THE ACIDOPHILIC MICROORGANISMS DIVERSITY PRESENT IN LIGNITE AND PIT COAL FROM PAROSENI, HALÂNGA, TURCENI MINES

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Abstract. Pollution from coal combustion is the largest problem in the current use of coal and the biggest constraint on the increased use of coal. When these fossil fuels are combusted, sulphur-di-oxide is released into the atmosphere causing acid rains which dissolves buildings, kills forest.

Knowing the physiological groups of microorganisms present in the coal samples has an ecological importance, completing the knowledge in the field of the microorganism's ecology and a practical importance, being a source of new microorganisms with biotechnological potential.

The microbial communities evidenced in such sites include both groups of chemolithotrophic microorganisms involved in the metals biosolubilization processes and groups of heterotrophic microorganisms involved in the processes of bioaccumulation or biofixation of metallic ions.

In this context, this paper presents the study regarding the main physiological groups of microorganisms present in the pit coal and lignite samples after the industrial processing of coal. The results revealed that the microorganisms belonging to the following physiological groups: aerobic heterotrophic acidophilic bacteria, strictly anaerobic heterotrophic (sulphur-reducing), nitrifying bacteria (nitrite and nitrate bacteria), denitrifying bacteria and acidophilic chemolithotrophic bacteria on Fe^{2+}, on S^0 and on S_2O_3.

Keywords: acidophilic bacteria, coal, biodesulphurization.

INTRODUCTION

During the past decade a special importance has been given to the microbial biodiversity, which could represent an ecological source for selecting adequate microorganisms for the environment bioremediation. The presence in the residual waters from the coal processing stations of metallic ions in higher concentrations raises an important problem about depolluting the environment.

After applying the classical procedure of coal manufacture and from the natural bacterial solubilization of the coal residual waters full of metallic ions results. The sulfur found in coal is either part of the molecular coal structure, is contained in minerals such as pyrite (FeS_2), or occurs in minor quantities in the form of sulfate and elemental sulfur [11, 17].

The microbial communities evidenced in such sites include both groups of chemolithotrophic microorganisms involved in the metals biosolubilization processes and groups of heterotrophic microorganisms involved in the processes of bioaccumulation or biofixation of metallic ions.

The development of the biotechnological processes, based mainly on the activity of the microorganisms, demonstrated the efficiency of bioremediation the polluted environment with residuum and toxic substances [18, 22].

A variety of ecological processes are affected and altered by air pollution. Such processes include community succession and retrogression, nutrient biogeochemical cycling, primary and secondary productivity, species diversity and community stability [1, 7].

Biodesulphurization is a process that involves a microbial system, heterotrophic and chemolithotrophic microorganisms that would remove sulphur from fuels without degrading the fuel value of the product [20].

The structural and physiological characterization of the species from microbial communities may lead to the discovery of new species of microorganisms, which could play an important role in bioremediation processes, due to their metabolic activity in environments polluted with metabolic ions [3, 8, 19].

The main microorganisms involved in the solubilisation of the metals in the sulphur ores are the sulphur-oxidizing bacteria which form a heterogenous group of microorganisms that belong to the following genera: *Thiobacillus, Thiomicrospira, Leptospirillum, Sulfobacillus, Sulfolobus.*

The main species of sulphur and iron-oxidizing bacteria involved in leaching of the sulphur ores are the mesophilic bacteria *A. ferrooxidans*, *A. thiooxidans*, *L. ferrooxidans*, the moderate thermophilic bacteria *Acidithiobacillus* sp., *Sulfobacillus acidophilus* şi *S. thermosulfidooxidans*, the extreme thermophilic microorganisms *Sulfolobus acidocaldarius*. These bacteria get their necessary energy by oxidizing the elemental sulphur and its different anorganic compounds. The *Acidithiobacillus* bacteria, especially *Acidithiobacillus ferrooxidans,* are prevalent in the acid mining waters, where the metallic sulphures and the oxygen appear in acid conditions, paarticipating to the natural leaching processes [5, 12, 15, 16].

The increasing pollution of the environment raised the interest for the resistance of the acidophilic microorganisms to metals and there is the potential recovery, but also in detoxifying the media polluted by heavy metals. They have a major contribution to the circuit of biogenic elements in the nature and on the global plane; they prevent the accumulation of residual materials of different environmental contaminants [9].

