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Simultaneous extraction, separation and purification of microbial genomic DNA and total RNA from acidic habitat samples†

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Although many studies have focused on DNA and RNA extraction, only a few have focused on the simultaneous extraction of high quality DNA and RNA from environmental samples, especially for acidic habitat samples. In this study, a widely used DNA extraction method (Zhou, J. Z., M. A. Bruns, and J. M. Tiedje 1996) was optimised for the simultaneous extraction of crude nucleic acid (including DNA and RNA), and a LiCl salt-based step-by-step precipitation method was also optimised for the separation of DNA and RNA. The results showed that nucleic acid extraction buffer (pH 7.0) containing piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) salt could effectively extract crude nucleic acid from acidic habitat samples, and the optimised LiCl salt-based step-by-step precipitation method could separate the nucleic acid into high quality DNA and RNA. The results also showed that the obtained DNA and RNA could be used for downstream molecular analysis. This study developed a useful method for the simultaneous extraction and separation of DNA and RNA from acidic habitat samples. This method has a great potential as a standard method to obtain high quality nucleic acid from biohydrometallurgy processes and extreme environmental samples.

1. Introduction

Acid mine drainage (AMD), which usually has a low pH (<2) and high concentrations of sulphate and metal ions, is a prevalent, international environmental problem since it is harmful for diatoms,[1] protozoans,[2] aquatic invertebrates,[3] piscivorous birds in freshwater lakes,[4] groundwater, rivers, streams[5] and surrounding vegetation.[6] However, due to the relatively simple microbial community and extreme characteristics, the microbial community in AMD is regarded as a model community to investigate the linkages between microbial communities and geochemistry and even to reveal the mechanism of extreme life.[7–9] A better understanding of the microbial ecology and dynamic shift could provide a foundation for the bioremediation of AMD contaminated environments. Moreover, it could be also accelerate the development of biohydrometallurgy (also known as bioleaching or biomining) technology, which is an energy-efficient recovery technology for valuable metals from mine waste.[7,10] Therefore, it is important to study the microbial community in AMD, and many studies have focused on microbial diversity in AMD during the past decades.[11–17]

Nowadays, culture-independent nucleic acid techniques such as restriction fragment length polymorphism (RFLP), quantitative reverse transcription polymerase chain reaction (qRT-PCR), Phylochip, GeoChip, pyrosequencing and metagenomic sequencing have greatly advanced environmental microbiology and have also been gradually applied to analyse the microbial community structure and dynamics in AMD.[17–24] However, the quality and quantity of the nucleic acid significantly affect the downstream molecular analysis, data processing and even the final conclusions.[25,26] Therefore, how to extract high quality nucleic acid from AMD is still a key issue.

Although several methods have been applied to extract genomic DNA and total RNA from extreme acidic habitats such as AMD and bioleaching pools,[15,17,27] the simultaneous extraction of DNA and RNA from extreme acidic habitat samples has not yet been well investigated. Only several reports have focused on the simultaneous extraction of DNA and RNA from soil,

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tissue, and so on.[28–33] There are two advantages of the simultaneous extraction of DNA and RNA: (1) it demands less sample since DNA and RNA are extracted simultaneously from one sample, and the total amount of sample required is therefore less than for extracting DNA and RNA separately, which is especially important for precious samples; and (2) it guarantees the results of the genomics and transcriptomics analyses as the data for DNA and RNA extracted simultaneously are more reliable than the data extracted separately from one sample. Due to these advantages, several simultaneous DNA and RNA extraction methods have been developed for different samples, especially for precious samples.[28–31,34,35] However, it is hard to apply these methods to acidic samples due to low pH and high concentrations of sulphide and heavy metal ions. Zammit[36] compared several nucleic acid extraction methods and developed an enzyme-based method to recover nucleic acid from biomining and AMD microorganisms by adapting Bond's method.[16] However, in Zammit's protocol, RNase or DNase was used to remove the residual RNA or DNA within total DNA or RNA. This operation causes nucleic acid loss. On the other hand, there is no standard method for the extraction of nucleic acid from the biohydrometallurgy microbial community. Therefore, it is hard to compare the results among different studies because different extraction methods might cause different results.[36,37] Thus, standard, feasible, affordable and efficient simultaneous DNA and RNA extraction, separation and purification methods need to be developed for the recovery of genomic DNA and total RNA from acidic habitat samples.

