Effects Of Phase Separation On Dewaterability Promotion And Heavy-Metal Removal of Sewage Sludge During Bioleaching

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Abstract

Bioleaching is of increasing interest due to its high efficiency for improving sludge dewaterability and removing heavy metals. However, a traditional single-phase bioleaching cannot run continuously at a high-efficiency status due to a destruction of the microbial synergistic effect in a low-pH environment. So, in this study, a series of multi-compartment baffled-flow trials were carried out to assess the effect of phase separation on sludge bioleaching by comparing the bioleaching process of a two-phase bioreactor with that of two single-phase bioreactors. Energy substrate and sludge reflux were introduced into two different compartments to form two phases, namely selection phase and leaching phase. The results show that phase separation obviously shortened the start-up duration for sludge bioleaching from 7 days in single-phase bioleaching to 4 days in two-phase bioleaching. The dewaterability improvement of bioleached sludge also was enhanced by phase separation with the relative decreases of 25.0 – 33.3% for specific resistance to filtration (SRF) and 14.2% for capillary suction time (CST), which was attributed to lower pH values, zeta potentials of closer to zero and less DOM of bioleached sludge after the two-phase bioleaching. Phase separation generally increased the dissolution ratio of heavy metals with the ratios of 56.3%, 49.1%, 29.6%, 19.9%, 16.0%, 15.5%, and 1.0% for Zn, Cd, Cu, Ni, As, Cr, and Pb from raw sludge. Phase separation also enhanced the enrichment of Acidithiobacillus and relieved the inactivation of acid-tolerant fungi, which could be conducive to producing a better synergy benefit and keeping a long-term stable operation in bioleaching phase during two-phase bioleaching.

Introduction

The production of sewage sludge has increased rapidly with improvement of sewage treatment capacity in China (Li et al. 2013). Since sewage sludge has a high value of moisture content after being gravimetrically condensed, subsequent dewatering is necessary to reduce sludge volume and facilitate its transportation and treatment. Moisture content of sewage sludge can be reduced to nearly 80% by mechanical dewatering (Lee and Liu 2000, Lo et al. 2001, Neyens et al. 2004). This moisture content is still high for subsequent sludge treatment, which results in a large overall cost. In order to decrease the overall cost of sludge treatment (Lee and Liu 2000, Mahmoud et al. 2011), it is necessary to improve dewaterability of sludge by pre-conditioning sewage sludge. Thus, effective sludge conditioning is important to enhance sludge dewaterability (Chen et al. 2001, Raynaud et al. 2012).

Generally, there are three classes of conditioning methods to enhance dewaterability of sludge: physical methods, chemical methods such as advanced oxidation (Masihi and Gholikandi 2018), and biological methods including microbial flocculants, and bioleaching (Liu et al. 2012, Lu et al. 2019). Among those methods, bioleaching is of increasing interest because of a low cost and high efficiency to promote sludge dewaterability. In addition, bioleaching also has a good advantage at removing heavy metals. During bioleaching, energy substrate such as $\text{Fe}^{2+}$ and $\text{S}^0$ is biologically oxidized by Acidithiobacillus spp. (Acidithiobacillus ferrooxidans, A. ferrooxidans; Acidithiobacillus thiooxidans, A. thiooxidans) to produce $\text{Fe}^{3+}$ or $\text{SO}_4^{2-}$ (Tyagi et al. 1994), which results to a low pH by hydrolysis of $\text{Fe}^{3+}$ or oxidation of...
S\(^0\). And then, sludge dewaterability is promoted via charge neutralization, destruction of EPS (extracellular polymer substances), and replacement of microbials at a low pH (Zhao et al. 2020, Zhou et al. 2015). Dewaterability improvement can be observed by a pronounced reduction of SRF (Liu et al. 2012, Song and Zhou 2008). Also, heavy metals can be removed from sewage sludge through acidification and dissolution at a low pH (Liu et al. 2021, Ye et al. 2021, Zheng et al. 2020), which can be indicated by the decreasing of total concentration of each heavy metal in sewage sludge.

