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ABSORPTION OF IONS Mn^{2+}, Zn^{2+}, Cu^{2+} AND Pb^{2+} BY CELLS OF *CHLORELLA VULGARIS* BEIJER.

We investigated the absorption characteristics of Mn^{2+}, Zn^{2+}, Cu^{2+} and Pb^{2+} by unicellular green algae *Chlorella vulgaris* Beijer. The accumulation of metals ions is fluctuating. There are 4 stages: the stage of protective self-isolation of cells as a result of the primary stress response, the stage of the active accumulation (decrease in resistance and destruction of outer membrane), the stage of inhibition of the accumulation (formation of the secondary concentric membrane), and the stage of uncontrolled accumulation (destruction of secondary concentric membrane). Kinetic parameters of accumulation (K_m, V_{max}, E_a) show that the process of absorption of ions Mn^{2+}, Zn^{2+}, Cu^{2+} and Pb^{2+} goes according to mixed type of inhibition and is determined by the affinity of metal-binding proteins to ions, and after saturation of their binding sites the process becomes uncontrolled.

Key words: absorption, kinetic parameters, Mn^{2+}, Zn^{2+}, Cu^{2+}, Pb^{2+}, Chlorella vulgaris *Beijer.*

Evolution of algae was in progress if in their habitat environment there were the ions of various metals, included in toxic concentrations. As a result, they formed mechanisms of toxin resistance that support their optimal level in cells. Ions of many metals in some concentrations are effective regulators of metabolism, which is an ecological factor in the regulation of their population [2, 10]. Unicellular algae of different taxonomic groups can accumulate microelements in concentrations that exceed their content in the water thousand times [7, 14]. However, excess of a certain level causes pathological changes of metabolism and their death [5].

The primary barrier for penetration of metal ions into the cells of algae is a cell membrane and plasmolemma [1]. The intensity of penetration of metal ions is determined by: their concentration in the environment, the interaction with membranes, and affinity of the components of cell membranes and intracellular components [4, 6]. It is believed that the penetration of metal ions into the cells is realized by diffusion and by using active transport [1]. Also it is shown [9] that the accumulation of heavy metals in aquatic organisms is a dynamic process that develops according to time gradient and is characterized by a certain intensity and specificity.

In this research we received the information about kinetic parameters of the penetration of ions Mn^{2+}, Zn^{2+},Cu^{2+} and Pb^{2+} into the cells of *Ch. vulgaris* Beijer.

Materials and methods

The object of the research was the unicellular green microalgae *Ch. vulgaris* Beijer., which was grown in the climate chamber with illumination at a temperature 20±1°C and lighting 2500 lx, in glass flasks (250 dm^3) in the Fitzgerald mineral medium in modification by Zehnder and Gorham [12], that contained among other cations 0,058 mg/dm^3 Mn^{2+} and 0,023 mg/dm^3 Zn^{2+} but without Cu^{2+} and Pb^{2+}. We added aqueous solutions of $MnSO_4$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $Pb(NO_3)_2$ at a rate per ion: Mn^{2+} − 0,1; 0,2; 0,5 mg/dm^3; Zn^{2+} – 1,0; 2,0; 5,0 mg/dm^3; Cu^{2+} − 0,001; 0,002; 0,005 mg/dm^3, Pb^{2+} – 0,1; 0,2; 0,3 mg/dm^3. The duration of the influence of metals on the algae was: 0,083; 0,25; 0,5; 0,75; 1; 3; 6; 12; 24; 48; 72; 168 hours. The control cells were the cells which were grown in nutrient medium without the salts of toxic metals.

The penetration of ions into the cells of chlorella was stopped by 2,5 mM EDTA. After centrifugation of the suspension of algae (2 000 r/min.) the sediment was washed with solution of nutrient medium for cultivation, then it was burned in the nitrate acid [2]. The content of metals was determined by atomic absorption spectrophotometer Selmi C-115 M. The concentration of proteins in the cells of chlorella was determined by Lowry's method.

