International Journal of Earth, Energy and Environmental Sciences
ISSN: 2517-942X
Vol:8, No:7, 2014

Isolation of Soil Thiobacterii and Determination of Their Bio-Oxidation Activity

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***Abstract*—**36 strains of sulfur-oxidizing bacteria were isolated in Southern Kazakhstan soda-saline soils and identified. Screening of strains according bio-oxidation (destruction thiosulfate to sulfate) and enzymatic (Thiosulfate dehydrogenises and thiosulfate reductase) activity was conducted. There were selected modes of aeration and culture conditions (pH, temperature), which provide optimum harvest cells. These strains can be used in bio-melioration technology.

***Keywords*—**Elemental sulfur, oxidation activity, Thiobacilli, fertilizers, heterotrophic S-oxidizers.

I. Introduction

THE fact that saline alkaline-soils cover wide area of Kazakhstan results in reduction of crops from 15 to 45%. This necessitates the constant search for the most promising methods for their melioration and chemical reclamation by adding plaster, phosphogypsum, pyrite, sulfuric acid, sulfur and industrial waste. Traditional soda-saline soils chemical melioration technologies are characterized by low efficiency and high costs [1], [2]. The elemental sulfur is one of the most promising methods of melioration of saline alkaline-soils [3]. However, sulfur due to the inertia as ameliorant is practically not used and the problem of increasing its melioration efficiency has almost not been recognized [4]. Therefore, there are demands in the development of additional techniques which can increase sulfur oxidation rates in the soil [5], [6].

The product of oxidation of the soil by widely distributed in it Thiobacteria is predominately elemental sulfur. These bacteria form sulfuric acid which acidifies the soil, helping to transfer some important for plants elements into appropriate forms. In this regard, a new direction in the field of land amelioration of solonets and alkaline soils- a joint introduction of elemental sulfur and Thiobacteria oxidizing sulfur during their life appeared [7].

The study found that the sulfur in the soil sulfur-oxidizing microorganisms with the scheme $S \rightarrow SO_2 \rightarrow SO_3$ slowly enters its sulfur di- and trioxides forms, where the latter combines with water to form sulfuric acid as a perfect agent for saline alkaline-soils. Thus, melioration process unlike the soil drench solution of sulfuric acid takes place in the "mild" conditions [9].

The purpose of this research is to obtain pure cultures of sulfur-oxidizing bacteria, to determine their sulfur-oxidizing activity and the level of biomass accumulation on selective medium.

Thus, oxidation rates of elemental sulfur can be introduced into the soil by biological products with soil sulfur-oxidizing microorganisms. Moreover, the use of microorganisms isolated from soda-saline soils of the area to endogenous microflora provides their existence and functioning in the ecological niche.

Soil association of sulfur-oxidizing bacteria was obtained by selection from soda-saline soils. To isolate the individual strains and to study physiological and biochemical culture properties was used Armbruster nutrient of the following composition: g/l: $Na_2S_2O_3$ – 10; KH_2PO_4– 4; $MgSO_4$ – 0, 8; NH_4Cl– 0,4; $CaCO_3$– 5; solution of microelements – 10 мл/л. Solution of microelements (300 g/ml distillated water): EDTA-15; $ZnSO_4$ - 0, 66; $CaCl_2$ - 1, 66; $MnCl_2$ -0, 75; $CoCl_2$ - 0, 15; $(NH_4)_6$ Mo_7O_{24}- 0, 15; $FeSO_4$ - 1,5; $CuSO_4$ - 0, 06; NaOH - 3, 3. Nutrients pH 6, 8 – 7, 0 were obtained with the help of 10% solution of $NaHCO_3$.

Cultivation was performed on the isolates Baalsruda medium of the following composition (g / l): $Na_2S_2O_2$– 5, 0; KNO_3 - 2, 0; NH_4Cl – 0, 5; $NaHCO_3$ – 1, 0; $MgCl_2$ – 0, 5; K_2PO_4 – 2, 0; $FeSO_4$ – 0, 01; H_2O – 1l; pH 7, 0. Growth of sulfur-oxidizing microorganism's biomass was evaluated by the change of protein content in the culture broth by the Lowry method and by counting the number of cells.