A few researches were reported about the effects of operation factors on conditioning efficiency of sludge with single-phase bioleaching. Ban et al. investigated the variation of sludge dewaterability with the different concentration of energy substrate, and they found that increasing concentration of energy substrate accelerated acidification of sludge but not stably improved sludge dewaterability (Ban et al. 2018). It also was reported that low pH was adverse for the stable operation of bioleaching, since it inhibited the growth of acid-tolerant heterotrophic microbes which is important for a positive microbial synergistic effect of acid-tolerant heterotrophic microbes and *Acidithiobacillus* spp. (Lin et al. 2020) For example, the decrease of SRF, CST, and EPS increased from to by 96.0%, 88.0%, and 73.0%, respectively, when Fe\(^{2+}\) concentration increased from 0 to 6 g/L. The bioleaching was run with from 2 g/L to at pH of 2.7, respectively (Wong et al. 2015). Even though sewage sludge can be effectively conditioned to improve its dewaterability under those optimal ranges of factors for single-phase bioleaching, it cannot be continuously operated at a stable and high-efficiency status, which is due to a destruction of the synergistic effect caused by growth inhibition of acid-tolerant heterotrophic microbes at a long-time low-pH environment. The low growth of acid-tolerant heterotrophic microbes causes a high concentration of DOM in bulk solution of sludge, which inhibits activity of *Acidithiobacillus* and results to deterioration of bioleaching system. So, it is important to maintain the stabilization of sludge bioleaching by phase separation which keeps optimal ranges of pH value for both acid-tolerant heterotrophic microbes and *Acidithiobacillus*.

However, the information is still lack about the effects of phase separation on bioleaching of sewage sludge. Therefore, in this study, two-phase (selection phase, bioleaching phase) baffled bioleaching was firstly designed by refluxing bioleached sludge and adding energy substrates in different compartments. And then, it was used to evaluate the feasibility of phase separation on sludge bioleaching via investigating its effects on physicochemical properties, dewaterability improvement and of heavy-metals removal sludge, and microbial community.

**Materials And Methods**

**Sewage sludge and standard samples**

The sewage sludge was collected from the sludge thickener of the Yanshan Municipal Wastewater Treatment Plant in Guilin, China. The collected sludge was diluted with tap water to attain a solid content of about 2.0%. Then, it was sieved using a 10-mesh nylon sieve (2 mm) to remove sand and fibers larger than 2 mm. The physicochemical properties of the sludge were: pH 7.2 ± 0.2, zeta potential 15.4 ± 1.2 mV,
soluble chemical oxygen demand (SCOD) 619.0 ± 18.8 mg/L, CST 31.5 ± 1.3 s, SRF 3.9 ± 0.3×10^{-13} m/kg. The mixed standard solution of heavy metal (100 mg/L) was purchased from Tmrm (Beijing, China), which included As, Cd, Cr, Cu, Pb, Ni and Zn.

**Single-phase and Two-phase baffled-flow bioleaching reactor**

The bioleaching setup mainly consisted of reactor, mixing tank, storage tank (Fig. 1). The bioreactor was a cuboid of a total effective volume of 15 L with length of 300 mm, width of 200 mm, and height of 300 mm, which was evenly divided into four compartments. Each compartment was divided to a downflow area of 15-mm width and an upflow area of 60-mm width by a baffle of inclined angle 45° and 14-mm length. An air pump was used to supply air into the bioreactor. After being mixed in the mixing tank, raw sludge without adding heavy metals was continuously transported into the 1st compartment, and then flew forward from 1st to 4th compartment. The bioleached sludge was discarded into the storage tank three times per day (9:00 am, 12:00 pm, and 21:00 pm), a part of which was returned as inoculum. For single-phase bioleaching, both refluxed sludge and energy substrate were added into the 1st compartment, while refluxed sludge and energy substrate were added into 1st, 2nd compartment, respectively, for two-phase bioleaching.

