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The Separation and Identify of SRB in the Experimental Research on the Disposition of Acid Coal Mine Drainage

Yongpeng Wang[1], Zhihuai Zhao[1]*, Xiuzhen Yin[2], Yuan Liu[3] and Huaai Liao[1]

[1]Taiyuan University of Technology,Taiyuan,Shanxi, China.
[2]Shandong Geological Prospecting Institute of China Chemical Geoology and Mine Bureau.
[3]Shanxi Geological Engineering Exploration Institute.

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The Separation and Identify of SRB is the basis of the Experimental Research on the Disposition of Acid Coal Mine Drainage, SRB can degradate many substances that other germs can not in the disposition of Acid Coal Mine Drainage. This paper introduces the culture, isolation and purification of the sulfate-reducing bacterias in detail, and eventually separated and identified two bacteria strains which are referred to as strain I and II through identifying various of characteristics of the strains.

Key words: Sulfate reducing bacteria (SRB), Acid coal mine drainage (ACMD), Strain of the bacteria, Separation, Identify.

Coal is our pillar of energy, accompanied by development of our coal industry, coal mines acidic waste water of the emissions also increasingly increased, not only polluted the clean surface water and groundwater resources and land resources, but also harm the crop's growth and water born organisms and the human's body health. So, the treatment and the research of use of mine acid waste water become an important element in the protection of water resources, which has been widespreadly concerned in domestic scholars.

Microbial treatment of coal mine acidic wastewater is meaning that the sulfate-reducing bacteria reduced the sulfate to H_2S by alienation sulfate biological reduction reaction, and oxidation the H_2S to elemental sulfur by the use of certain microorganisms.

Microbial treatment of sulfate-reducing bacteria mine acidic waste water is low cost, applicability, no secondary pollution, important material elemental sulfur can also be recycled, so it is widespreadly concerned by environmental workers, and it becomes mine acid waste water's treatment technology [1] topics at the forefront.

In the process of biological desulphurization, the key is to obtain high conversion activity of strains, in this paper, the isolation, purification and identification of sulfate-reducing bacteria belonging to the basic research for further research and application basis.

The materials and apparatus of test

Testing mud source

The SRB distributes broader and can be obtained in most soil and water, the testing vaccination mud is taken from secondary sedimentation tank return sludge in sewage purification plant in Yang Jiapu Taiyuan.

The enrichment and separation of the medium

Liquid A: distilled water: 980 ml, NH_4Cl:2.0g, $MgS0_4 \cdot 7H_20$: 2.0g, Na_2S0_4:1.0g, sodium lactate (70%): 3.5ml, yeast paste: 1.0g, $CaCl_2 \cdot H2O$: 0.1g, K_2HPO_4:0.5g.

Liquid B: $FeS0_4$:10ml

Liquid C: distilled water: 10ml, ascorbic acid: 0.1g

The pH of Liquid A and C was adjusted to 7.4 by

* To whom all correspondence should be addressed.
E-mail: 317157931@qq.com;
Zhihuai Zhao, College of Water Conservancy Science and Engineering of Taiyuan University of Technology, Yingze West Street No. 79, Taiyuan, Shanxi, China, 030024.

washing with 0.lmol / l NaOH, the final pH is neutral.

Test instruments and reagents

Instruments: constant temperature water bath, UNICO-PC2102 UV-visible spectrophotometer, pHS-3C precision pH meter, insulation incubator, vertical clean benches, TGL-16G high-speed desktop centrifuge, electric portable high-pressure steam sterilization pot culture dish test tube, ring vaccination, alcohol lamp,graduated cylinder, electric stove, PSH-200 incubator, microscope, nitrogen cylinders.

Reagents: distilled water, alkaline Meilan dye, safranin solution, capsular staining solution.

The method of test

Enrichmental culture of sulfate-reducing bacteria

Using 500ml glucose injection bottles, dispensing a certain volume of non-sterile enrichment medium, inoculated with black-odor characteristics of anaerobic sewage and sludge inoculum in size of 20% to the full state, culturing in 28 ° C ~ 30 ° C dark thermostat box. About a week later, culture medium shows a very strong mozi black, the bottleneck of H2S exudes the smell of rotten eggs, indicating sulfate-reducing bacteria has blooms. and then re-inoculated into another bottle of medium culture in inoculums' size of 20% continue to be in an incubator at 28 ° C ~ 30 ° C, and so forth operation for four times, the last media must be sterilized(Fan, *et al*.,1988).

Separation of sulfate-reducing bacteria

Preparation of dilution series

The1ml enrichment of ready broth is sterile operated to a 10-fold dilution, sequentially 10-1, 10-2, 10-3, 10-4, 10-5, 10-6, 10-7, 10-8, 10-9 concentration of bacterial suspension.

