JOURNAL OF PURE AND APPLIED MICROBIOLOGY, APRIL 2014. Vol. **8**(2), p. 1361-1367

Separation and Characterization of Dominant Strain in SBBR Denitrifying Phosphorous Removal System

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(Received: 09 January 2014; accepted: 18 March 2014)

A SBBR reactor using nitrate as an electron acceptor was operated under anaerobic/anoxic condition to prove the existence of denitrifying phosphate-accumulating organisms and to investigate their characteristics of denitrification and phosphorus removal. Dilution coating method and plat streaking method were used for the separation and purification of denitrifying phosphate-accumulating organisms from sludge extracted on the stable operation duration. Metachromatic granules staining test, gram staining test and nitrate reduction test were used for the identification of purified strains. In this study, three screened strains, which were capable of denitrification and phosphorus removal, all had black metachromatic granules inside and showed negative reactions to Gram staining test. The nitrate removal rate of three strains was 75.82%,78.26% and 62.84%,respectively. It is proved that there were a large amount of denitrifying phosphate-accumulating organisms in SBBR denitrifying phosphorus removal system and the organisms had good performance on phosphorus storage and denitrifying nitrogen removal.

Key words: SBBR; denitrifying phosphate-accumulating organisms; Dilution coating method; Plat streaking method;metachromatic granules staining; Gram staining; nitrate reduction

Biological nutrient(N and P) removal is essential in the field of water pollution control and key links in wastewater biological treatment technology are phosphorus removal and denitrification[1]. In 1993, Kuba et al[2] discovered a new bacteria genera which was capable of simultaneous nitrogen and phosphorus removal—-(Denitrifying Phosphate-Accumulating Organisms,DNPAOs). DNPAOs could oxidize the intracellular stored PHB using O_2, NO_2^--N and NO_3^--N as electron acceptors, N_2 was produced and discharged into the environment[3]. Compared to the traditional nutrient removal processes, such as A^2/O, Bardenpho, oxidation ditch, denitrifying phosphorus removal technology solved the problem of carbon source competition between denitrifying bacteria and phosphorus accumulating organisms and saved 50% of the organic compounds consumption. At the same time, the release of CO_2 into air decreased 20% because organic compounds were not only oxidized into CO_2 in this technology[4,5]. Furthermore, denitrifying Phosphate-Accumulating Organisms could uptake phosphorus in anoxic conditions, oxygen consumption decreased about 30% and the volume of surplus sludge decreased sharply[6,7].

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In recent years, many researches has been developed about denitrifying phosphorus removal technologies all over the world, but most of them choose sludge containing denitrifying phosphate-accumulating organisms as the research subjects. Due to little focus on single strain, it needs further discussion to get clearer understanding about denitrifying phosphorus removal mechanism and energy efficiency[8~13]. In this study, denitrification specific culture medium was used to separate and purify the dominant strain in SBBR denitrifying phosphorus removal systems. Metachromatic granules staining, gram staining and nitrate reduction test were used to identify purified strain. This study investigated phosphrous storage and denitrifying nitrogen removal characteristics of DNPAOs from the perspective of microbiology and identified the effect of DNPAOs in SBBR system. It aimed at providing theoretical basis for engineering application of denitrifying phosphorus removal technology.

MATERIALS AND METHODS

Testing materials

Testing instruments

Instruments for strains separation: sterile triangular flask, sterile pipette, inoculating loop, glass beads, magnetic stirrer; a) Instruments for sterilization: high-pressure steam sterilizer, spirit lamp; b) Instruments for strains cultivation: culture dish, constant temperature unit; c) Instruments for strains observation: biological electron microscope, electronic analytical balance, digital camera, UV-Visible Spectrophotometer.

Culture medium

Specific culture medium: seignette salt 20g, agar 20g, $MgSO_4.7H_2O$ 2g, potassium nitrate 2g, dipotassium hydrogen phosphate 0.5g, distilled water 1000mL, the pH was controlled between 7.2 to7.4.

