The Method of Sterile Anaerobic Centrifugation

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Abstract: *The method of sterile anaerobic centrifugation was developed. The essence of the method is centrifuging the suspensions of bacteria or insoluble metal compounds in sealed flasks immersed in a water layer. Dipping flasks in water prevents destruction of the flasks under the centrifugal force. The method provides the possibility to centrifuge in anaerobic (inert gas) atmosphere. Thus, the method allows obtaining biomass of both aerobic and anaerobic microorganisms in a sterile conditions and preparing sterile insoluble metal compounds.*

Keywords: *Anaerobic sterile centrifugation, Anaerobes, Solid reductants, Metals compounds preparation.*

Introduction

Method of centrifugation is used in microbiology for concentrating microorganisms, their separation in a density gradient, washing the biomass, etc [7]. Despite the widespread use of this method for a hundred years, there is still no method to centrifuge microorganisms in sterile conditions. Moreover, there is no method to centrifuge obligate anaerobes in inert gas atmosphere.

In microbiology solid (insoluble) reductants such as iron sulfide [6], iron(II) hydroxide are used to cultivate anaerobic microorganisms because both FeS and $Fe(OH)_2$ create low redox potential values [6, 11]. Thus, it is necessary to develop a method of sterile deposition, washing (resuspension and precipitation) of FeS and $Fe(OH)_2$ in an atmosphere of inert gas.

Interaction of microorganisms with insoluble metal compounds is important question of fundamental and applied microbiology. It clarifies the role of the microorganisms in biogeochemical cycles of metals in the biosphere [2]. Furthermore it gives the possibility to develop microbial methods of metals extraction from ore deposits [5, 8] and to predict biogeochemical pathways of metals transformation in the areas of man-made disasters [4, 10]. To obtain insoluble metal compounds (carbonates, hydroxides etc.) the method of centrifugation in sterile conditions is also needed.

The aim of this work is to develop the method that provides a centrifugation of microorganisms in sterile conditions in aerobic and anaerobic atmosphere (argon, etc.), and the preparation of solid reductants and insoluble metal compounds.

The methods of microbial biomass (aerobic, anaerobic) precipitation by centrifugation and precipitation of metals compounds have different sequences of operations. For this reason we describe separately these two methods.

Materials and methods

The objects of study were the microorganisms and insoluble metal compounds (IMC).

Microorganisms

Cultures of aerobic (*Bacillus subtilis* 316M), facultative anaerobic (*Escherichia coli* K12), and obligate anaerobic (*Clostridium lituseburense* K-1) microorganisms were used (Ukrainian Collection of Microorganisms).

Insoluble metal compounds

Precipitates of metals sulfides (FeS, CuS, CoS, NiS) and copper hydroxide were obtained due to the method of sterile anaerobic centrifugation. Ferric sulfide (FeS) is used as a solid reductant for the cultivation of anaerobic microorganisms [1, 11]. $Cu(OH)_2$, CuS, CoS and NiS are used for investigation of microbial mobilization of toxic metals.

Cultivation of microorganisms

Aerobic and facultative anaerobic microorganisms were grown on nutrient meat broth (HiMedia Laboratories Pvt. Ltd) in 0.5 l shake flasks at 220 turns/min. In sterile flasks 250 ml of nutrient meat broth was added. Then microorganisms were injected to the flasks. The final optical density of suspension was 0.05 units (λ = 540 nm, the optical step = 0.5 cm). Cultivation temperature was 30°C. The cultivation time was 24 hours.

Obligate anaerobic microorganisms were cultivated in a 0.5 l sealed flasks in argon atmosphere with hydrogen sulfide as the reductant. In sterile flasks 300 ml of meat broth was added. Then the medium was bubbled by argon for 15 min (argon flow rate – 0.5 l/min). The flasks were hermetically sealed with rubber plugs and metal retainers. Next, suspension of *C. lituseburense* was added to the flask with the help of syringe to a final optical density 0.05 units. The reductant was added as well: 0.1 ml of sterile H_2S was injected via syringe to the flask. Sodium resazurin was used as an indicator of the redox potential of solution. Discoloration of sodium resazurin indicated the obligate anaerobic conditions in flask ($Eh \leq 100$ mV, pH = 7.2-7.4). Anaerobic microorganisms were cultivated without mixing. Cultivation temperature was 30°C. The cultivation time was 24 hours.