Biooxidation process of the model substrate (thiosulphate) while the development of sulfur-oxidizing microorganisms was carried out under batch culture flasks in a thermostatic shaker. Changes in the concentration of substrate - thiosulfate and bio-oxidation products – sulfates were under control. Thiosulfate was determined by cyanolized. Sulphate was determined by liquid chromatography Biotronik LC 500. To control the chemical oxidation of reduced sulfur concentration of the main substrate was simultaneously analyzed without introducing microbial environment.

To determine the activity of enzymes sulfur metabolism there were used bacteria of an exponential growth end. Cells were separated from the medium by centrifugation for 20 min at 10,000 g washed twice with fresh medium containing no source of energy then they were washed with 0, 05 M Tris-HCl buffer, pH 7, 4. Cells were disrupted by sonication at 22 kHz in a buffer for 3 min at 1 min intervals for cooling. The homogenate was centrifuged for 25 min at 40,000 g and the supernatant was used to determine enzymes by spectrophotometric methods or colorimetric spectrophotometer.

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Thiosulfate dehydrogenase was measured according to the rate of ferricyanide recovery at 420 nm in the presence of sulphite or thiosulphate. Thiosulfate dehydrogenase activity was measured by the rate of hydrogen sulfide formation from thiosulfate at the presence of recovered glutathione and ditiotrietola.

II. Results and Discussion

Sulphur-oxidizing bacteria were isolated from samples from alkaline environments including soda soil (genus *Thiobacillus*).

Referred to as "sulfur-oxidizing" microorganisms if they are capable of model substrate thiosulfate oxidation a nutrient medium comprising to sulfate (S^{6+}).

Elective conditions for the development of sulfur-oxidizing microorganisms were created by incorporation into the culture medium reduced sulfur source, thus the pH conditions in the range of neutral and slightly alkaline values. Microorganisms have been cultivated out in flasks on a mixing device at a temperature of 28°C.

The sterile nutrient media formed a stable association representing after several passages collecting a culture of bacteria capable of oxidation of reduced sulfur compounds.

Sulfur-oxidizing microorganisms obtained Association HC1 – NC-23 is representatives of biogenesis soda- saline soils.

It is known that the development of sulfur-oxidizing microorganisms related to oxidation of reduced sulfur compounds to the final product - sulfates or partially oxidized to form , for example polythionates, accompanied by a change in pH environment [7]. In order to determine the bio-oxidation activity of the microorganisms were investigated associations biooxidation processes model substrate (thiosulfate).

Based on data obtained, the culture of the association of NK -1, NK- 4, NK- 6, NK - 7, NK - 12, NK - 18, NK - 21, are able to oxidize thiosulfate only to polythionates that accompanied alkalinity of the nutrient medium.

Development of microorganisms includes to the cumulative culture of NK -2, NK - 5, NK - 8, NK - 10, NK - 13, NK - 14, NK - 17, NK- 19, NK - 23 is associated with an initial increase of the pH to 7, 0-8, 0 units and its further reduction to 6,2-5,8, which is a consequence of the accumulation of sulfate as the final oxidation product [6].

In the process of bio-oxidation was observed accumulation of intermediates products- polythionates that formed in the oxidation of thiosulfate and molecular sulfur, in that case, evidenced by the increasing pH of the culture medium from 6, 6 to 8, 1 units on the 5th day of cultivation.

Polythionates accumulation in the medium firstly is associated with the microbiological oxidation of thiosulfate to tetrathionate, which is accompanied by alkalinization of the medium by the reaction:

$$2S_2O_3^{2-} + ½\ O_2 + H_2O \rightarrow 2S_4O_3^{2-} + 2OH^-$$

It must, however, be considered and ways of chemical oxidation of thiosulfate ions, and in chemical polythionates disproportionate of sulphite and thiosulphate reciprocal action [5]. First accumulation polythionates replaced by the reaction of decreasing concentration in the medium that may be associated with the partial reduction of microbial sulfur to form thiosulfate, and the oxidation of sulphite to sulphate. Alternately increasing and decreasing concentrations of the intermediates in the process of bio-oxidation characterizes the equilibrium nature of the transitions and polythionates thiosulfate.