The values of Michaelis constant (K_m) and maximum speed of penetration of metal ions (V_{max}) into the cells of algae were calculated by the graphic method of double return values in the

Lineweaver – Burk coordinates and energy of activation (E_a) was determined by Arrenius's graphical method [13].

Results

As a result of research it was revealed the fluctuating character of the process of accumulation of the investigated metal ions by cells of *Ch. vulgaris* Beijer. in time and depending on the concentration.

The intensity of accumulation of Mn^{2+} by cells of algae under metal concentrations 0,2 and 0,5 mg/dm^3 is reduced during 30 min. (Fig. 1) and under ion concentration of 0,1 mg/dm^3 − at first it is increased (to 0,25 h.), and than also is decreased (to 0.75 h.). Later it is observed an active accumulation of metal ions (to 24 h.) under action of all investigated concentrations, what can be explained by a breach of resistibility of the cell membrane. After this the process is inhibited (to 48 h.). With increase of duration of the cultivation of chlorella with Mn^{2+} to 72 and 168 hours it is observed the restoration of ions accumulation during actions of 0,1; 0,5 and 0,2 mg/dm^3 respectively, with further decrease of intensity.

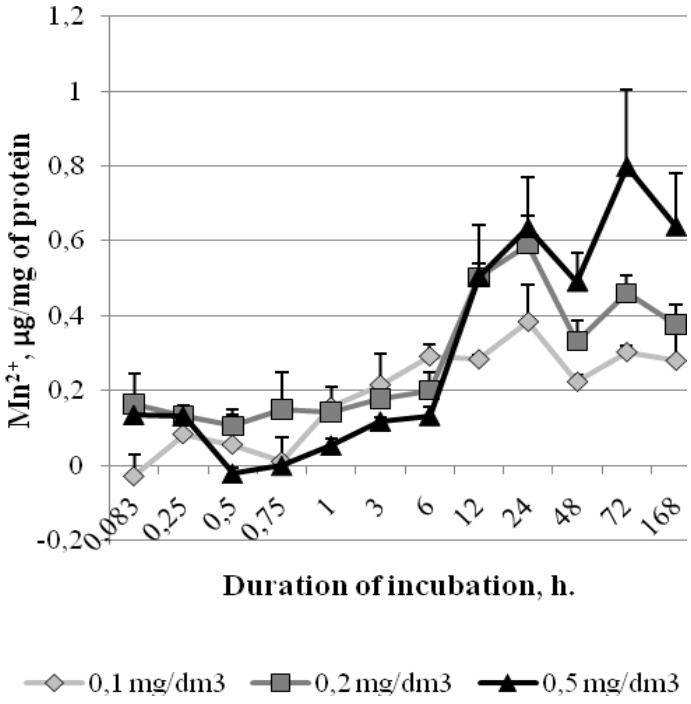

Fig. 1 The accumulation of Mn^{2+} by cells of *Ch. vulgaris* Beijer.

This process is a subject to kinetic laws of Michaelis − Menten only during 0,083-0,5 h. and 12-168 h. (table 1). Thus, the values of V_{max} and K_m of the accumulation of Mn^{2+} are reduced by 22% and 82% respectively (during 0,25 h.), then increased by 3% and 38% (to 0,5 h.), later V_{max} is increased by 11% (from 12 to 24 h.), reduced by 17% (to 48 h.), and again increased by 30% (to 72 h.) and reduced by 12% (to 168 h.). K_m is decreased by 4% during 12-24 h., increased by 52% (to 72 h.) and reduced by 25% (to 168 h.). The activation energy of binding of Mn^{2+} is reduced by 77% (to 0,25 h.), increased by 36% (to 0,5 h.), reduced by 15% (from 12 to 24 h.), increased by 44% (to 72 h.) and again reduced by 14% (to 168 h.).

Table 1

Kinetic parameters of the accumulation of Mn^{2+} by cells of *Ch. vulgaris* Beijer.