Preparation of IMC

The following metals salt solutions were used to prepare solid reductants and IMC: $CuCl_2 \times 2H_2O$ (20.0 g/l), $FeSO_4 \times 7H_2O$ (20.0 g/l),
$NiCl_2 \times 2H_2O$ (10.0 g/l), $CoCl_2 \times 2H_2O$ (10.0 g/l).

Metal sulfides and hydroxides were obtained by adding to metals salt solutions saturated solutions of Na_2S or NaOH respectively. To obtain a saturated solution of sodium sulfide 15 g of $Na_2S \times nH_2O$ and 35 ml of distilled water were added in 100 ml flask, then heated to 80°C and cooled to temperature 20°C. Upon cooling crystals of redundant Na_2S precipitated on the bottom of the flask. To prepare a saturated solution of sodium hydroxide 60.0 g of NaOH was added in 100 ml flask and dissolved in 50 ml of distilled water at 50°C. Redundant NaOH precipitated on the bottom of the flask after cooling the solution to temperature 20°C.

Saturated solution of Na_2S was added in a fume hood to solutions of $CuCl_2$ and $FeSO_4$ to precipitate the metals in form of sulfides. Metal sulfides (CuS and FeS) were obtained according to the following reactions (1), (2) [9]:

$$Cu^{2+} + S^{2-} = CuS\downarrow \quad (1)$$

$$Fe^{2+} + S^{2-} = FeS\downarrow \quad (2)$$

Herewith the clear bright green solution of $CuCl_2$ transformed to black suspension of insoluble CuS. Suspension of insoluble black FeS formed during the sulfide precipitation of the Fe^{2+} cation. It is important to control the pH of solutions during the preparation of both CuS and FeS, as very toxic H_2S forms in the case of acidification of these solutions. In a similar manner, sulfides of Ni^{2+} and Co^{2+} were obtained.

Copper hydroxide $Cu(OH)_2$ was precipitated by alkaline (NaOH) according to the reaction:

$$Cu^{2+} + 2OH^{-} = Cu(OH)_2\downarrow \quad (3)$$

To prepare $Cu(OH)_2$[1] sterile solutions of NaOH and $CuCl_2$ are needed. Solution of NaOH was added fractionally (1 ml) to a solution of $CuCl_2$. The pH value was controlled with universal indicator paper. Color of the solution changed from bright green to blue because light-blue crystals of $Cu(OH)_2$ had formed. The pH value of the solution should not exceed 12, because above this pH value $Cu(OH)_2$ dissolves with forming of bright blue cuprates. At slightly acidic and neutral pH values (6.4-7.4) Cu^{2+} does not completely precipitate from the solution [8].

Centrifugation and washing the precipitate
Biomass culture fluids containing *B. subtilis*, *E. coli*, *C. lituseburense* and suspension of IMC were aseptically dispensed into transparent colorless 50 ml flasks with screw thread on the neck. Biomass and suspension of IMC were precipitating on the RS-6 centrifuge at 12 000 g during 15 min.

Supernatant was removed from the flask with plastic disposable 10 ml syringes after centrifuging. Sterile physiological saline solution (0.9% NaCl) was used for washing cell biomass. Sterile distilled water was used to wash the insoluble metal compounds. Physiological solution and distilled water for washing the biomass and insoluble metal compounds were previously purged with argon to remove O_2. The pH value (7.0) was controlled with universal indicator paper (pH range from 0 to 14 with step 1.0 pH unit).