**Experimental design Acclimation and enrichment of inoculum**

Two sequential steps were applied to acclimate and enrich the bioleaching inoculum. Firstly, 3.3 L of raw sludge was put into four compartments with adding 10 g/L (substrate mass / sludge volume) of FeSO\(_4\)·7H\(_2\)O as energy substrate. The sludge was inoculated to a stable pH value of about 2.0–3.0 with an aeration rate of 3.8 L/min at 28 ± 2°C, and then the inoculation maintained for another 2 – 3 days to acclimate corresponding microbes. Secondly, the similar inoculation of raw sludge was run to enrich microbes with the acclimated sludge as inoculum at a portion of 25.0% (volume of inoculum / volume of raw sludge). Repeated the enrichment three times, and then the final enriched culture was applied as the inoculum for the following bioleaching trails.

**Bioleaching trials**

Three trials of 15 days were run to investigate the effects of phase separation on bioleaching of sewage sludge. In each trail, the enriched sludge as inoculum was introduced into the 1st compartment at an inoculation portion of 40.0%. And then, raw sludge was bioleached continuously with an aeration rate of 3.8 L/min in each compartment at 28 ± 2°C. The bioleached sludge was refluxed from the storage tank twice a day (9:00 am and 21:00 pm) with a total proportion of 40%, when FeSO\(_4\)·7H\(_2\)O was added into the bioreactor as energy substrate with the total dosage of 6 g/L (substrate mass / raw sludge volume). Before sludge refluxing and adding energy substrate, sludge samples were collected from each compartment for pH measurement at 9:00 am every day. Three trials were carried out as follows: trial 1 for two-phase bioleaching with four compartments (labeled as TP-4), trail 2 for single-phase bioleaching with four compartments (labeled as SP-4), and trail 3 for single-phase bioleaching with three compartments (labeled as SP-3). In trial 1, 7.5 L/d raw sludge was treated in four compartments with
adding energy substrate into 2nd compartment, while adding energy substrate into 1st compartment in trial 2. For trial 3, 5.6 L/d raw sludge was treated in three compartments with adding energy substrate into 1st compartment, when stopping the aeration in 4th compartment to make it a storage tank. In each trial, there were two steps: start-up and stabilization. Start-up was considered to have finished when the pH value of each compartment was almost no longer decrease for 2 – 3 days. During the stabilization process, each trial was carried out for another 7 – 10 days, in which period sludge samples were collected from all compartments every 3 – 5 days to measure the physicochemical properties, dewaterability, heavy-metals concentration and microbial community. For each trial, two replicates were run.

Analysis methods

The sludge morphology and *Acidithiobacillus* distribution were observed with an optical microscope (N-10E, Novel, China) and a confocal laser scanning microscope (CLSM; Revolution XD, Andor, UK), respectively, when both under 10 × 10 magnification. For both analysis, sludge samples were collected from the last compartment of each bioreactor. The spatial distribution of *A. ferrooxidans* was identified by observing blue spots when using 450 – 490 nm as the exciting wavelength in CLSM (Bai et al. 2011).

The pH was measured using a pH meter (PB-10, Sartorius, China). The solid content of the sludge was determined by the gravimetric method. After being settled in a centrifuge tube for 2 hours, the supernatant of each sludge sample was collected to measure zeta potential using a particle size and zeta potential analyzer (Zetasizer Nano ZS90, Malvern, UK). The bioleached sludge samples were collected from each compartment to analyze SCOD, which was employed to represent dissolved organic matter (DOM) (Qiao et al. 2008). Sludge samples were centrifuged with 4000 r/min for 5 min, and then SCOD of the supernatant was determined using a fast catalytic digestion method with a graphite furnace digester (SH220F, Hanon, China). SRF was measured by the Buchner funnel-vacuum suction method with a neutral quantitative filter paper (0.45 µm) under a negative pressure of 0.03 – 0.04 MPa (Chen et al. 2010, Velmuzhov et al. 2020), while CST was measured by a CST Meter (304M, Triton, UK) with a 1.8-cm diameter funnel (Yu et al. 2010). With a digestion method of nitric acid-perchloric acid method, heavy-metal concentrations of sludge were measured by an inductively coupled plasma mass spectrometry (NexION 350X, PerkinElmer, America), in which the recoveries were 72.2%, 70.6%, 75.1%, 77.4%, 71.2%, 73.9%, and 108.1% for As, Cd, Cr, Cu, Pb, Ni, and Zn, respectively.