Culture dishes operation

With a ready 200ml nutrient agar solid medium, agar concentration of 2.0%, bacteria insulate around 45 ° C, using five 60mm × 12mm Culture dishes (large culture dish medium required amount should increase accordingly), sterile pour a layer of nutrient agar to culture dish after 121 ° C sterilization, the thickness of culture dish seeming about half of the height is appropriate. Until the agar was cooled, take respectively after the 10-5 to 10-9 dilutions of the bacterial suspension 0.1ml aseptic coating in each agar plate. The broth permeate five minutes after coating(Mu, *et al*.,1998).

Anaerobic inflatable

Tagged the coating culture dish which was sealed, placed in a vacuum desiccator and placed in a desiccator for a certain amount of oxygen absorbing agent, and then filled deoxy-purity argon with a vacuum pump repeatedly operation three times, until the vacuum desiccator is filled with argon gas, and seal the vacuum desiccator with a gas sealing membrane.

Culturing

Cultured vacuum dryer in the dark at 28 ° C ~ 30 ° C incubator within 8 to 10 days, until it grows small black colonies in the culture dish.

Pick single colony

Picked an appropriate dilution culture dishes (the number of colonies growing preferably 1-20) in sterile operating table, aseptic kick shelters, hydrogen sulfide of the colonies its own metabolism react with ferrous salt to the black deposit and black spherical colonies, it can easily be designated picked. Single colony was picked and inoculated into the already sterilized medium by vaccination ring, cultured in a 28 ° C ~ 30 ° C incubator in the dark, until the liquid turns rich mozi black after sealed.

Identification of sulfate-reducing bacteria

Isolates purity testing

Using phase contrast microscope or an ordinary optical microscope observe the morphology, size of the two strains cells of consistency, preliminarily to determine whether contamination by bacteria. Cultured on nutrient agar plates containing glucose and peptone, and check whether contaminated with aerobic bacteria by observing whether the plates grow aerobic bacteria colonies.

Check whether there are other non-sulfate-reducing bacteria pollution anaerobes by using the following medium: peptone (0.4%), glucose (1.0%), Na_2S0_4 (0.2%), $MgS0_4$ (0.1%), agar (1.5%),added Fe $(NH_4)_2 (S0_4)_2 \cdot 6H_20$ of separate sterilization to the ultimate concentration of 0.05% after sterilization, adjusted pH to7.0—7.6 Diluted cultured using agar the oscillation dilution method at 30 ° C incubation 3 to 4 days after the observation(Chen, *et al*.,2007). If it appears non-black colonies, gas production, or forming a halo, it turn out to be polluted other non-sulfate-reducing bacteria in anaerobic bacteria.

The bacteria can only enter the next identification after be confirmly purified.

Bacterial staining and morphological characteristics observing

Two strain cultures in the logarithmic growth phase were Gram stain, flagella, spore staining, observed various strains of shape, size, Gram stain results under an optical microscope, and whether have presence and absence of flagella or not and observe whether live bacteria move or not and movement characteristics by using an ordinary optical microscope or phase contrast microscopy.

Resistance to heat checking of strain

For Strain I ,keep the temperature of different growth phase liquid cultures, respectively, in the 100 ° C water bath for 5min, check whether it is formed the endogenous spores of the heat resistant in the growth process. The method is as follows: expelled air with a stream of nitrogen in a few pieces of a test tube at the same time that moved into 2ml ~ 3ml in different periods of the bacterium, and after 100 ° C for 5min conditional processing, dumped it into a certain amount liquid medium of sterile for disgust oxygen culturing, whether the growth of the tested strains in the culture medium was observed after one week. If growth, it indicates that this strain can be formed on the heat resistant spores.

Desulphurization green ding qualitative detection

Qualitatively detect the strain I of desulphurization green ding. Inoculated tested strains in medium, thermostat culture 3d ~ 6d at 30!, take 30ml ~ 40ml broth centrifuging for 20min at 8000 r / min, discard the supernatant and drop a few drops of 2.0 mol / 1 NaOH on the precipitated bacterial, and then move it on filter paper by a straw and observe it under 365nm UV light. If the bacteria can emit red fluorescence, the bacteria body contains desulphurization green ding, otherwise the desulphurization green ding is negative.