Results and discussion

Microbial suspension or suspension of IMC (35 ml) was transferred in the argon steam to 50 ml flask (colorless transparent glass) with a screw thread on the neck. Suspensions were bubbled with argon (Fig. 1) to remove O_2 (O_2 concentration should not be higher than 0.02%). Argon was brought in the closed with cotton-gauze plugs flasks through a 15 cm steel needle. Basalt wool was inserted in cannula of each needle for the sterilization of argon. Needles with basalt wool were sterilized in a flame of gas-burner and after cooling were attached to the hose of argon supply system. Flasks were bubbled for 3 min with argon flow rate 0.5 l/min.

[1] $Cu(OH)_2$ decomposes to CuO and H_2O during the heat treatment (to +70°C) [9]. The temperature of $CuCl_2$ and NaOH solutions should be no higher than 30°C when using to avoid thermal decomposition of $Cu(OH)_2$.

After O_2 removing flasks were sealed with the elastic rubber plugs and fixed with 1 mm retainers made of extruded aluminum for tight fixing rubber plugs to the neck of the flask.

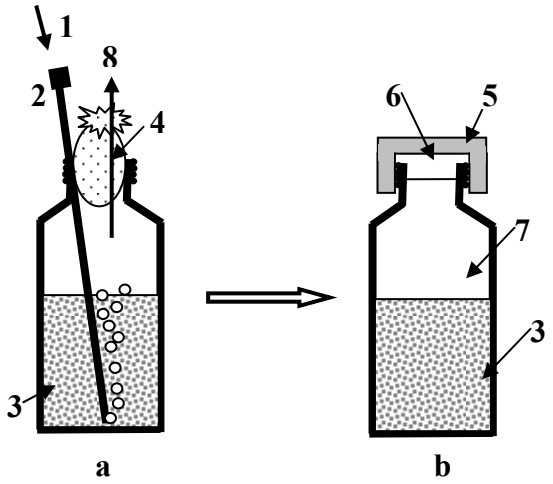


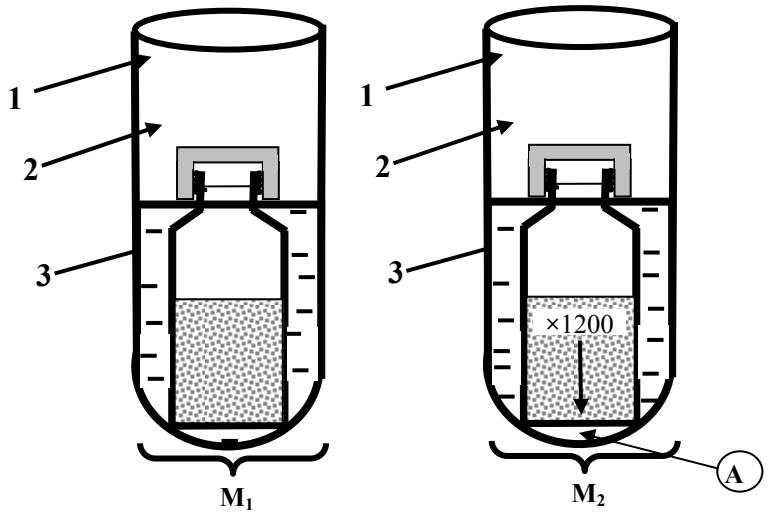
Fig. 1 Creation of anaerobic conditions in flasks:
a) removing of air from the flasks by argon bubbling; b) anaerobic conditions in the flask with bacterial suspension; 1 – argon flow, 2 – steel needle, 3 – bacterial suspension, 4 – cotton-gauze plug, 5 – retainers, 6 – rubber plug, 7 – argon, 8 – O_2 removing.

Insoluble metal compounds can be also prepared in sealed flasks in argon atmosphere with the help of syringe. Equivalent amount of reagents – i.e. solutions of metal salts, sodium sulfide or alkaline – are successively brought via syringe injection in the empty sealed flask. The precipitate is formed directly in flask. In this case one can obtain exactly calculated amount of the insoluble metal compounds.

The standard procedure of centrifugation provides the usage of open tubes. Obviously, it is impossible to keep sterility in such conditions, and moreover it is impossible to provide protection of obligate anaerobes and low-potential reductants from negative action of atmospheric oxygen.