During stabilization process, sludge samples were collected from the raw sludge and each compartment and stored at – 20 °C for measuring the microbial community, which were labeled as A0 (raw sludge), A1 and A3 (1st, 3rd compartments in SP-3), B1, B2, B4 (1st, 2nd, 4th compartments in SP-4), C1, C2, C4 (1st, 2nd, 4th compartments in TP-4). For each sludge sample, DNA of bacteria and fungi were extracted with the kit from Novogene (Beijing, China) using CTAB and SDS methods, respectively. The extracted DNA was PCR-amplified with the primers 341F (5, -CCTAYGGGRBGCASCAG-3) and 806R (5, -GGACTACNNNGGTATCTAAT-3) for 16S rDNA of bacterial and ITS3-2024F (5, -GCATCGATGAAGAACGCAGC-3) and ITS4-2409R (5, -TCCTCCGCTTATTGATATGC-3) for ITS of fungal.
The 16S RNA/ITS amplicons were sequenced on the Illumina NovaSeq 6000 platform. The GreenGene Database was applied to annotate taxonomic information of microorganisms.

Statistical analysis

Correlation analysis of the results was carried out to analyze the relationship of physicochemical properties and dewaterability indicators of bioleached sludge using R software (Team 2018) with at a significance level of (p = 0.05).

Results And Discussion

Morphology of sewage sludge and distribution of *A. ferrooxidans*

Fig. 2 shows the morphology of the raw and bioleached sludge in three trials. In this figure, the shadow represented sludge flocs, which reflected the size and compactness of flocs. Compared with the raw sludge, bioleached sludge had larger and aggregated flocs in each trail, and the phase separation enhanced this trend. Along with sludge-flow direction, sludge gradually became to be more aggregated. Changes of sludge morphology were due to the flocculation enhancement caused by hydrolysis of Fe$^{3+}$ during bioleaching (Li et al. 2012, Lin, et al. 2020, Mohammadi et al. 2016, Yu et al. 2015). Since the blue spot was excited by alive *A. ferrooxidans* (Fig. 3), more spots in sludge meant a larger amount of *A. ferrooxidans* in high activity. In each trail, bioleached sludge had more blue spots than that from the raw sludge, which showed bioleaching enriched more *A. ferrooxidans* in sludge (Lin, et al. 2020). More blue spots were observed in compartments from two-phase bioleaching, especial for the 2nd compartment in TP-4 (Fig. 3(C4)), which indicated that phase separation was beneficial to cultivate more *A. ferrooxidans* in a bioleaching bioreactor. The higher abundance of *A. ferrooxidans* in TP-4 could be illustrated by relief of toxicity inhibition of SCOD to *A. ferrooxidans* caused by phase separation (Mohammadi, et al. 2016).

Physicochemical properties

Figure 4 shows the changes of pH during the bioleaching process in three trails. For each trial, this trial was considered to have been stable and finished the start-up, when the temporal variation of pH values was no less more than 0.2 per day in every compartment. Phase separation pronouncedly shortened the period of start-up for sludge bioleaching, which was 4 days for TP-4, much less than 7 days for two trials (SP-3, SP-4) with single-phase bioleaching. During start-up, pH values rose rapidly in 1st compartment, while relative smaller changes were observed in the other compartments (Fig. 4a). That increase was due to gradual dilution of high H$^+$ activity from inoculums via continuously adding raw sludge of nearby neutral pH mentioned previously (Liu et al. 2012, Misra et al. 2015). During the stabilization period, pH values generally declined along with sludge-flow direction in each trial with the similar order as 1st compartment > 2nd compartment > 3rd compartment ≈ 4th compartment, which was caused by the growth of *A. ferrooxidans* Meanwhile, there was an observable phenomenon of phase-separation in TP-4, which had higher pH values of 6.4 – 6.6 in 1st compartment and low pH values of 2.7 – 2.9 in 3rd ~ 4th compartments than those of two single-phase bioreactors. It could be beneficial for a long-period
synergistic effect of acid-tolerant heterotrophic microbes and *A. ferrooxidans*, because the slight acidity in the selection phase (1st compartment in TP-4) is a suitable environment for the growth of acid-tolerant heterotrophic microbes (Xu et al. 2020, Ye, et al. 2021).