Nitrate reduction culture medium: potassium nitrate 1g, glucose 1g, peptone 20g, dipotassium hydrogen phosphate 2g, agar 1g, distilled water 1000mL,the pH was controlled between 7.2 to7.4.

Staining solution

Metachromatic granules staining solution (solution A and solution B):

Solution A: malachite blue 0.2g, toluidine blue 0.15g 95% alcohol 2mL, glacial acetic acid 1mL,distilled water 100mL.

Solution B: potassium iodide 3g, iodine 2g, distilled water 300mL.

Gram staining solution: crystal violet staining solution, Gram's iodine, 95% alcohol and safranin counterstain.

Steps and methods

Dilution coating method and plat streaking method were used for the purification and separation of strains which were capable of denitrifying nitrogen removal in SBBR system. The use of denitrifying specific culture medium could achieve obvious observation of bacteria colonies, shorten time and workload of plat streaking. Metachromatic granules staining was applied for staining purified strains and separating strains with the ability of phosphorus accumulating. The positive and negative reaction to Gram staining method was used for the identification of the existence of denitrifying phosphate-accumulating organisms in SBBR system. Nitrate reduction test could detect the nitrogen removal ability of extracted strains and ensure that this strain was the dominant strain with the characteristic of denitrifying phosphate accumulating.

Separation and purification of DNPAOs in SBBR system

Pipettes, triangular flasks, culture dishes, glass beads, distilled water, weighed drug, coater and some other instruments used for the test were autoclaving at the temperature of 120°C for 20 minutes in high-pressure sterilizer. The following steps were operated in sterile environment.

10mL of sludge was pipetted into a 250 mg/L sterile triangular flask which contained several glass beads at the end of a cycle of SBBR system. Concentration grads of bacteria suspensions in triangular flask turned out to be 10^{-1} after adding 90mL of sterile water.In order to scatter the zoogloea in sludge, the triangular flask was stirred on the magnetic stirrer for 20 minutes. 1mL of bacteria suspensions with the centration grads of 10^{-1} was pipetted into a tube which contained 9mL of sterile water. Cover cotton plug and mix up the bacteria suspension in the tube, the concentration grads of bacteria suspension became 10^{-2}. Repeat the operation above until the concentration grads of bacteria suspensions become 10^{-5}.

The diluted bacteria suspension was pipetted on several culture dishes, each concentration grads was made into 2 plates, inject denitrifying specific culture medium on the plates at the temperature of 40! and shake the plates then invert them.0.5mL of bacteria suspensions of five different concentration gradient was added to 10 plates, use sterile coater to finish evenly coating on each culture dish.

Ten prepared plates were inverted and cultivated in constant temperature unit at 30!. After three days of cultivation,plates were taken out from the unit for bacteria colonies observation. A evenly dispersed plate with numerous species of bacteria colonies was chosen, different bacteria colonies were picked from this plate to several specific culture medium for plat streaking by sterile inoculating loops and it should be careful not to prick the culture medium in this process.

Metachromatic granules staining test

Smearing

Operation should be sterile. First step was to add a drop of sterile water on slides, then choose a strain on the streaking plate by sterile inoculating loops and mix it up with sterile water evenly,finally draw it into appropriate size of circular and square on the slides.

Immobilization

Put slides back and forth through the spirit lamp flame several times until they dried up, it should be careful not to put the slides on the outer flame of the spirit lamp, in case the high temperature of outer flame lead to the charred strains which would affect the observation.

Staining

First step of staining was to add solution A on the slides,after five minutes, rinse it off with solution B.1 minute later, wash it away with distilled water and blot it with filter paper.

Microscopic examination: Prepared slides were observed under the microscope.

Gram staining test

Stearing

Operation should be sterile. First step was to add a drop of sterile water on slides, then choose a strain on the streaking plate by sterile inoculating loops and mix it up with sterile water evenly,finally draw it into appropriate size of circular and square on the slides.

Immobilization

Put slides back and forth through the spirit lamp flame several times until they dried up, it should be careful not to put the slides on the outer flame of the spirit lamp, in case the high temperature of outer flame lead to the charred strains which would affect the observation.

