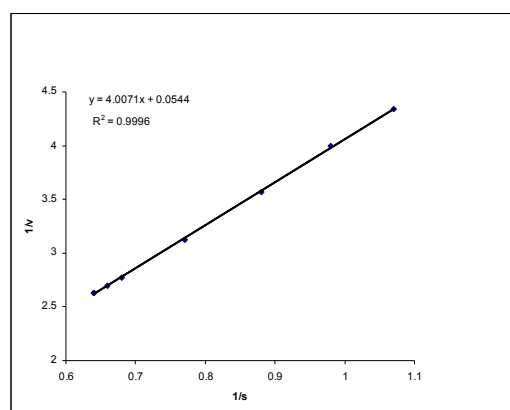


b)

Figure 9 (a). Speed variation of enzymatic reaction depending on substrate concentration for the shielded magnetic field culture containing 0.012% EDTA; (b). Lineweaver-Burk representation for the 0.012% EDTA containing shielded magnetic field culture



a)



b)

Fig. 10 (a). Speed variation of enzymatic reaction depending on substrate concentration for the control culture containing 0.012% EDTA; (b). Lineweaver-Burk representation for the 0.012% EDTA containing control culture

In the Table 7 the parameters of Michaelis-Menten equation are represented ( $K_m$  and  $V_{max}$ ) both for the control samples and the samples incubated under shielded magnetic conditions. It may be seen that, depending on the concentration of the chelator (EDTA), the studied parameters vary in both normal and shielded magnetic field conditions.

Table 7

The parameters of Michaelis-Menten equation,  $K_m$  and  $V_{max}$

Chelation agent concentration	Parameters	Normal geomagnetic field	Shielded magnetic field
0.008% EDTA	$V_{max}$	36.65 min <sup>-1</sup>	96.13 min <sup>-1</sup>
	$K_m$	141 μM/ml	387 μM/ml
0.01% EDTA	$V_{max}$	24.13 min <sup>-1</sup>	102 min <sup>-1</sup>
	$K_m$	89.43 μM/ml	412 μM/ml
0.012% EDTA	$V_{max}$	19.21 min <sup>-1</sup>	111 min <sup>-1</sup>
	$K_m$	77.15 μM/ml	425 μM/ml

*Azotobacter chroococcum* 2286<sup>T</sup> developed well on selective liquid medium, both in normal laboratory conditions and in shielded chamber, regardless the type of the inoculum, solid or liquid. After 14 incubation days, the number of bacteria/ml culture medium was of 10<sup>6</sup> level. The small difference between the bacterial number/ml culture medium determined by plate culture method and the one obtained through microscopic examination reflects the viability of bacterial cells present in the medium after the incubation period.

In the shielded chamber a slight inhibitory effect on the bacterial cultures development manifested, reflected by the smaller quantity of total nitrogen present in the culture medium after 14 incubation days. Compared to the control cultivated in normal laboratory conditions, the inhibition percentages are 1.01% (solid inoculum) and 3.86% (liquid inoculum). The same effect is marked out also by the slightly bigger bacterial number/ml culture medium incubated in normal laboratory conditions.

In the shielded chamber the bacterial culture obtained by inoculation from solid medium developed better than the one obtained through inoculum from liquid environment. At the cultures incubated in normal laboratory conditions no significant difference was noticed from this point of view.

In the culture media oxidation processes of reduced nitrogen compounds did not occur, fact indicated by the absence of NO<sub>2</sub><sup>-</sup> și NO<sub>3</sub><sup>-</sup> ions, both in the control samples and the samples incubated in the shielded chamber.

At the medium with solid inoculum incubated in the shielded chamber a bigger number of bacteria in decaying state, released higher ammonium quantities in the medium, compared to control.

**Conclusions.** Shielded magnetic field induces a diminishing of *Azotobacter chroococcum* 2286<sup>T</sup> cells viability, the downhill period of growth curve under such conditions occurring sooner compared to normal geomagnetical field. As compared to control, the life span of microorganisms was shorter in shielded environment their nitrogenase activity was lower.

At the samples incubated in normal conditions the values of total nitrogen quantities are very close and higher than those recorded in the media incubated in the shielded chamber.

A higher affinity of the enzyme for the substrate can be noticed at the control samples compared to the samples incubated under shielded magnetic field where nitrogenase has an much lower affinity for bonding acetylene.

Over the threshold of 0.01% EDTA in normal geomagnetic field a dramatically growth of enzyme affinity for the substrate occurs. In shielded magnetic field the affinity of the enzyme for bonding acetylene reduces dramatically and an about 3-5 fold growth of reaction speed compared to the normal geomagnetic field is noticed, suggesting that

the cells under normal geo-magnetic field showed higher nitrogenase activity than cells exposed to low magnetic fields.

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