During the last decade a special attention has been given to investigating acid media with high concentrations of metallic ions. The study of the microbial biodiversity present in these media is justified in the context of the problems regarding the environmental pollution [6, 9, 23].

The structural and physiological characterization of the species from microbial communities bioremediation processes, due to their metabolic activity in environments polluted with metabolic ions may lead to the discovery of new species of microorganisms, which could play an important role in [4, 21].

In this context, it become necessary studying the main physiological groups of microorganisms, chemoorganotrophic and chemolithotrophic, present in pit coal and lignite samples from Paroseni, Halânga, Turceni mines.

MATERIALS AND METHODS

One of the purposes of this project was the quantitative determination of microorganisms from six samples of pitcoal (from Lupeni, Paroşeni, Lonea, Mintia, Vulcan, Petrila) in 2006 year and two samples of lignite (from Turceni and Halânga) taken in 2007 year. Also, in our experiments were used six samples of lignite (from Halânga and Turceni), three sample of pitcoal (from Paroseni) taken in 2008 year.

The microbiological analysis meant revealing and quantitative estimating of the following physiological groups of microorganisms: aerobic heterotrophic acidophilic bacteria, nitrifying bacteria (nitrite and nitrate bacteria), denitrifying bacteria, sulphur-oxidizing bacteria and sulphur-reducing bacteria.

The working technique was that of decimal dilutions with inoculation on selective media, specific to each physiological group analyzed, incubation at 28^0C on a period of time. The quantitative estimation I was made through MPN technique (Most Probable Number) using Mc Cready tables.

The isolated strains were characterized regarding the Gram staining, cellular morphology and biochemical features (oxidaze and catalaze activity, biosynthesis of organic acids). The determination of catalase production by the isolated strains was performed by emulsified of a loopful of the culture in few drops of 3% hydrogen peroxide. If the hydrogen peroxide effervesces, the microorganism tested is catalase positive.

The strains and populations of acidophilic heterotrophic bacteria, *Acidiphilium* sp., were analyzed having in view the growth of the bacterial cultures in the specific culture media. The presence of acidophilic heterotrophic bacteria was revealed after the incubation for 21 days at 28^0C on the liquid Manning medium by the appearance of sediment, by the medium turbidity and the decrease under 2.0 of the initial pH value of the medium (2.5) [10].

The presence of the sulphur-reducing bacteria is revealed in Postgate medium after 10 incubation days through the forming of a black precipitate, sa a result of the sulphate in the medium to iron sulphurs [10].

The growing of the sulphur-oxidizing bacteria, *Acidithiobacillus thiooxidans*, in liguid Waksman medium (pH=4.0) is revealed after 21 incubation days by the lowering under 2.0 of the initial pH value. For *A. intermedius* was used the Postgate medium (pH 5.0 and 7.5), having as an energetic substratum the thiosulphate which by oxidation to elementary sulphure or reduced compounds of the sulphure determin the high decrease (2.0-2.8) of the initial pH value [10].

For the moderate termophilic acidophilic sulphur-oxidizing and iron-oxidizing bacteria of the *Acidithiobacillus* type it was used the March complex medium (pH 2.5), which contains ferrous sulphure, elementary sulphure and yeast extract. The presence of these bacteria determines the oxidizing both of Fe^{2+} (reddish colour) and the S^0, having as a result the decrease under 2.0 of the initial pH value of the medium [10].

In a view to obtaining populations of acidophilic chemolithotrophic bacteria were got using isolated colonies on agarized selective culture media, following the dynamics of the physiological activity in inorganic media specific (9K medium). Isolated colonies obtained are coloured brownish red. Using these technique 10 strains of *Acidithiobacillus ferrooxidans* were isolated [10].

The presence of the nitrifying bacteria in media containing ammonium (for nitrite bacteria) and sodium nitrite (for nitrate bacteria) was revealed by producing nitrite and nitrate, using diphenylamine as an indicator.

The denitrifying bacteria are revealed after 7 incubation days at 28^0C by changing of theGiltay specific culture medium (asparagyn, KNO_3 – N source and blue brom-tymol as indicator) colour from blue to yellow because of the metabolical activity through which the nitrates are reduced to nitrites [10].