Staining

First step was to add crystal violet staining solution on the slides, wash it off with distilled water after 1 minute, dry the smears on the spirit lamp flame; Secondly, add Gram's iodine and wash it off with distilled water, dry the smears on the spirit lamp flame; Add several drops of alcohol and shake it gently for 30 seconds to make the solution fully destained, then wash it with distilled water and blot it with filter paper; Finally, add safranin counterstain, after 10 seconds, wash it off with distilled water and dry the smears on the spirit lamp flame.

Microscopic examination

Prepared slides were observed under the microscope.

Nitrate reduction test

Every tube contained 8mL of nitrate reduction culture medium. All the tubes were autoclaved at the temperature of 120°C.

Pick purified strains which was proved to be capable of phosphorus removal by sterile inoculating loop, put it into a tube which contained nitrate reduction culture medium, use sterile liquid paraffin to seal the tubes, then put them into oscillatory incubator at the temperature of 30! and keep several blank tubes as control groups.

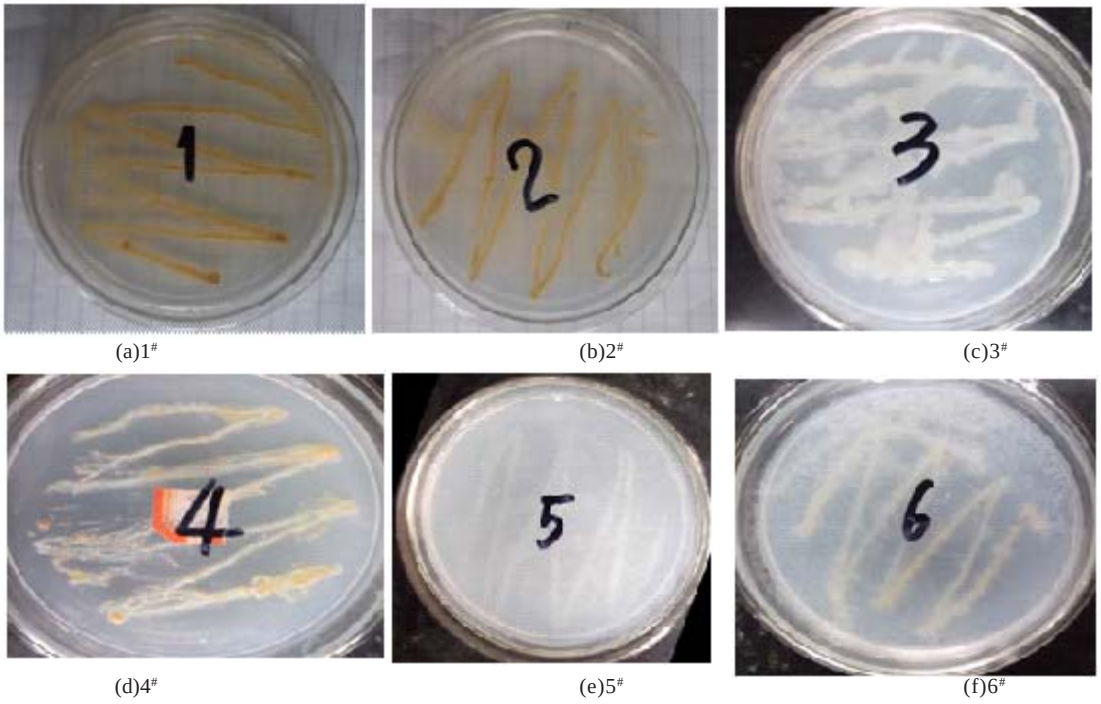
RESULTS AND DISCUSSION

Separation and purification of DNPAOs in SBBR system

After the observation of strains which were separated from bacteria suspensions of five different concentration grads, it is discovered that bacteria colonies on 10^{-4} diluent plates had more obvious colony characteristics and better dispersity; bacteria colonies on 10^{-1},10^{-2} and 10^{-3} diluent plates were difficult to observe characteristics and had bad dispersity. Bacteria colonies on 10^{-5} diluent plates had good disperisity but inconspicuous colony characteristics. From further observation, 10^{-4} diluent plates had 6 bacteria colonies with different morphological

characteristics, and the growth conditions and morphological characteristics of various colonies were shown in fig.1 and table 1. Choose two 10^{-4} diluent plates which had numerous single bacteria colonies and even dispersity as the base and start separating strains by plat streaking.The growth conditions of strains of each plate were shown in fig.2.