Determination of the ability of organic carbon utilization

The measurement as the for one of the major identification basises of inoculums, using the medium that remove the organic carbon source and yeast extract, and add four kinds of vitamin VB1, VB2, biotin, and the amino benzoic acid as a basic medium, respectively supplemented with the tested carbon source (supplemented with the same molar amount of sodium lactate, etc.), the measured carbon source of strain I are: lactic acid, pyruvic acid, acetic acid, butyric acid, malic acid, and a carbon source as measured by the strain II: lactic acid, pyruvic acid, ethanol, formic acid , acetic acid(Zhao, *et al*.,1997). Inoculated with the strains to be tested in the inoculation amount of the addition of 2% (V / V) above medium and then cultured continuous adapter 2 to 3 days after cultured one week, check the last growth of the cultures and compare the culture liquid of different carbon sources.

Measuring of the thallus growth curves

Configurate 700ml of sulfate-reducing bacteria medium to sterilizate, packed into fourteen 50ml vials (without Fe2 +) ,inoculated into 10% cultured broth under aseptic conditions, anaerobic culture by placing in 30! incubator. Take the broth every 4 hours, suspend precipitation with an equal volume of distilled water after 12000r/min centrifuged for 5 minutes and colorimetric it under UNICO-PC2102 UV-visible spectrophotometer at 400nm, measure OD value of the thallus to water as the reference.

RESULTS AND DISCUSSION

Pick out single colonies, observe each colony under an optical microscope, separate the two bacteria, strains '! identified to species while the strain a! only identified to genus.

The strains' Purification and purity testing results The two strains were coating cultured respectively on nutrient agar plate containing glucose and peptone, aerobic colony growth is not found on the tablet; growing at medium that containing Fe2 + salt ,colonies appear all black for the sake of that H2S and Fe2 + generate FeS black precipitate whose diameter is 1 ~ 3mm, the central of the colonies of projection of 0.5 mm diameter, the colonies can be grown in the medium surface and the inner layer. This result features explain that the two isolates bacteria belong to SRB, and have high purity.

Observing results of bacterial staining and morphological characteristics

Through the test, The shape of the resulting strainsis the curved or S-shaped, some chains, Gram stain was negative, flagella stain displays with a single pole born flagellum, bacillus stain was negative; observing with motility in the light microscope, the feature of motion is straight

movement instead of somersaults or twisting-like motion, strains is size of 0.5 ~ 0.8 μm × 2.5 ~ 5ìm. The cell morphology of the strain after staining under an optical microscope is shown in Fig. 1.

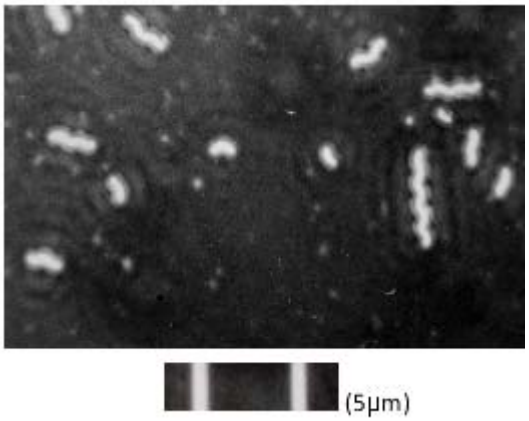

Fig. 1. Strain cell morphology

The shape of the resulting strainsa! is the rod-shaped, Gram stain is negative, Flagellum dyeing display with a single pole born flagellum, observing with motility in light-microscopy with, sports characteristics is roll type sports, strain size is 0.5 ~ 0.7 u m × 3 ~ 5 μ m. The cell morphology of the strain after staining under an optical microscope is shown in Figure 2:

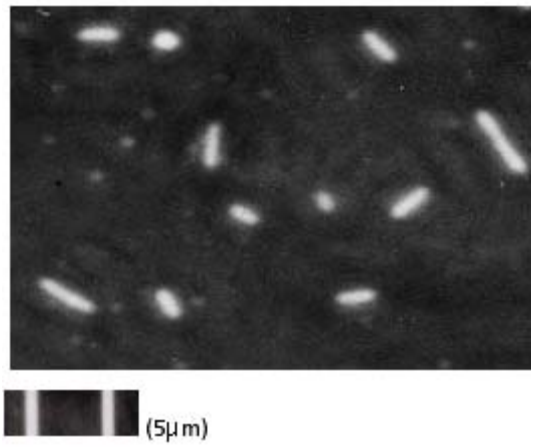

Fig. 2. Straina cell morphology

Heat-resistant testing results of strain

Strains After heat preservation for 5 min at 100 ° C, the Strains'!can't restorablely grow, the strain body in the surface do not form heat-resistant spores and are inactivated by high temperature.

Inspection results of activity of the desulphurization green ding

Strainsis issue strong red fluorescence in excitation of 365 nm ultraviolet, explaining that the strain cell containing sulfite reductase of desulphurization green ding.