RESULTS

The microbiological characterization of the coal samples evidenced a big variety of microorganisms, which presented a higher resistance to extrem conditions. In the pit coal sample come from Lupeni, Paroseni, Zonea, Mintia, Vulcan and Petrila mines and the lignite sample come from Halânga and Turceni mines was revealed a large release of acidophilic chemoorganotrophic and chemolithotrophic bacteria (Table 1).

The microbiological analyses of pit coal and lignite samples taken in 2006-2007 were evidenced a higher numerical density of sulphur-oxidizing bacteria comparated to the heterotrophic acidophilic bacteria. Among acidophilic heterotrophic microorganisms was identificated, on Manning selective medium, bacteria belonging to *Acidiphilium* genus from the morpho-physiological point of view: non-spore bacili, Gram-negative, strict aerobe, mesophilic, chemoorganotrophic nutrition, extremely acidophilic (pH 2.5).

After microbiological analyses of lignite samples from Turceni and Halânga mines taken in 2006-2007 was obtained differences in the presence and the numerical distribution of acidophilic bacteria, chemoorganotrophic and chemolithotrophic, because in higher measurements differents between the concentrations of sulphate from the lignite sample (Table 1).

A prove about the influence of sulphate concentrations on the growth and development of acidophilic bacteria, chemoorganotrophic and chemolithotrophic, is decreasing the numerical density by the representatives of two physiological groups (2.0×10^4 bact./ml, respectively 9.5×10^4 bact./ml) in lignite sample, compared to the pit coal sample (1.1×10^3 bact./ml , respectively 4.5×10^4 bact./ml).

Table 1. The quantitative estimation of the physiological groups of microorganisms present in the coal sample taken in 2006-2007.

Coal sample	The physiological groups of microorganisms analyzed					
	C cycle	N cycle		S cycle		
	Heterotrophic acidophilic bacteria (/ml /g)	Nitrifying bacteria (/ml /g)		Sulphur-oxidizing bacteria (/ml /g)		Sulphur-reducing bacteria (/ml /g)
		Nitrite bacteria	Nitrate bacteria	grow on sulphur (pH 6.6)	grow on thiosulphat (pH 6.6)	
Pit coal Lupeni (1)	9.5×10^2	4.5×10	3.5×10^3	2.5×10^4	4.5×10^4	4.5×10
Pit coal Paroseni (2)	3.5×10^3	1.1×10	2.0×10^4	0.4×10	0	1.5
Pit coal Zonea (3)	1.5×10^2	4.5×10	2.5×10^4	4.5×10^3	4.5×10^4	2.5×10
Pit coal Mintia (4)	4.5×10^2	1.5×10	1.5×10^4	2.5×10^4	2.5×10^4	9.5×10
Pit coal Vulcan (5)	1.1×10^3	1.5×10	1.4×10^3	7.5×10^3	9.5×10^3	2.5×10^2
Pit coal Petrila (6)	4.5×10	9.5	1.1×10^5	2.0×10^3	2.5×10^3	1.5×10^2
Lignite Turceni (7)	1.5×10^4	2.5×10^3	4.5×10^2	9.5×10^4	9.5×10^4	1.1×10^3
Lignite Halânga (8)	2.0×10^4	0.9×10	7.5×10	9.5×10^2	1.5×10^3	4.5×10^4

The experiments revealed that all analyzed samples contain heterotrophic acidophilic bacteria having correlation with chemical content of nutrient media. The lowest content of heterotrophic acidophilic bacteria had been registered in the pit coal sample from Petrila mine (4.5×10 bact./ml) and the biggest in the lignite sample from Turceni and Halânga mines (1.5×10^4 - 2.0×10^4 bact./ml) taken in 2006-2007.

The quantitative estimation of the physiological groups of heterotrophic bacteria present in the lignite sample prelevate from Turceni and Halânga mines showed the numerical distribution of acidophilic heterotrophic bacteria (2.0×10^4 bact./ml) compared with the nitrifying bacteria (0.9×10 bact./ml).

In the pit coal sample prelevated from Lupeni, Zonea and Mintia mines was revealed in the high number of sulphur-oxidizing bacteria from 2.5×10^4 bact./ml to 4.5×10^4 bact./ml, compared to the sulphur-reducing bacteria (2.5×10 - 9.5×10 bact./ml).