In each trial, bioleaching pronouncedly increased the zeta potential of sludge in the last compartment compared with that of the raw sludge, which meant a higher flocculation capability (Fig. 5a). Zeta potentials in the last compartments were −2.6, −3.4, and −3.7 mV for TP-4, SP-4, SP-3, respectively, which meant that phase separation could enhance the dewaterability improvement of bioleached sludge on account of the zeta potential of closer to zero. Meanwhile, more subphases in SP-4 slightly increased the zero potential of bioleached sludge with single-phase bioleaching. This variation of zeta potentials was corresponded to the trend of pH values among three trials (Fig. 4, 5a), which was explained by neutralization of negative charge outside surface of sludge flocs with H⁺ and Fe³⁺ (Mikkelsen and Keiding 2002). The concentration of SCOD was decided together by cell lysis, EPS decomposition and biodegradation of acid-tolerant heterotrophic microbes (Zhou, et al. 2015). During bioleaching, SCOD of raw sludge was pronounced removed from 619.0 ± 18.8 mg/L to 63.6 ± 7.5 – 135.3 ± 16.3 mg/L in all three trails (Fig. 5b), which meant that most of SCOD from the raw sludge was biodegraded by acid-tolerant heterotrophic microbes. Among these trails, SCOD gradually increased along with the sludge-flow direction, which was due to more production of SCOD from cell lysis, EPS decomposition than its biodegradation. Each compartment in TP-4 had lower concentrations of SCOD in the range of 63.6 ± 7.5 – 112.0 ± 26.2 mg/L than that in the other two trails, which showed that phase separation could be more effective to biodegrade DOM in sludge and relieve its inhibition to *A. ferrooxidans* (Wong et al. 2004).

Lower pH can be achieved by better growth of *A. ferrooxidans* after the relief of DOM inhibition (Fig. 4).

**Dewaterability**

Figure 5c, 5d show SRF and CST of sludge in three trails during the stabilization period of bioleaching. In each trial, bioleaching markedly reduced the SRF from 3.9 × 10⁻¹³ m/kg of raw sludge to 0.12 – 0.18 × 10⁻¹³ m/kg of bioleached sludge in the last compartment (Fig. 5c), which meant a large improvement of sludge dewaterability (Liu, et al. 2012, Huang et al. 2020). Compared with two trials of single-phase bioleaching, the bioleached sludge in TP-4 had a lower SRF, which indicated that phase separation enhanced the dewaterability improvement of bioleached sludge. Meanwhile, more subphases in SP-4 slightly decreased the SRF of bioleached sludge with single-phase bioleaching. After bioleaching, sludge CST had a similar trend as SRF, which also sharply decreased from 31.5 s of raw sludge to 9.4 – 13.9 s of bioleached sludge in the last compartment (Fig. 5d). For TP-4, CST pronouncedly declined from sludge flew from 1st compartment to 2nd compartment but an only small reduction for SP-4 and SP-3, which meant that phase separation enlarged this difference. These variations of SRF and CST were related to differences in pH values and zeta potentials among three trials (Fig. 4, 5c), which was mainly explained by denser floc structure caused by better flocculation performance when the zeta potential was close to zero under high activity of H⁺ and Fe³⁺ (Citeau et al. 2011).
Phase separation also manifested decreases of SRF by decreasing the sludge SCOD from lysis cell in the bioleaching phase with acid-tolerant heterotrophic microbes. These results were consistent with those from correlation coefficient matrix (Fig. 6). There was a strong positive correlation among SRF, CST, and pH at a significant level of $p = 0.05$, while the strong negative correlation between zeta potential and SRF or CST. As for SCOD, it was weak positively related to SRF but no significant relationship with CST. Therefore, the factors affected the dewaterability of bioleached sludge with the following sequence: $\text{pH} \approx \text{zeta potential} > \text{SCOD}$.