Kinetic parameters of accumulation of Mn^{2+}			
Duration of incubation, h.	V_{max}, μmol/h.*mg of protein	$K_м$	E_a, $*10^{-3}$ kJ/μmol
0,083	34,5	0,100	2,90
0,25	27,0	0,018	0,67
0,5	27,8	0,029	1,05
0,75	–	–	–
1	–	–	–
3	–	–	–
6	–	–	–
12	54,1	0,055	1,02
24	60,6	0,053	0,87
48	50,0	0,067	1,33
72	71,4	0,111	1,55
168	62,5	0,083	1,33

Footnote: "–" in the table 1, 3, 4 – the process isn't a subject to kinetic laws of Michaelis – Menten.

The accumulation of Zn^{2+} by the cells of *Ch. vulgaris* Beijer. is active (Fig. 2) (under action of 1 mg/dm^3 − to 0,5 h., under action of 2 and 5 mg/dm^3 − to 0,75 h.), which can be explained by their use in the active vital functions of algae; and it is changed by the oppression of the accumulation process (under action of 1 and 2 mg/dm^3 − to 3 h., under action of 5 mg/dm^3 − to 1 h.). Then accumulation of metal ions is activated (under action of 1 and 2 mg/dm^3 – to 168 h., under action of 5 mg/dm^3 – to 72 h.) with the following decrease of the absorption under concentration of 5 mg/dm^3.

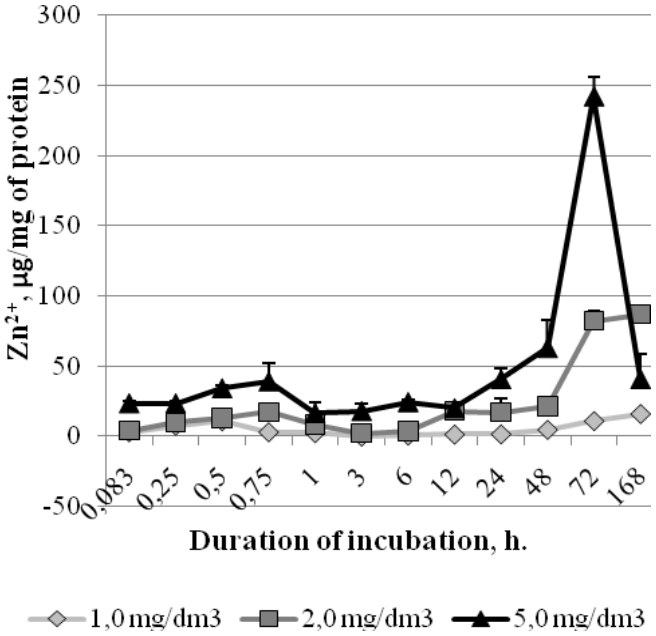

Fig. 2 The accumulation of Zn^{2+} by cells of *Ch. vulgaris* Beijer.

The absorption of Zn^{2+} is characterized by such indicators (table 2): V_{max} accumulation of metal ions is increased by 28%, 60%, 38% and 76% during 0,083-0,5 h., 0,75-1 hours., 3-6 h. and 24-168 h. respectively, and is decreased by 28%, 75% and 50% during 0,5-0,75 h., 1-3 hours and 6-24 h. respectively, K_m is decreased by 63%, 50%, 62% and 63% during 0,083-0,25 h., 1-3 hours., 6-24 h. and 48-168 h. respectively, and increased by 89%, 25% and 25% during 0,25-1 h., 3-6 h. and 24-48 h. respectively. E_a of binding of Zn^{2+} changes: during the first 0,5 h. it is decreased by 65%, then (to 3

h.) increased by 88%, than (to 12 h.) again decreased by 44%, to 24 h. than increased by 10% and again decreased by 88% to 168 h.

Table 2

Kinetic parameters of the accumulation of Zn^{2+} by cells of *Ch. vulgaris* Beijer.