In the lignite samples come from Turceni and Halânga mines taken in 2006-2007, when the pH value is neutral to low basis, were representative in a numerical distribution by the sulphur-oxidizing bacteria (9.5×10^4 bact./ml) compared with the nitrifying bacteria (4.5×10^2 - 2.5×10^3 bact./ml).

The quantitative estimation of the physiological groups of microorganisms present in pit coal samples from Lupeni, Paroseni, Zonea, Mintia, Vulcan, Petrila mines and lignite samples from Turceni and Halânga mines showed a higher numerical distribution of the sulphur-oxidizing bacteria from *Acidithiobacillus* genus, compared to the sulphur-reducing bacteria. Although, in the lignite sample from Turceni by inoculation on two selective medium, which has as an energetic substratum like sulph and tiosulphat, the quantitative estimation of the bacteria showed present the sulph-oxidizing bacteria in number of 9.5×10^4 bact/ml, compared with the sulphur-reducing bacteria in number of 1.1×10^3 bact/ml.

The quantitative estimation of the physiological groups of microorganisms present in pit coal samples from Lupeni, Paroseni, Zonea, Mintia, Vulcan, Petrila mines and lignite samples from Turceni and Halânga mines showed a higher numerical distribution of the sulphur-oxidizing bacteria from *Acidithiobacillus* genus, compared to the sulphur-reducing bacteria. Although, in the lignite sample from Turceni by inoculation on two selective medium, which has as an energetic substratum like sulph and tiosulphat, the quantitative estimation of the bacteria showed present the sulph-oxidizing bacteria in number of 9.5×10^4 bact/ml, compared with the sulphur-reducing bacteria in number of 1.1×10^3 bact/ml.

The quantitative estimation of physiological groups of microorganisms present in coal samples taken from Halânga, Turceni and Paroseni mines, illustrated in Tables 2 - 4. The microbiological analysis showed differences in the numerical distribution, the acidophilic chemoorganotrophic bacteria (1.3×10^5 bact./ml) being better represented in the lignite sample from Halânga mine than in the pit coal sample from Paroseni mine (8.4×10^3 bact./ml).

The acidophilic heterotrophic bacteria has been a numerical distribution more higher in the raw lignite sample (4.5×10^4 - 1.3×10^5 bact./ml) compared with the raw pit coal sample (8.4×10^3 bact./ml). They are present in a low number from 8.4×10 bact./ml to 6.3×10^2 bact./ml, in the non-magnetic sample with the decrease of the pH value and higher concentrations of metallic ions.

On the Manning medium it was revealed the existence in analysed samples of the heterotrophic acidophilic bacteria belonging to the *Acidiphilium* genus. It is obvious the influence of the increased pH value of the samples analysed on the presence and numerical density of the acidophilic heterotrophic bacteria of the *Acidiphilium* genus. The acidophilic heterotrophic bacteria were representative in numbers in all samples analyzed (8.4×10 - 5.4×10^4 bact./ml).

The sulphur-oxidizing acidophilic bacteria of the genus *Acidithiobacillus* are strongly influenced by the neutral pH of the analysed samples, both as number (0.4×10 - 2.5×10^6 bact./ml) and as dominant species. Thus, the chemolithotrophic sulph-oxidizing bacteria belonging to *Acidithiobacillus thiooxidans* were present in all analysed samples. To reveal these bacteria we used one specific media Waksman which has as an energetic substratum elementary sulphur in an optimum concentration, the presence of the bacteria being revealed by the apperance of colour of the medium and the decrease of the pH value under 2.5.

By the inoculation of dilutions in two selective medium, which has as an energetic substratum like as sulph and tiosulphat, the numerical estimation of the

Table 2. The assessment of total number of heterotrophic acidophilic bacteria from coal samples taken in 2008.

No.	Coal sample	Total number of heterotrophic acidophilic bacteria (CFU / g)	Isolated strains number	Strains or mixed cultures with organic acids biosynthesis activity
1	Raw lignite Halânga	1.3×10^5	13	N/A
2	Mixed 2 lignite Halânga	2.7×10^3	14	N/A
3	Non-magnetic Carpco Halânga	6.3×10^2	10	6
4	Raw lignite Turceni	5.4×10^4	12	N/A
5	Mixed 2 lignite Turceni	3.8×10^3	9	N/A
6	Non-magnetic Carpco Turceni	8.4×10	1	1
7	Raw pi coal Paroşeni	8.4×10^3	13	N/A
8	Mixed pi tcoal Paroşeni	2.7×10^2	10	3
9	Non-magnetic Carpco Paroşeni	6.3×10^2	5	N/A
Number of isolated heterotrophic acidophilic bacterial strains			92	10

Table 3. The assessment of total number of sulphur-oxidizing and sulphur-reducing bacteria from coal samples taken in 2008.