**Removal of heavy metal from sludge**

Generally, heavy metals in sludge can be reduced in an acidic environment, which is dissolved out with the form of anion from the forms of acid-soluble fraction and oxidizable fraction (Liu et al. 2021, Zheng et al. 2021). Throughout the bioleaching, heavy metals were removed from raw sludge with the ratios of $13.5 - 16.0\%$, $34.1 - 19.1\%$, $4.5 - 15.5\%$, $6.1 - 29.6\%$, $10.6 - 19.9\%$, $1.0 - 10.2\%$, and $45.6 - 56.3\%$ for As, Cd, Cr, Cu, Ni, Pb and Zn, respectively (Table 1). Pb in sludge was almost not reduced, which could be attributed to the chemical sedimentation of lead sulfate. Compared with single-phase bioleaching, there were more removal of Cd, Cr, Cu, Ni, and Zn, less removal of Pb, and close for As, which meant that phase separation could generally increase the dissolution ratio of heavy metals from sludge. Zhu et al. also found that a lower value of pH promotes removal of heavy metals from sewage sludge during bioleaching process (Zhu et al. 2013). Moreover, for the two-phase bioleaching, the bioleached sludge satisfied the requirement about the concentration limits of heavy metals for Level A sludge-compost in the agricultural utilization (State Administration for Market Regulation and Standardization administration, 2018).
Table 1
Concentrations of heavy metals in the raw sludge and bioleached sludge following different trails

|                  | As          | Cd          | Cr          | Cu          | Ni          | Pb          | Zn          |
|------------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Raw sludge       | 12.69 ± 0.74 | 2.26 ± 0.13 | 46.05 ± 1.78 | 148.02 ± 13.40 | 30.70 ± 2.16 | 26.80 ± 0.04 | 1394.66 ± 60.21 |
| bioleached sludge in TP-4b | 10.66 ± 0.22 | 1.15 ± 0.08 | 38.91 ± 2.93 | 104.22 ± 5.56 | 24.59 ± 1.74 | 26.54 ± 4.35 | 610.10 ± 29.34 |
| bioleached sludge in SP-4c | 10.56 ± 0.05 | 1.40 ± 0.11 | 43.99 ± 1.62 | 138.95 ± 9.05 | 26.80 ± 0.89 | 24.07 ± 4.72 | 703.87 ± 44.34 |
| bioleached sludge in SP-3d | 10.99 ± 0.37 | 1.49 ± 0.11 | 40.96 ± 0.98 | 130.83 ± 5.97 | 27.44 ± 1.60 | 25.88 ± 0.50 | 759.09 ± 54.09 |

a indicates the mean ± standard deviation, and the same as the others, n = 3

b indicates bioleached sludge in the last compartment in TP-4

c indicates bioleached sludge in the last compartment in SP-4

d indicates bioleached sludge in the last compartment in SP-3

Microbial community

For each sludge samples, there was a high number of sequences, which meant the measured sample contained enough information to analyze of microbial community (Table 2). During bioleaching, specie numbers (OTU) generally decreased along the sludge-flow direction in each trial for both bacteria and fungi, while the decreases of fungi were more obvious than bacteria. The similar trends also were investigated when considering the community diversity (Shannon index, Simpson index) and the community richness (Chao1 index, ACE index). Compared with single-phase bioleaching, sludge samples in two-phase bioleaching had more fungal species, higher community diversities and higher richness, which meant that phase separation did relieve the inactivation of acid-tolerant fungi. Taking into consideration that fungi is one key part of the acid-tolerant heterotrophic microbes (Yang et al. 2015), the relative higher species of fungi could produce better synergy benefit in TP-4.
Table 2
Relative abundance of bacteria and fungi in samples of raw sludge and bioleached from three trials