Organic carbon utilization of strains

Strainsis showed good growth in the following nutritional composition: (1) lactic acid + sulfate, (2) pyruvic acid (without sulfate), (3) malic acid + sulfates; it can not grow in the following nutritional composition: (1) acetic acid + sulfates, (2) butyric acid + sulfate, (3), malic acid (without sulfate).

Strain II shows good growth in the the following nutritional composition: (1) lactic acid + sulfates, (2) pyruvate + sulfate, (3) formic acid + sulfate, (4) Ethanol + sulfates; this strain cannot grow normally in the nutritional composition: acetate + sulfate(Su, *et al*.,2006).

The appraisal of sulfate reducing bacteria strain genus

According to the description of sulfate strain genus' identification characteristics, the shape of strain '!are all cambers0S-shaped and centenariansÿthey don't produce heat-resistant sporesÿcontaining desulphurization green ding, and have straight movement(Li, *et al*.,1999). Four main features coincides with desulfovibrio desulfuricans genus, therefore the strain are all classified as desulfovibrio desulfuricans genus; Based on the identification of strain '!results about using of the organic carbon energy, comparing with various kinds of the identification characteristics' description in sulfate reducing desulfovibrio desulfuricans genus, all of the strain's morphological characteristics0physiological and biochemical characteristics all coincide with desulfovibrio desulfuricans, so the strain should be identified as desulfovibrio desulfuricans.

The shapes of strain a! are all rod-shaped, and have tumbling motion. Based on the identification results about using of the organic carbon energy, comparing with the identification characteristics' description in sulfate reducing desulfovibrio desulfuricans genus, the main features coincides with desulfotomaculum sp genus, then the strain should be identified as desulfotomaculum sp(Mu,*et al*.,2000;Holt,1994).

The growth curve of thallus

Through the sampling in different times, using UNIC0 - PC2102 uv-vis spectrophotometer in the 400 nm place for testing, the growth curves of strain and are shown as below in figure 3 and figure 4.

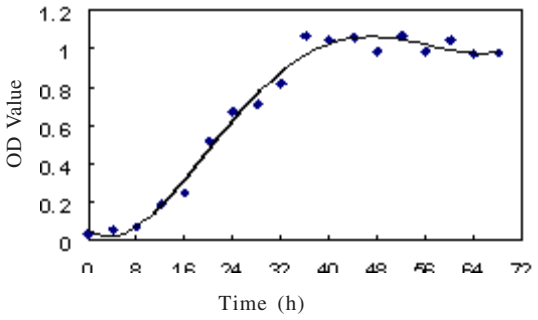

Fig. 3. The growth curves of strain I

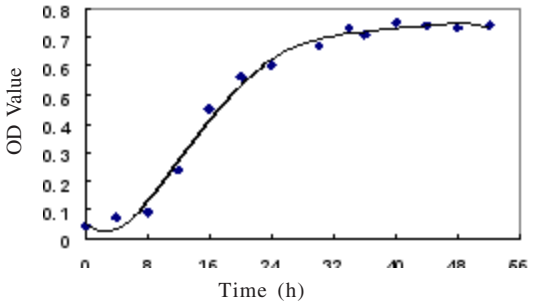

Fig. 4. The growth curves of strain II

According to the figure 3, strain‘!is in the lag phase of growth when the desulfovibrio desulfuricans is in the former 8 hours of growth, 8 hours later the thallus begins to enter logarithmic growth phase and last 32 hours or so, after another 40 hours the thallus enters stable phase.

According to the figure 4, strain a! is in the lag phase of growth when the desulfotomaculum sp is in the former 8 hours of growth, 8 hours later the thallus begins to enter logarithmic growth phase and last 28 hours or so, after another 36 hours the thallus enters stable phase.

The conclusion of testing

´ Through the separation and purification of the second sedimentation tank's return sludge of Yangjiapu sewage purification factory in Taiyuan city, we get two sulfate reducing bacteria strains. After the purity test, the two strains' purity are pure.

´ Strain '! is identified as desulfovibrio desulfuricans through the observation of strain's morphological characteristics0checking of heat resistance0 desulphurization green ding0utilization of organic carbon. We know through the testing of the growth curve, strain'!is in the lag phase of growth when the desulfovibrio desulfuricans is in the former 8 hours of growth, 8 hours later the thallus begins to enter logarithmic growth phase and last 32 hours or so, after another 40 hours the thallus enters stable phase.

´ Strain a! is identified as desulfotomaculum sp through the observation of strain's morphological characteristics0checking of heat resistance0 desulphurization green ding0utilization of organic carbon. We know through the testing of the growth curve, strain a! is in the lag phase of growth when the desulfotomaculum sp is in the former 8 hours of growth, 8 hours later the thallus begins to enter logarithmic growth phase and last 28 hours or so, after another 36 hours the thallus enters stable phase.

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