TABLE I
Change in pH of the Culture Medium during Cultivation Associations Sulfur-Oxidizing Microorganisms

Cumulative culture	pH				
	Cultivation's time, day				
	0	5	7	10	18
NK-1	7,1	7,4	7,6	7,3	7,0
NK-2	6,4	6,7	6,4	6,2	6,1
NK-3	6,4	5,8	5,1	4,8	4,3
NK-4	7,0	7,9	8,2	8,2	8,1
NK-5	6,4	6,7	6,3	6,3	5,6
NK-6	7,1	7,5	7,5	7,4	7,1
NK-7	7,0	7,8	8,1	8,2	8,1
NK-8	6,4	6,7	6,2	6,1	6,0
NK-9	6,4	5,8	5,1	4,7	4,5
NK-10	6,4	6,7	6,3	6,3	5,7
NK-11	6,4	5,8	5,1	4,9	4,3
NK-12	7,1	7,4	7,5	7,2	7,2
NK-13	6,4	6,7	6,4	6,1	5,8
NK-14	6,4	6,7	6,3	6,2	6,2
NK-15	6,4	5,8	5,1	4,7	4,4
NK-16	6,4	5,7	5,1	4,8	4,3
NK-17	6,4	6,7	6,4	6,2	6,1
NK-18	7,1	8,0	7,5	7,1	7,3
NK-19	6,4	6,7	6,4	6,1	6,0
NK-20	6,4	5,8	5,1	4,7	4,1
NK-21	7,0	7,8	8,3	8,1	7,9
NK-22	6,4	5,8	5,1	4,6	4,3
NK-23	6,5	6,8	6,4	6,3	5,8

Sulfur-oxidizing bacteria for associations that make up the savings culture NK- 3, NK- 9, NK- 11, NK- 15, NK- 16, NK-20, NK-22, isolated from soda-saline soils in the metabolism of thiosulfate is a direct education sulfate without accumulation polythionates, as evidenced by a decrease of pH from 6,4 to 4,3 without alkalizing.

It should be noted that the association of NK- 2, NK- 5, NK- 8, NK- 10, NK- 13, NK- 14, NK- 17, NK- 19, NK- 23, the importance of economic education sulfate ratio YR p/ s power 0, 86, which is the result of accumulation in the environment of products of incomplete oxidation of reduced sulfur compounds (polythionates, molecular sulfur, sulfites).

Association of NK- 3, NK- 9, NK -11, NK- 15, NK- 16, NK-20, NK-22 is a complex of sulfur-oxidizing microorganisms involved in the complete oxidation of thiosulphate to sulphate, as evidenced by high value:

$$YR\ p/\ s = 1,47.$$

Importance of the economic factor Yx p/ s for these

associations was more typical for the bacteria *Thiobacillus ferooxidans*, which vary in the range of 2, 2-6, 6 x 10- 3 g or 3, 5-5, 3 × 10- 3 ASB/Fe^{2+}. Odds Comparison of microbial growth and development in this case is very conditional, since the values for pure cultures cannot be compared with the values for the association of microorganisms.

Thus, the final product of the bio-oxidation of reduced sulfur compounds for associations NK- 2, NK- 5, NK- 8, NK-10, NK- 13, NK- 14, NK- 17, NK- 19, NK- 23, and NK -3, NK-9, NK- 11, NK- 15, NK- 16, NK- 20, NK- 22 are sulfates, as evidenced by their accumulation in the culture medium, leading to a decrease in pH to 5, 6 at day 18 units culturing. However, it should be noted that increasing the concentration started on day 10 and cultivation was then continued throughout the experiment. This may mean simply that the data consist of the association of microorganisms on their various physiological characteristics and bio-oxidation. Isolation of pure cultures and their development processes Trials will confirm this assumption.