| Sludge Sample a | Sequence | OTU | Shannon | Simpson | Chao1 | ACE |
|----------------|----------|-----|---------|---------|-------|-----|
| **Bacteria**    |          |     |         |         |       |     |
| A0             | 62140    | 2710| 8.998   | 0.995   | 2583.860| 2645.064|
| A1             | 62015    | 2310| 8.373   | 0.991   | 2258.154| 2290.812|
| A3             | 62918    | 2088| 7.707   | 0.980   | 2024.849| 2066.619|
| B1             | 62689    | 2524| 8.632   | 0.993   | 2497.466| 2534.517|
| B2             | 60460    | 2182| 7.944   | 0.984   | 2182.298| 2204.748|
| B4             | 65452    | 2160| 7.431   | 0.969   | 2122.291| 2173.776|
| C1             | 64994    | 2565| 8.725   | 0.993   | 2537.618| 2573.380|
| C2             | 67664    | 2381| 8.349   | 0.989   | 2346.213| 2394.293|
| C4             | 64571    | 2236| 7.817   | 0.973   | 2176.655| 2208.463|
| **Fungi**      |          |     |         |         |       |     |
| A0             | 64723    | 314 | 3.781   | 0.856   | 311.218 | 309.409 |
| A1             | 65567    | 61  | 0.194   | 0.038   | 68.548  | 74.239  |
| A3             | 64713    | 61  | 0.210   | 0.045   | 67.450  | 73.103  |
| B1             | 69892    | 90  | 0.505   | 0.144   | 82.882  | 89.162  |
| B2             | 63054    | 61  | 0.285   | 0.060   | 62.416  | 66.259  |
| B4             | 66230    | 59  | 0.349   | 0.085   | 63.950  | 74.115  |
| C1             | 65583    | 215 | 2.235   | 0.603   | 222.188 | 221.231 |
| C2             | 62595    | 133 | 1.397   | 0.393   | 130.205 | 136.027 |
| C4             | 67090    | 141 | 1.915   | 0.606   | 135.571 | 143.213 |

a except raw sludge (A0), all other sludge samples were collected during the stabilization process, and A, B, C meant SP-3, SP-4, TP-4, respectively, while the number after the capital letter (such as 3 in A3) meant the compartment number in the corresponded trial.

In each sludge sample, the four main phyla were Proteobacteria, Bacteroidetes, Acidobacteria, Firmicutes for bacteria, in which Proteobacteria was majority with the relative abundance of more than 50% (Fig. 7a). The similar phenomenon also was found by Huang (Huang, et al. 2020). At the genus level, *Acidithiobacillus* was the largest abundance of the identified types in most of bioleached sludge other than the raw sludge and the bioleached sludge from the selection phase (C1) in TP-4, which meant that bioleaching improved the growth of *Acidithiobacillus* in the bioleaching phase (Fig. 7b). Throughout the
bioleaching, the relative abundance of *Acidithiobacillus* gradually increased in the bioleached sludge along with the sludge-flow direction, and the values were 20.7%, 18.1%, and 13.8% in the corresponded last compartments for TP-4, SP-4, and SP-3, respectively. Higher relative abundances of *Acidithiobacillus* were observed in the bioleaching phase (C2, C4) of TP-4 than that from two trials of single-phase bioleaching, which indicated that phase separation enhanced the enrichment of *Acidithiobacillus* and was beneficial for a long-term and stable operation of the bioleaching reactor. Those results were consistent with the change trends of physicochemical properties and dewaterability of sludge. Even though fungi are important for releasing the activity inhibition of *Acidithiobacillus* caused by DOM via biodegrading DOM (Zheng et al. 2016), most of the fungi were not identified in the sludge samples at the levels of phylum and genus (Fig. 7c, 7d). Based on the identified fungus, phase separation resulted into a dramatically succession that the major phylum and genus were changed from Basidiomycota, *Candida* to Chytridiomycota, and *Boothiomyces*, respectively, while the fungal succession only happened at the genus level for two trails with single-phase bioleaching. That difference could be related to the different pH levels in those bioleaching reactors resulted by the phase separation. Anyway, it still is necessary to obtain more detailed information about fungal community for elucidating the relationship among phase separation, fungal community, physicochemical properties and dewaterability improvement of sludge.