No.	Coal sample	S cycle		
		Sulphur-oxidizing bacteria (/ml /g)		Sulphur-reducing bacteria (/ml /g)
		grow on sulphur (pH 6.6)	grow on thiosulphat (pH 6.6)	
1	Raw lignite Halânga	4.5×10	4.5×10^6	2.0×10^5
2	Mixed 2 lignite Halânga	2.5×10^6	1.5×10^5	0.7×10^4
3	Non-magnetic Carpco Halânga	0.4×10^2	0.9×10	0.7×10^4
4	Raw lignite Turceni	2.5×10^6	2.5×10^6	2.5×10^3
5	Mixed 2 lignite Turceni	2.5×10^6	2.5×10^6	1.1×10^4
6	Non-magnetic Carpco Turceni	2.5×10	0.4×10	0
7	Raw pitcoal Paroşeni	0.4×10^3	2.5×10	9.5×10
8	Mixed pitcoal Paroşeni	0.9×10	2.5×10	1.5×10^3
9	Non-magnetic Carpco Paroşeni	0.4×10	0.9×10	2.5×10

chemolithotrophic bacteria was evidenced the presence of these bacteria at similare value. Thus, in the case of lignite samples taken from Halânga and Turceni mines, on the two selective media were estimated 4.5×10^6 bact./ml on medium with sulph and 9.5×10^4 bact./ml in medium with tiosulphat (Table 3).

The chemolithotrophic bacteria that belonging to *Acidithiobacillus* genus are strongly influenced by the sulphate and metallic ion concentration value of samples, both as numerical distribution and as dominant species. In the microbiological analysis determined the present of the thiobacilli species, which were representative by the following species: *A. ferrooxidans, A. thiooxidans, A. neapolitanus, A. intermedius,* their growth and development was realized on the elementary sulphur and thiosulphate.

Another physiological groups of the bacteria were represented by the sulphur-reduction bacteria, which has in a number influenced by the highest content of sulphate present in the coal sample. This bacteria were present in the sample of raw lignite analyzed from Halânga mine taken in 2008 in a high number, reaching the maximum value of 2.0×10^5 bact/ml compared in the case of the non-magnetic from Halânga mine.

The sulphur-reduction bacteria were present a high tolerance to specific nutrient conditions and higher concentrations of sulphate in lignite and pit coal samples, in a moderat number from 4.5×10^4 bact./ml to 2.5×10^4 bact/ml.

The results obtained in the microbiological analysis of lignite and pit coal samples from Paroseni, Halânga, Turceni mines taken in 2008 after applying the classical procedure of coal manufacture, have been in correlation with the highest concentrations of sulphate and metallic ions resulted from the oxidative activity of the chemolithotrophic bacteria (Table 3).

Another groups of chemical autotrophic microorganisms is represented by the nitrifying bacteria, that are strongly influenced by the pH value of all analyzed samples, both as number (0.9×10 – 2.5×10^4 bact./ml) and dominant species. Although, in all the samples analyzed taken in 2008 showed differences in the numerical distribution, the nitrifying bacteria (2.5×10^3 - 2.5×10^6 bact/ml) being better represented than the denitrifying bacteria from 0.4×10 to 2.5×10^4 bact/ml. The nitrifying bacteria were better represented numerically reaching in samples of lignite (2.5×10^6 bact/ml) comparated with samples of pit coal (2.5×10^3 bact/ml), this fact is corelated with the presence of nitrate in samples of lignite (Table 4).

Table 4. The assessment of total number of nitrifying and denitrifying bacteria from lignite and pitcoal samples taken in 2008.