Microbial biomass and suspension of insoluble metal compounds can be precipitated in sterile conditions in sealed centrifuge flasks. However, in this case the problem arises: the layer of liquid under the centrifugal force destroys the flask (the bottom of the flask is “pressed out” and crushed). We solved this problem as follows. It is well known, that water is practically incompressible. Therefore, if the flasks in the centrifuge tubes are covered with water to the neck, equal pressure will act on the entire surface of the flask. Clearly, in such conditions flasks will remain unbroken during centrifugation regardless of the high *g* value.

Flasks (50 ml) with biomass or suspension of IMC were inserted into 150 ml plastic centrifuge tubes and poured with water so that the flasks were immersed in water (Fig. 2). There is a water layer between the bottom of the flask and the tube (in Fig. 2 marked as "A"). Water is not compressed during centrifugation. Thus, water created equal pressure on the glass flask, which provided its integrity.



$M_1 = M_2$

Fig. 2 Equilibration of centrifuge tubes with flasks in the water layer:
1 – centrifuge tubes, 2 – water layer, 3 – sealed flasks with bacterial or insoluble metal compounds suspension.

Then the tubes with the flasks were equilibrated on the scales with distilled water. Balanced tubes were placed in a centrifuge rotor opposite to each other for equal distribution of the load during rotation.

The metal sulfides form after centrifugation a dense black or dark brown precipitate on the bottom of flasks. The supernatant is clear (Fig. 3).

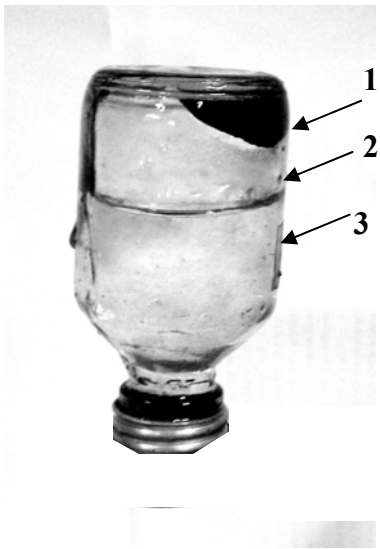


Fig. 3 Sterile precipitate of ferric sulfide in the argon atmosphere after centrifugation:
1 – FeS precipitate, 2 – gas phase (argon), 3 – supernatant.

The metal precipitate and biomass have to be washed for three times by centrifugation. Metals are washed in order to remove alkaline from the solution and to obtain suspension of the metal hydroxide or metal sulfide at neutral pH. Biomass is washed to remove exametabolites. Sterile distilled water or physiological saline solution were added into flasks with syringe for precipitate washing.