Kinetic parameters of the accumulation of Zn^{2+}			
Duration of incubation, h.	V_{max}, μmol/h.*mg of protein	$K_м$	E_a, $*10^{-3}$ kJ/ μmol
0,083	2000	5,9	2,6
0,25	2000	2,2	1,1
0,5	2778	2,5	0,9
0,75	2000	6,7	3,3
1	5000	20,0	4,0
3	1250	10,0	8,0
6	2000	13,3	6,6
12	1250	5,7	4,5
24	1000	5,0	5,0
48	2500	6,7	2,6
72	2941	4,3	1,5
168	4167	2,5	0,6

An active absorption of Cu^{2+} (Fig. 3) occurs during 0,75 and 1 h. during chlorella cultivation with 0,001 and 0,005 mg/dm^3 respectively. During action of 0,002 mg/dm^3 it is observed an inhibition of absorption of the ions (to 0,5 h.), which can be explained by self-isolation of cells from metal with following activation (to 0,75 h.). Later the intensity of accumulation of metal ions during action of the investigated concentrations is decreased (to 3 h.), then it is activated (12 h.), inhibited (to 24 h.), activated for the second time (to 72 h.) and inhibited (up to 168 h). In this case during the action of the investigated concentrations it is observed the accumulation of metal ions to 0,75 and 1 h. and it is changed to inhibition of absorption of ions to 3 h.

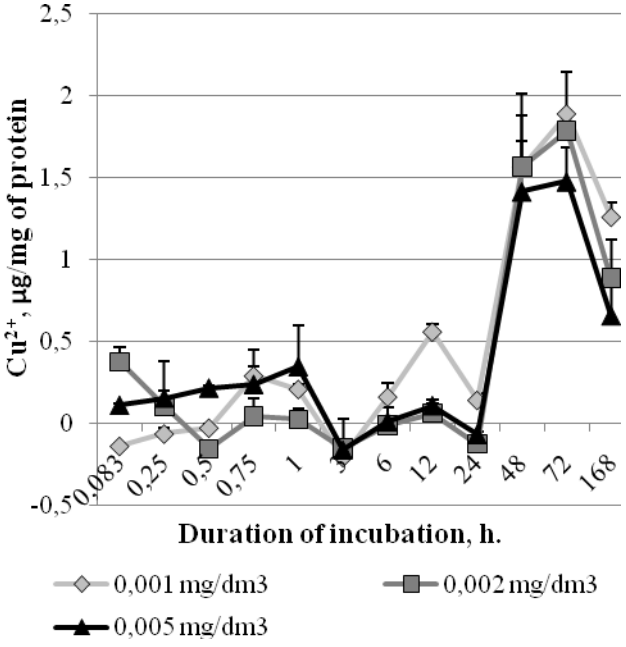

Fig. 3 The accumulation of Cu^{2+} by cells of *Ch. vulgaris* Beijer.

For Cu^{2+} (table 3) value of V_{max} is increased by 20% (to 0,75 h.), decreased by 60% (to 3 h.), again increased by 42% (to 12 h.) and decreased by 23% (for 24 h.); values of K_m and E_a are reduced by 22% and 33%, 92% and 91%, 50% and 33% respectively during 0,083-0,5 h., 0,75-1 h., 3-6 h., and

increased by 50% and 45%, 50% and 67%, 80% and 73% respectively during 0,5-0,75 h., 1-3 hours., 6-24 h. The accumulation of metal ions during 48-168 h. is not a subject to laws of Michaelis − Menten.

Table 3

Kinetic parameters of the accumulation of Cu^{2+} by cells of *Ch. vulgaris* Beijer.

Kinetic parameters of the accumulation of Cu^{2+}			
Duration of incubation, h.	V_{max}, μmol/h.*mg of protein	$K_м$	E_a, $*10^{-3}$ kJ/ μmol
0,083	50,0	0,0009	0,018
0,25	53,0	0,0007	0,013
0,5	55,5	0,0007	0,012
0,75	62,5	0,0014	0,022
1	48,78	0,0001	0,002
3	25,0	0,0002	0,006
6	37,0	0,0001	0,004
12	43,5	0,0003	0,006
24	33,3	0,0005	0,015
48	–	–	–
72	–	–	–
168	–	–	–

The accumulation of Pb^{2+} (Fig. 4) depends on the concentration: during action of 0,1 mg/dm^3 it is observed the decrease of the absorption of the ions of metal (to 0,25 h.), then increase (to 6 h.), decrease (to 24 h.), again increase (to 72 h.) and decrease (168 h.), during the action of 0,2 mg/dm^3 - initial accumulation (to 1 h.) is decreased (to 3 h.), then gradually increased (to 168 h.) during the action of 0,5 mg/dm^3 − the intensity of the absorption of metal ions is decreased (to 0,25 h.), then activated (6 h.), inhibited (to 12 h.), activated one more time (to 48 h.) and inhibited (to 168 h.).