Fig. 1. Growth conditions of bacteria colonies on 10^{-4} diluent plates



(a)1# (b)2# (c)3#

(d)4# (e)5# (f)6#

Fig. 2. Growth conditions of strain 1#~6#

Table 1. Morphological characteristics of bacteria colonies on 10^{-4} diluent plates

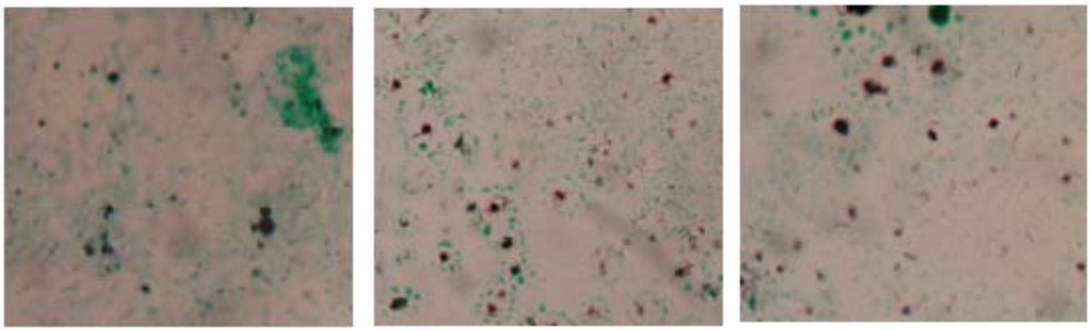
Number	Intuitive features of bacteria colonies	Color	Size
1#	Regular round,raised in the middle,yellow overall,shiny	yellow	big
2#	Concentric circles,raised in the middle,a little milky white edges,shiny	Light yellow	medium
3#	Regular round,smooth and transparent edges,raised in the middle,shiny	Milky white	small
4#	Jagged edges,small bump in the middle,shiny	Milky yellow	medium
5#	Concentric circles,small circle in the middle,shiny,dark milky white edges	Milky white	big
6#	Irregular shape,conspicuous bump in the middle,shiny	Milky white	medium

Metachromatic granules staining test and Gram staining test

Metachromatic granules staining test

Metachromatic granules are ubiquitous storage of high-energy phosphates and the main component of high-energy phosphate is poly metaphosphate[14]. Part of the poly metaphosphate are conversed from ATP and could become larger as the extension of bacteria age,its main feature is strong basophilla and specific reaction to certain

stainings,it could present a different color with stainings[15]. According to this characteristic, obvious observation could be achieved by staining microorganisms. By this method,not only could the phosphorus accumulating performance be identified, but also the morphology characteristics of microorganisms could be observed obviously. From metachromatic granules staining results, in 6 purified strains,strain 1#,3# and 5# contain a black substance, the rest of them is green, indicating that strain 1#,3# and 5# contain metachromatic granules so that they have phosphorus storage ability. Metachromatic granules staining results of three strains are shown in Fig. 3.



(a)1# (b)3# (c)5#

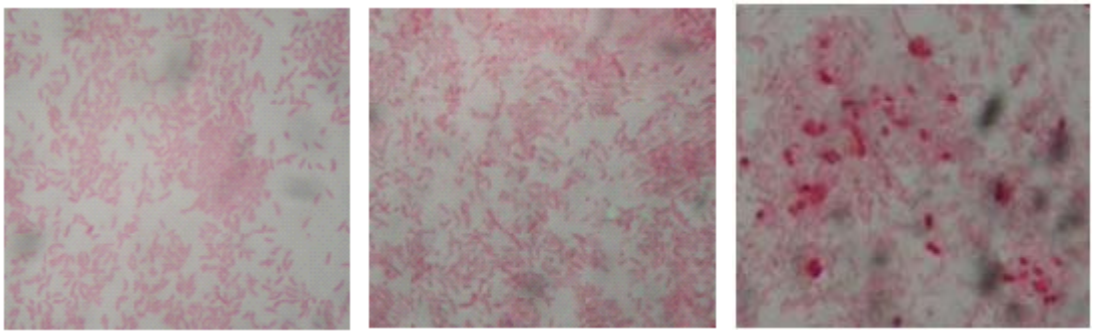
Fig. 3. Results of metachromatic staining of strain 1#, 3#and 5#