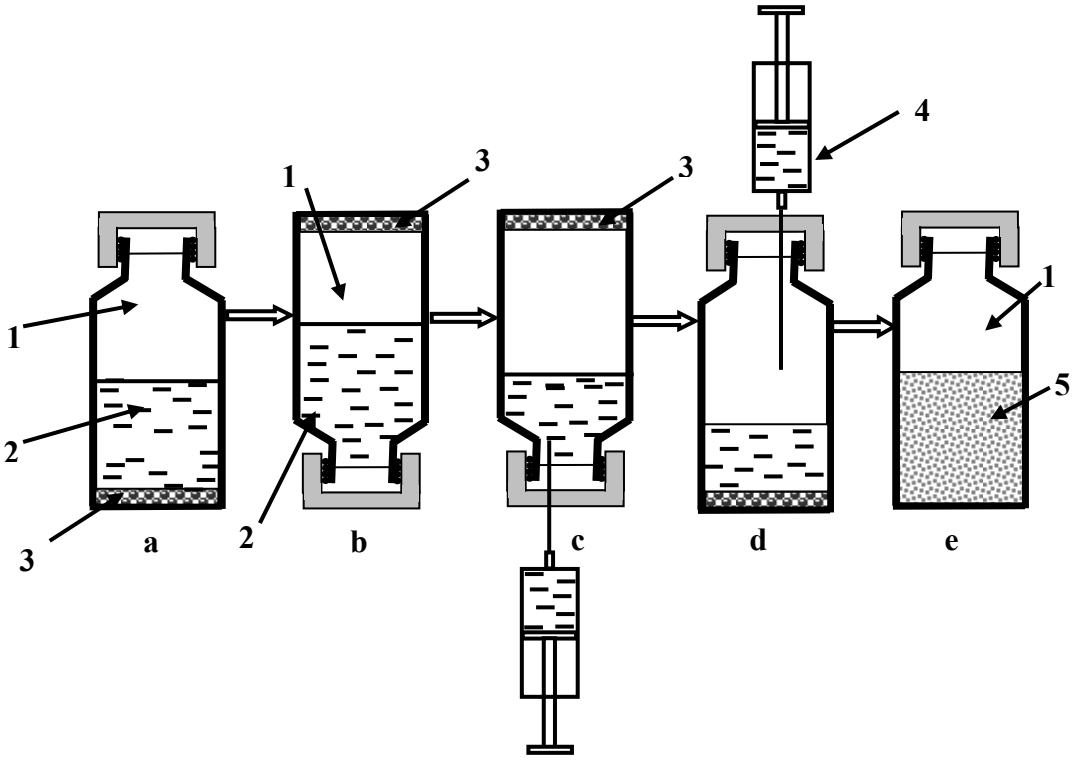


Fig. 4 Precipitate washing: a) biomass or metal precipitate on the bottom of the flask; b) separation the precipitate and liquid phase; c) removing of supernatant; d) injecting sterile distilled water or physiological solution; e) suspending of bacteria or metal precipitate; 1 – argon; 2 – supernatant; 3 – precipitate; 4 – sterile solution; 5 – resuspended biomass or insoluble metal compounds.

To control the pH value a little volume (0.1 ml) of supernatant was withdrawn from the flask with the syringe. After three times washing by centrifugation, the pH was 7.0 (measurement with universal indicator paper). Sterile saline solution (0.9% NaCl) was added into flask with syringe for washing biomass. The sequence of precipitate washing is shown on Fig. 4. Precipitate formed on the bottom of the flask after centrifugation (biomass or insoluble forms of metals) (Fig. 4a). The flask was turned upside down. The precipitate and supernatant were separated by gas phase (Fig. 4b). Then, the supernatant was removed from the flask with a syringe (Fig. 4c). Sterile physiological saline solution for biomass washing or sterile distilled water for IMC washing was added to the flasks with syringe after removing the supernatant (Fig. 4d). The precipitate was resuspended by shaking the flask to homogeneous consistence of suspension (Fig. 4e).

The $Cu(OH)_2$ transforms into CuO and H_2O in one week. It is evidenced by the appearance of black and gray areas in the thickness of light blue $Cu(OH)_2$ precipitate. Therefore, $Cu(OH)_2$ should be prepared immediately before use.

It should be emphasized that biomass and IMC precipitation by the method of anaerobic centrifugation provides full tightness of the process. The suggested method provides the sterility of centrifugation procedure of both microbial biomass and insoluble metal compounds.

Conclusion

1. Suitability of our method for obtaining concentrated biomass in sterile conditions of aerobes and facultative and obligate anaerobes was shown. An important advantage of this method is the ability to centrifuge in an inert gas atmosphere. It makes possible to obtain biomass of anaerobic microorganisms without contact with toxic for anaerobes oxygen.
2. Anaerobic centrifugation method can also be used to obtain a sterile precipitate of metal compounds, which are further used for microorganisms' cultivation. For example, FeS is used as the solid reductant. Ferric sulfide creates redox potential –250 mV, which is suitable for the cultivation of anaerobic microorganisms [1, 11]. Metal compounds, such as CuO, CuS, $Cu(OH)_2$, $CuCO_3$, are used in biological researches for studying the mechanisms of microbial mobilization of insoluble metal compounds [3, 12].

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