Microscopic studies have yielded their association microbiological characteristics was performed using preparations of living cells on a microscope equipped with phase-contrast device. Type flagellation was determined by the nature of the motion of bacterial cells in the microscope.

Below are presented the morphological properties investigated associations:

- The community of bacterial cells, which is dominated by small straight rod-shaped bacteria (1, 2 x0, 4 mic), moreover found curved rod-shaped bacteria (1, 6 x0, 8 mic), round (coccid) sticks 0, 4 mic;
- Dominated by the bacterial cells in the form of rod (2, 0 x 0, 8 mic) with and fixed polar flagellation. In association with them rod detected size of 0, 4 mic;
- The association rod-shaped bacteria. Dominate the cells sizes: 5, 0 x1, 2 mic and 1, 2 x0, 8 mic; noted the presence of cocci rod 0, 4 mic;
- Rod-shaped bacteria, which dominated of thin elongated bacilli 2, 2 x 0, 8 mic). Rod-shaped bacterium found 0, 1-2, 4 and 3, 0 mic x 0, 4 mic, and curved rod-shaped cells;
- The bacterial cells predominate in the form coccoid rods size of 0, 4 mic with a polar flagellation. In association with them, rod-shaped bacterium found two types: fixed (1, 6 x0, 4 mic), and cells capable of movement, the dimensions of 2, 0 x0, 8 mic;
- The association represented rod-shaped bacteria. It is noted the presence of cell size: 1, 0 x0, 4 mic; 2, 5 x0, 6 mic, 1, 2 x0, 8 mic and 0, 4 microns coccid rods. Are the dominant bacteria, which have the form of small rods (1, 2 x0, 4 mic);
- Rod-shaped bacteria: small (1, 2 x0, 4 mic) and coccid (0, 4 mic) with polar flagellation, still elongated sticks. When the microscope fixed cells stained preparations were found Gram-positive filamentous forms.

Sulfur-oxidizing bacteria studied associations are Gram-negative, represented by single cells or form clusters of two or more units.

TABLE II
CULTURAL PROPERTY OF ASSOCIATIONS OF SULFUR-OXIDIZING MICROORGANISMS

Associated	Type of colony	Description colonies			
		forms	size	optical properties	color
Type 1	1	round	d=2 mm	translucent	White
	2	round	point	translucent	without color
	3	Round	point	matt	white
	4	Round	d=2 mm	opaque	grey
Type 2	1	Round	point	translucent	without color
	2	round with wavy edge	d=2 mm	matt	white
	3	Irregularly shaped inner part thickened, surrounded by translucent ringe	d=3-4 mm	opaque	white and grey
Type 3	1	Round	d=1,5 mm	matt	white
	2	Round	point	opaque	white
Type 4	1	Round	d=2 mm	opaque	white and yellow
Type 7	1	Round	point	opaque	white

Thus, the examined associations are represented by rod-shaped bacteria mainly differing in the size of cells and the presence of flagella.

Pure cultures of sulfur-oxidizing bacteria were obtained from collecting soil microbial associations at their growth on medium with thiosulfate (sulfur source) and NH_4CI (nitrogen source). In the result, there were isolated 36 strains of sulfur-oxidizing bacteria. According to the results of the phenotypic identification bacteria were assigned to 6 species: *Thiobacillus thioparus; Thiobacillus thiooxidans; Thiobacillus denitrificans; Thiobacillus thiocyanoxidans; Thiobacillus novellus; Thiobacillus ferrooxidanas.*

The development of all the strains on nutrient medium was characterized by its full thiosulfate to form disposing sulfate on the 8 day. 10 strains were accumulated up to 3900-4300 mg / dm 3900-4300 mg/ CFU/sulfate in liquid culture. In this case the substrate is not detected on the third day of cultivation.

To quantify bio-oxidation activity of *Thiobacillii* strains there was performed a special study of their enzymatic activity (Table III).