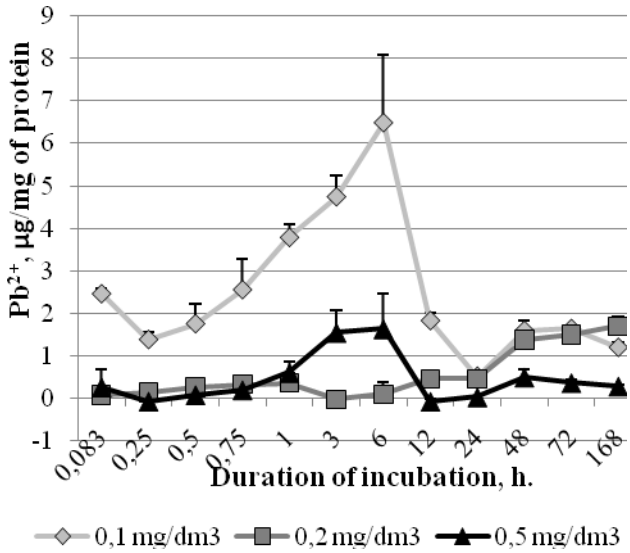

Fig. 4 The accumulation of Pb^{2+} by cells of *Ch. vulgaris* Beijer.

The accumulation of Pb^{2+} is a subject to laws of Michaelis – Menten only during 0,083 h. and 1-6 h. (table 4).

Table 4

Kinetic parameters of the accumulation of Pb^{2+} by cells of *Ch. vulgaris* Beijer.

Kinetic parameters of the accumulation of Pb^{2+}			
Duration of incubation, h.	V_{max}, μmol/h.*mg of protein	$K_м$	E_a, $*10^{-3}$ kJ/ μmol
0,083	200	0,50	2,5
0,25	–	–	–
0,5	–	–	–
0,75	–	–	–
1	238	0,22	0,9
3	50	0,50	10,0
6	125	0,67	5,3
12	–	–	–
24	–	–	–
48	–	–	–
72	–	–	–
168	–	–	–

The value of V_{max} of the absorption of Pb^{2+} is decreased by 79% within 1-3 h. and increased by 60% to 6 h. K_m during this time is increased by 67% and E_a of binding of metal ions is increased by 91% within 1-3 h. and decreased by 47% to 6 h.