Gram staining test

Gram staining method, which was founded in 1884 by a Danish physician,Gram, is one of widely used differential staining methods in bacteriology and belongs to counterstaining. Because of the small differences of refractive idex between the unstained microorganisms and the surrounding environment, it is difficult to observe the unstained bacteria by the microscope; on the contrary, stained bacteria is a sharp contrast to the environment, the morphological, arrangement and some structure characteristics can be observed clearly. Thus, Gram staining were chosen to classify and identify microorganisms. Stain 1#, 3# and 5# were tested by Gram staining. The test results are shown in fig.4 and table 2.

Table 2. Results of Gram staining of strain 1#,3#and 5#

Strain	Morphology of strain	Gram staining result
1#	rod	negative
3#	rod	negative
5#	spherical	negative

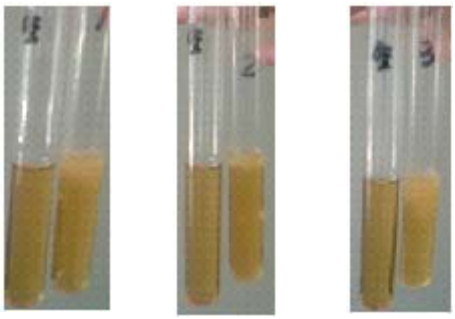


(a)1# (b)3# (c)5#

Fig. 4. Results of Gram staining of strain 1#, 3#and 5#

Nitrate reduction test

On anaerobic condition, bacteria containing nitrate reduction enzyme could utilize organic compounds to reduce nitrate into nitrogen by denitrification process. Tube 1#, 2# and 3# were cultivated for seven days and represented the nitrate reduction test of strain 1#, 3# and 5# respectively. The test results are shown in fig.5.



(a)1# (b)3# (c)5#

Fig. 5. Results of nitrate reduction test of strain 1#, 3# & 5#

Table 3. Nitrate concentration detection results

Number of strain	Nitrate concentration/mg·L^{-1}		
	After 3 days	After 5 days	After 7 days
blank	176.75	171.78	173.8
1#	89.87	55.27	42.02
3#	78.28	50.67	37.79
5#	122.63	91.71	65.21

As shown in fig.5, bubbles and turbidity of culture medium all appeared in tube 1#, 2# and 3#, suggesting that the three strains had reducibility on nitrate. In order to increase the accuracy of test, strain 1#, 3# and 5# were divided into three parts respectively and added separately into tubes which contained nitrate reduction culture medium, three other tubes were kept blank as the control groups. Each part of strain 1#, 3# and 5# was cultivated for 3, 5 and 7 days respectively. The test results are shown in table 3 and 4.

Table 4. Nitrate removal efficiency

Number of strain	Nitrate removal rate/%		
	After 3 days	After 5 days	After 7 days
1#	49.15	67.83	75.82
3#	55.71	70.50	78.26
5#	30.62	46.61	62.48

CONCLUSIONS

1. Dilution coating method and plat streaking method were used for the separation and purification of DNPAOs in stably operated SBBR system. Six purified strains with different morphological characteristics were screened by specific denitrifying culture medium.
2. Metachromatic granules staining test identified the phosphrous storage ability of strain 1#,3# and 5#, Gram staining test all showed a negative reaction.
3. Nitrate reduction test identified the nitrate reduction ability of strain 1#,3# and 5#. It also proved the nitrogen removal ability of these strains,the nitrate removal rate was 75.82%, 78.26% and 62.48%, respectively.

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