Dehydrogenase enzymes of oxidoreductases participate in the process of thiosulfate biooxidation. Tiosulfatdehydrogenase and tiosulfatreductase are two of the key enzymes of energy metabolism sulfur-oxidizing microorganisms [9].

The highest value of dehydrogenase activity in the disposal of sulfate was found in 7 strains. (1, 5, 10, 11, 16, 24, 31 - in Table III). High values of enzyme activity were achieved on the third day of cultivation, when the bacteria were in the logarithmic growth phase, which is characterized by intensive consumption of the substrate, and hence a superior enzymatic activity.

Cells harvest in those same seven strains was twice higher than that of the middle-strains dehydrogenase activity. It is

also found that the yield of cells depends on the oxygen regime of cultivation. The oxidation of sulfur compounds in increasing the oxygen content to 20 % is accompanied by two-fold increase in cell concentration.

TABLE III
SULFUR METABOLISM ENZYME ACTIVITY OF *THIOBACTERII* AFTER 72 HOUR BATCH CULTIVATION

Strain	Protein concentration mg/ml	Enzyme activity , nmol/(min * mg protein)	
		Thiosulfate dehydrogenase	*Thiosulfate Reductase*
1	130	1532	1834
5	100	1547	1509
10	150	1611	2762
11	105	1509	1956
13	60	929	1150
15	90	1207	1497
16	110	1394	2003
24	120	1592	2398
25	70	1023	1248
31	145	1603	2679

The presence of reduced compounds of sulfur in the culture medium also contributes significantly stability and increase in the harvest of culture cells in 1, 2 times.

Thus, the general result of this study is to select the most active strains of thiobacteria. These are: *Thiobacillus ferroxidans* 10, 16, 24; *Thiobacillus thiooxidans* 5, 11, 31; *Thiobacillus denitrificans* 1.

TABLE IV
CELLS HARVEST (LG CFU / ML) OF BACTERIA OF THE GENUS *THIOBACILLUS* ON CONDITIONS OF BATCH CULTIVATION IN SELECTIVE CULTURE MEDIUM

Strain	Cultivation time, hours			
	24	48	72	96
1	1,9±0,1	4,7±0,1	8±0,1	7,9±0,2
5	2±0,2	5±0,2	7,9±0,1	7,9±0,3
10	2±0,3	5±0,1	8±0,2	8,1±0,1
11	1,8±0,1	4,3±0,2	7,7±0,2	7,8±0,3
13	1,5±0,3	3,5±0,2	6±0,1	6,1±0,1
15	1,6±0,1	3±0,1	5,5±0,1	5,6±0,1
16	1,8±0,1	4,6±0,2	7,5±0,1	7,4±0,2
24	1,9±0,1	4,1±0,2	7,6±0,2	7,6±0,1
25	1,5±0,1	3,2±0,1	6,1±0,2	6,2±0,3
31	2±0,2	4,9±0,2	8±0,2	8±0,3

Cells harvest in those same seven strains was twice higher than that of the middle-strains dehydrogenase activity. It is also found that the yield of cells depends on the oxygen regime of cultivation. The oxidation of sulfur compounds in increasing the oxygen content to 20% is accompanied by two-fold increase in cell concentration. The presence of reduced compounds of sulfur in the culture medium also contributes significantly stability and increase in the harvest of culture cells in 1, 2 times.

The general result of this study is, therefore, to select the most active strains of thiobacteria. These are: *Thiobacillus ferroxidans* 10, 16, 24; *Thiobacillus thiooxidans* 5, 11, 31; *Thiobacillus denitrificans* 1.

These strains are expected be used for introduction into soil to raise the level of sulfur biooxidation. To do this it is necessary to obtain cells biomass. In this regard, optimal spacing of pH and temperature for cultivation were adjusted. Two of them were chosen as the most active strains: *Thiobacillus thiooxidans* 10 and *Thiobacillus ferroxidans* 31 (Table V).

It has been established that the optimum pH range is from 6, 6 to 9 units. The greatest increase in biomass in the stationary phase of growth was observed at pH 7. Maintaining neutral pH is requirement for effective biomass accumulation of sulfur-oxidizing bacteria.