Discussion

The cells of *Ch. vulgaris* Beijer. actively accumulate Mn^{2+} to 24 h. of incubation, Zn^{2+} − to 0,5 h. (1,0 mg/dm^3) and to 0,75 h. (2 and 5 mg/dm^3), Cu^{2+} − to 0,75 pm. (0,001 mg/dm^3) and to 1 h. (0,005 mg/dm^3), Pb^{2+} − to 1 h. (0,2 mg/dm^3) and to 6 h. (0,1 and 0,5 mg/dm^3), thus controlling the penetration of ions. Later accumulation is inhibited. The mechanism of accumulation of metal ions is fluctuating and has 4 stages: the stage of self-isolation (stress reaction) of cells, stage of the active accumulation, stage of the inhibition, stage of the second accumulation. The stage of the self-isolation is the response of the cellular organism to the action of stress factor, in our case the ions of metals. The best isolation function of the cells appears during the action of Mn^{2+} (0,2 and 0,5 mg/dm^3 − to 0,5 h.), Cu^{2+} (0,002 mg/dm^3 − to 0,5 h.), and Pb^{2+} (0,1 and 0,5 mg/dm^3 − to 0,25 h.). Decrease of resistance of the primary cellular membrane to the investigated concentrations of metal ions is characterized by the stage of active accumulation of Mn^{2+} (0,1 mg/dm^3 − from 0,75 to 24 h., 0,2 and 0,5 mg/dm^3 − from 0,5 to 24 h.), Zn^{2+} (1mg/dm^3 − to 0,5 h., 2 and 5 mg/dm^3 − to 0,75 h.), Cu^{2+} (0,001 mg/dm^3 − to 0,75 h.; 0,002 mg/dm^3 − from 0,5 to 0,75 h.; 0,005 mg/dm^3 − to 1 h.), Pb^{2+} (0,1 and 0,5 mg/dm^3 − from 0,25 to 6 h.; 0,2 mg/dm^3 − to 1 h.), which is followed by the destruction of the primary membrane [6]. Later the cells of chlorella try to control the absorption of the ions at the stage of secondary inhibition. The observed fluctuating type of accumulation of the ions corresponds with our previously established structural and functional reconstructions of the cellular membrane during the action of metal ions, which consists in formation of double concentric membrane with changes in concentration and duration of action of metal ions [6, 10]. Also in the cells of *Ch. vulgaris* Beijer. during the action of metal ions there is a thickening of the membranes, which is observed on the first day of the action of Zn^{2+} and Pb^{2+}. The stage of the reactivation process of accumulation, that is observed during the actions of Mn^{2+} (0,1 and 0,5 mg/dm^3 − from 48 to 72 h.; 0,2 mg/dm^3 − from 48 h. to 168 h.), Zn^{2+} (1 and 2 mg/dm^3 − from 3 to 168 h., 5 mg/dm^3 − from 1 to 72 h.), Cu^{2+} (0,001; 0,002 and 0,005 mg/dm^3 − from 24 to 72 h.), Pb^{2+} (0,1 mg/dm^3 − 24 to 72 h.; 0,2 mg/dm^3 − from 3 to 168 h., 0.5 mg/dm^3 − from 12 to 48 h.), is characterized by destruction of the secondary concentric membrane [6].

Restructurings of the membranes during the action of metal ions are also consistent with violation of the functioning of the membranous ATP-ases [2], particularly Na^+/ K^+ ATP-ase, that participates in the regulation of ions [11]. Thus, Zn^{2+} do not practically impact on the membranous ATPases, except for high concentrations (5 mg/dm^3), because they have high permeability, mobility in the cell, and complexing ability [3]. Pb^{2+} inhibit the activity of ATPase [2], because they are characterized by high affinity to proteins and strong restraining of this metal within metallothioneins

[3]. A certain number of Cu^{2+} can bind with cellular membranes, and other ones make the complexes with low-molecular organic substances and proteins up to the saturation of their centers of binding [8]. The highest affinity to the proteins among investigated ions is Cu^{2+}, and the least − Zn^{2+}. Mn^{2+} supplant Ca^{2+} from cellular membranes [15], and therefore their accumulation is limited by duration of antyport.

The kinetic indicators conform to the regularity of absorption of metals. The increase of V_{max} accumulation of Mn^{2+} (0,25-0,5; 12-24; 48-72 h.), Zn^{2+} (0,083-0,5; 0,75-1; 3-6; 24-168 h.), Cu^{2+} (0,083-0,75, 12.03 h.), Pb^{2+} (3-6 h.) shows that metal ions are bound with molecules of the cellular walls of alga and molecules- carriers of membranes according to the noncompetitive type [13]. The absorption of ions is also characterized by the decrease of value of V_{max}: Mn^{2+} (0,083-0,25; 24-48; 72-168 h.), Zn^{2+} (0,5-0,75; 1-3; 6-24 h.), Cu^{2+} (0 ,75-3, 12-24 h.), Pb^{2+} (1-3 h.), that indicate the competitive inhibition.