**Conclusion**

(1) Phase separation obviously shortened the duration of start-up for sludge bioleaching, which was decreased from 7 days in single-phase bioleaching to 4 days in two-phase bioleaching. During the stabilization process, the final bioleached sludge had a slighter lower pH value 2.7 – 2.9 after two-phase bioleaching than that from single-phase bioleaching 3.2 – 3.4.

(2) The results of SRF and CST showed that phase separation also pronouncedly enhanced the dewaterability improvement of bioleached sludge with the relative decreases of 25.0 – 33.3% for SRF and 14.2% for CST, while more subphases slightly promoted the dewaterability improvement. This enhancement is attributed to lower pH values, zeta potentials of closer to zero and less DOM of bioleached sludge during the two-phase bioleaching, and the factors affected the dewaterability of bioleached sludge with the following sequence: pH ≈ zeta potential > SCOD. Additionally, phase separation generally increased the dissolution ratio of heavy metals from sludge, and after two-phase bioleaching heavy-metals were removed from raw sludge with the ratios of 56.3%, 49.1%, 29.6%, 19.9%, 16.0%, 15.5%, and 1.0% for Zn, Cd, Cu, Ni, As, Cr, and Pb, respectively.

(3) Phase separation enhanced the enrichment of *Acidithiobacillus* and relieved the inactivation of acid-tolerant fungi, which could produce better synergy benefit in bioleaching phase during two-phase bioleaching of sewage sludge and be conducive to keeping a long-term and stable operation of the bioleaching reactor.

**Declarations**
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Authors’ contributions Yulan Lu: conceptualization, data curation, writing (original draft) and writing (review and editing). Rongjun Wu: Data curation and validation. Jun Zhang: validation, guidance and editing. Hongtao Liu: conceptualization, writing (review and editing). Yu Dai: data curation, and writing (review).

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Compliance with ethical standards

Ethics approval and consent to participate Not applicable.

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Competing interests The authors declare that they have no competing interests.

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**Figures**
Figure 1

Baffled-flow bioleaching reactor: 1, 2, 3, and 4 represent 1st, 2nd, 3rd, and 4th compartment, respectively.

Figure 2

Images of raw sludge and bioleached sludge in three trials by optical microscopy: A0, Raw sludge; A1 – A3, 1st, 2nd, and 3rd compartment of SP-3; B1 – B4, 1st, 2nd, 3rd, and 4th compartment of SP-4; C1 – C4, 1st, 2nd, 3rd, and 4th compartment of TP-4.

Figure 3

Images of raw sludge and bioleached sludge in three trials by a confocal laser scanning microscope: A0, Raw sludge; A1 – A3, 1st, 2nd, and 3rd compartment of SP-3; B1 – B4, 1st, 2nd, 3rd, and 4th compartment of SP-4; C1 – C4, 1st, 2nd, 3rd, and 4th compartment of TP-4.
Figure 4

Changes of pH with time during the sludge bioleaching process: a, 1st compartment; b, 2nd compartment; c, 3rd compartment; d, 4th compartment

Figure 5

- Zeta potential (mV)
- SCOD (mg/L)
- SRF (x 10^{-13} m/kg)
- CST (s)
Changes of zeta potentials (a), SCOD (b), SRT (c), and CST (d) of the bioleached sludge in different compartments during stabilization period from the single-phase and two-phase baffled-flow reactors

Figure 6

Pearson's correlation coefficient matrix with color-coded correlation coefficients. Multiply sign indicates a nonsignificant correlation defined by a p-value of > 0.01
Figure 7

Relative abundances of bacteria and fungi of sludge samples at the phylum and genus level in each compartment of three trails: a, bacterial phylum, b, bacterial genus, c, fungal phylum, d, fungal genus. Except raw sludge (A0), all other sludge samples were collected during the stabilization process, in which A1, A3 were from 1st, 3rd compartments of SP-3, while B1 – B4 and C1 – C4 from 1st, 2nd, 4th compartments of SP-4, and TP-4, respectively.