Decrease of pH of the culture medium to 3 units is characterized by minimal microbial growth (25-30 % for 5 - day culture). It is noted that Thiobacteria development at pH 9 was also accompanied by increase of biomass.

TABLE V
CHANGE IN THE NUMBER OF CFU IN THE GROWTH OF SULFUR-OXIDIZING MICROORGANISMS IN THE DIFFERENT PH CONDITIONS OF CULTURE MEDIUM

Time	Number of CFU $x10^5/sm^3$ Strain 10			Number of CFU $x10^5/sm^3$ Strain 31	
	pH 3	pH 7	pH 9	pH 3	pH 7
0	5,17±0,72	6,72±1,01	4,48±0,58	5,60±0,78	7,36±0,72
1	5,90±0,77	7,36±0,88	5,92±0,65	6,18±0,87	7,87±0,94
5	6,48±0,71	12,48±0,72	9,78±0,67	7,28±0,82	13,06±0,51

Increase of biomass allows to recommend these strains or their associations be implemented as biological products for soda- saline soils. Seasonal temperature decline 7°C in winter can lead to significant limitation for using the strains in systems of soda-saline soils melioration. Most bacteria capable of oxidation of sulfur compounds are mesophyll cells. Range of optimal temperatures for sulfur-oxidizing bacteria of genus *Thiobacillus* is from 15 to 42°C.

Due to the seasonal fluctuations in soil temperature, growth of strains *Thiobacillus thiooxidans* 31 and *Thiobacillus ferooxidans* 10 was analyzed in the following temperature ranges: 4 - 5°C, 23°C (average soil temperature in summer) and 28°C (optimal range for development of sulfur-oxidizing bacteria of genus *Thiobacillus*).

Development of sulfur-oxidizing microorganisms at temperatures of 23-28°C was characterized by maximum biomass growth on the number of CFU (88-122% on the 5^{th} day of experiment), which suggests the feasibility of cultivation and use of strains and their associations in the specified temperature range.

The temperature factor, accordingly, has a significant influence on the development of these organisms and one of the limiting their effective in the soil (biotransformation sulfur). In this regard, while using methods of reclamation of soda- saline soils by using sulfur-oxidizing bacteria, the necessary condition to keep the bacteria activity is to maintain the temperature at 23-28°C.

TABLE VI
CHANGE IN NUMBER OF CFU IN THE GROWTH OF STUDIED SULFUR OXIDIZING MICROORGANISMS ASSOCIATIONS IN DIFFERENT TEMPERATURE CONDITIONS

Time of cultivation, days	Number of CFU x10^5/sm^3 Strain 10			Number of CFUx10^5/sm^3 Strain 31	
	4°C	23°C	28°C	4°C	23°C
0	5,28±0,74	5,92±0,53	4,67±0,51	4,18±0,45	5,67±0,39
1	5,99±0,72	9,01±0,42	8,21±0,37	4,64±0,33	8,23±0,74
5	6,30±0,71	11,14±0,49	10,37±0,43	5,06±0,35	10,24±0,81

III. CONCLUSION

Sulphur-oxidizing bacteria were isolated from samples from alkaline environments including soda soil and identification 36 strains. According to the results of phenotypic identification they are assigned to the following taxa: *Thiobacillus thioparus; Thiobacillus thiooxidans; Thiobacillus denitrificans; Thiobacillus thiocyanoxidans; Thiobacillus novellus; Thiobacillus ferrooxidanas.*

Activity of enzymes of sulfur metabolism of Thiobacteria after 72 hour batch cultivation was determined. Activity level of thiosulfate dehydrogenase is 929-1611 nmol / (min * mg protein). Thiosulfate dehydrogenase activity ranges from 1150 to 2762 nmol / (min * mg protein). From the results 7 active strains are selected.

It was found that the greatest increase in biomass strains was observed on the 5th day batch culture in Baalsruda nutrient at pH 7, 0. The temperature optimum was from 23 to 28°C.

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