The energy of the activation of binding of Mn^{2+} (0,25-0,5; 24-72 h.), Zn^{2+} (0,5-3; 12-24 h.), Cu^{2+} (0,5-0,75; 1-3 ; 6-24 h.), Pb^{2+} (1-3 h). by surface membrane of algae and penetration through the membrane show that the process of accumulation of metals within this period is energy-dependent.

The absorption of metal ions goes according to mixed mechanism and is determined by the affinity of metal-binding components of membranes, formation of secondary concentric membrane and its resistance to metals, the duration of its structural and functional activities, and after loss of it and after saturation of the centers of binding of metal ions by cytoplasmic components − the process of accumulation becomes passive and uncontrolled.

Conclusions

Thus, accumulation of the ions of Mn^{2+}, Zn^{2+}, Cu^{2+} and Pb^{2+} by the cells of *Ch. vulgaris* Beijer. is fluctuating and is determined by the concentration of ions in the environment and duration of its action on the cells. There are 4 stages: the stage of protective self-isolation of cells as a result of the primary stress response, the stage of the active accumulation as a result of decrease in resistance and destruction of outer membrane, the stage of inhibition of the accumulation as a result of formation of the secondary concentric membrane [6]; the stage of uncontrolled accumulation as a result of destruction of the secondary concentric membrane. The absorption of Mn^{2+}, Zn^{2+}, Cu^{2+} and Pb^{2+} goes according to mixed type of inhibition and is determined by the affinity to the ions of the metal-binding components of the membranes and the cytoplasm.

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НАКОПИЧЕННЯ ІОНІВ Mn^{2+}, Zn^{2+}, Cu^{2+} і Pb^{2+} КЛІТИНАМИ *CHLORELLA VULGARIS* Beijer

Досліджували особливості поглинання Mn^{2+}, Zn^{2+}, Cu^{2+} і Pb^{2+} одноклітинною зеленою водорістю *Chlorella vulgaris* Beijer. Накопичення іонів металів є флуктуаційним. Можна виділити 4 його етапи: етап захисної самоізоляції клітин як результат первинної стрес-реакції, етап активного накопичення (зниження опірності і руйнування зовнішньої мембрани), етап пригнічення накопичення (утворенням вторинної концентричної мембрани), етап неконтрольованого накопичення (руйнування вторинної концентричної мембрани). Кінетичні показники накопичення (Km, Vmax, Eact.) свідчать про те, що процес поглинання іонів Mn^{2+}, Zn^{2+}, Cu^{2+} і Pb^{2+} відбувається за змішаним типом інгібування та визначається спорідненістю до іонів метал-зв'язуючих білків, після насичення їх сайтів зв'язування процес стає неконтрольованим.

Ключові слова: Mn^{2+}, Zn^{2+}, Cu^{2+} і Pb^{2+} , накопичення, кінетика, Chlorella vulgaris *Beijer.*

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НАКОПЛЕНИЕ ИОНОВ Mn^{2+}, Zn^{2+}, Cu^{2+} и Pb^{2+} КЛЕТКАМИ CHLORELLA VULGARIS Beijer

Исследовали особенности накопления Mn^{2+}, Zn^{2+}, Cu^{2+} и Pb^{2+} одноклеточной зеленой водорослью *Chlorella vulgaris* Beijer. Накопление ионов металлов является флуктуационным. Можно выделить 4 его этапы: этап защитной самоизоляции клеток как результат первичной реакции стресса, этап активного накопления (снижение сопротивляемости и разрушение внешней мембраны), этап уменьшения накопления (образованием вторичной концентрической мембраны), этап неконтролируемого накопления (разрушение вторичной концентрической мембраны). Кинетические показатели накопления (K_m, V_{max}, $E_{act.}$) свидетельствуют о том, что процесс поглощения ионов Mn^{2+}, Zn^{2+}, Cu^{2+} и Pb^{2+} происходит по смешанному типу ингибирования и определяется сродством к ионам металл-свезующих белков, после насыщения их сайтов связывания процесс становится неконтролируемым.

Ключевые слова: Mn^{2+}, Zn^{2+}, Cu^{2+} и Pb^{2+}, накопление, кинетика, Chlorella vulgaris *Beijer.*

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