No.	Coal sample	N cycle		
		Nitrifying bacteria		Denitrifying bacteria (/ml /g)
		Nitrite bacteria (/ml /g)	Nitrate bacteria (/ml /g)	
1	Raw lignite Halânga	2.5×10^6	0.9×10^6	0.4×10^4
2	Mixed 2 lignite Halânga	2.5×10^6	2.5×10^6	2.5×10^4
3	Non-magnetic Carpco Halânga	2.5×10^6	2.5×10^6	0.9×10
4	Raw lignite Turceni	0.4×10^6	0.9×10^6	0.9×10^4
5	Mixed 2 lignite Turceni	0.9×10^6	2.5×10^6	2.5×10^4
6	Non-magnetic Carpco Turceni	2.5×10^6	0.9×10^6	2.5×10
7	Raw pit coal Paroşeni	2.5×10^6	2.5×10^6	0.9×10^2
8	Mixed pit coal Paroşeni	2.5×10^6	0.9×10^6	0.4×10
9	Non-magnetic Carpco Paroşeni	2.5×10^3	2.5×10^3	0.4×10^3

DISCUSSIONS

The research regarding microbiological analysis of the samples from the processing plant revealed a big variety of microorganisms from different physiological groups: aerobe heterotrophic acidophilic bacteria, optional anaerobe bacteria (denitrifying), strict anaerobe heterotrophic bacteria (sulphur-reducing) and chemoautotrophic bacteria (sulphur-oxidizing and nitrifying), confirms the data in the speciality literature [3, 8, 16] about the increased capacities of the bacterial population to adapt to the extreme medium conditions (acid pH and high concentrations of metal ions) (Tables 1-4).

For identification the strains and populations of microorganisms in the lignite sample were analyzed having in view inoculated into specific medium and consideration the following features: morphology of colonies, rate of growth, morphology and size of microscopically structures, potential of growth into nutrient medium containing different concentration of substratum, termotolerance. On the obtained basis were preliminary characterized that belonging to *Acidithiobacillus*, *Acidiphilium* genera or species reprezentative in the analysed samples, confirm the data in the speciality literature [10, 20].

The results revealed that the higher number of aerobic heterotrophic acidophilic bacteria was determined from raw coal comparated with non-magnetic and mixed samples. The acid production activity by acidophilic heterotrophic bacteria increased with cellular growth, the lowest pH values being obtained after 14 days of incubation at 28^0C.

It is obvious the influence of the increased pH values of the samples analysed on the presence and numerical density of the acidophilic heterotrophic bacteria of the *Acidiphilium* genus, confirm the data in the speciality literature [15, 21] about the higher abilities of the bacterial populations to adapt to extreme medium conditions (Tables 1 & 2).

The quantitative estimation of heterotrophic acidophilic bacteria showed a numerically abundance in samples of lignite from Hălânga mine (9.5×10^5 bact/ml) and Turceni mine (4.5×10^5 bact/ml), comparated with samples of pit coal from Paroşeni mine (1.5×10^4 - 2.0×10^4 bact/ml).

The sulphur-oxidizing acidophilic bacteria of the genus *Acidithiobacillus* are strongly influenced by the neutral and basis pH of the analysed samples, both as number (0.4×10 - 2.5×10^6 bact/ml) and as dominant species. Thus, the acidophilic chemolithotrophic iron-oxidizing bacteria belonging to *Acidithiobacillus ferrooxidans* were absent in all the predominant samples analyzed.

The heterotrophic strict anaerobe, sulphur-reduction bacteria were representative in numbers in samples of lignite from Halânga mine (9.5 - 2.0×10^5 bact/ml) compared with samples of pit coal from Paroseni mine (1.5×10^3 bact/ml).

The quantitative estimation of sulphur-oxidizing bacteria present in samples taken from Turceni mine showed a numerically distribution of acidophilic chemolithotrophic bacteria (2.5×10^6 bact./ml), compared with sulphur- reducing bacteria (1.1×10^4 bact/ml).

The sulphur-reducing bacteria were better represented numerically in samples of lignite (2.5×10^3 - 2.0×10^5 bact./ml), comparated with samples of pit coal (9.5×10 – 1.5×10^3 bact/ml).

The chemical autotrophic bacteria, although present in all the samples analyzed, showed differences in the numerical distribution, the nitrifying bacteria from 2.5×10^3 to 2.5×10^6 bact./ml) being better represented than the denitrifying bacteria (0.4×10 - 2.5×10^4 bact./ml).

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