The objective of this study was to develop a robust, effective, economic, simple and rapid method for the simultaneous extraction, separation and purification of intact genomic DNA and total RNA from acidic habitat samples. A high-salt piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) buffer containing 0.1 M PIPES salt, 0.1 M EDTA, 1.5 M NaCl and 1% CTAB was used for total nucleic acid extraction. The nucleic acid extraction procedure was optimised based on a widely used DNA extraction method that shows excellent recovery of high-molecular-weight DNA from soil with diverse compositions.[28,38,39] The DNA and RNA separation procedure was optimised using different kinds of salts and separation conditions. Five bioleaching strains, *Acidithiobacillus ferrooxidans* (*A. f*), *Acidithiobacillus caldus* (*A. c*), *Acidithiobacillus albertensis* (*A. a*), *Leptospirillum ferrooxidans* (*L. f*) and *Ferroplasma Thermophilium* (*F. t*), and several AMD samples from three different locations, Daye copper mine (Daye), Xiangxi gold mine (Xxi) and Axi gold mine (Axi), were used to confirm the optimised extraction and separation methods. Our results showed that genomic DNA (gDNA) and total RNA could be simultaneously extracted and separated from the same sample, contributing to the quantitative analysis of biohydrometallurgy microbial community structure and finally accelerating the efficiency of biohydrometallurgy.

2. Materials and methods

2.1 Preparation of reagents and materials

All solutions, water, glassware and utensils used for nucleic acid extraction and separation were treated with 0.1% diethylpyrocarbonate (DEPC) overnight at 37 °C and autoclaved at 121 °C for 30 min. The glassware and utensils used for nucleic acid extraction were then baked at 105 °C for 8 h. The disposable plastic ware was RNase-free, and the working bench was treated with RNase removal reagent (Sigma, Shanghai, China).

2.2 Preparation of type strains and acidic environmental samples

Five strains (*A. f*, *A. c*, *A. a*, *L. f* and *F. t*) were used to optimise the nucleic acid extraction and separation conditions. All strains were obtained from the Ministry of Education Key Laboratory of Biometallurgy Strain Library and grown in 9 K liquid medium containing 3.0 g L^{-1} $(NH_4)_2SO_4$, 0.1 g L^{-1} KCl, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.01 g L^{-1} $Ca(NO_3)_2$, 0.5 g L^{-1} K_2HPO_4, 44.2 g L^{-1} $Fe_2SO_4 \cdot 7H_2O$ or 10 g L^{-1} elemental S. The pH of the culture medium was adjusted to 2.0, and the volume of the medium was 200 mL for each flask. To obtain enough biomass, a total of 2 L of liquid medium was cultured in 10 flasks for each time. Cells were harvested by centrifugation at 10 000× *g* for 10 min when cell density was around 10^7 to 10^8 cell per mL, and the cell pellet was immediately stored at −20 °C prior to total nucleic acid extraction. Several AMD samples (25 L) were obtained from the Hubei Daye copper mine (Daye I and Daye II), the Hunan Xiangxi gold mine (Xxi), and the Xinjiang Axi gold mine (Axi I and Axi II). Each AMD sample was filtered using 0.22 μm nylon filters, and the filters were stored at −20 °C until being used for total nucleic acid extraction.

2.3 Nucleic acid extraction and separation protocol

The collected cell/sediment samples were mixed with 2 g of sterilised silica sand and then ground three times from freezing to thawing using liquid nitrogen. The mixture was transferred into an RNase-free tube containing 16.5 mL of PIPES extraction buffer (containing 0.1 M PIPES sodium salt, 0.1 M EDTA, 1.5 M NaCl and 1% CTAB) and mixed gently several times. The pH of the mixture was checked and adjusted to 7. Then, 1.83 mL 20% sodium dodecyl sulphate (SDS) was added to the above mixture and incubated at 65 °C for 1 h with gentle inversion every 15 min. When 61 μL of proteinase K was used, the mixture was first incubated at 37 °C for 30 min before SDS was added. After incubation, the mixture was centrifuged at 6000× *g* for 10 min, and the supernatant liquid was transferred to a new centrifuge tube and extracted with chloroform–isoamylol (v/v = 24 : 1) for 10 min. The mixture was centrifuged at 6000× *g* for 10 min, and the supernatant liquid containing DNA and RNA was transferred to a new centrifuge tube. Nucleic acids were then precipitated with 0.6 volume isopropanol and then collected by centrifugation at 12 000× *g* for 30 min at room temperature. The nucleic acid pellet was air dried for around 10 min and resuspended with 100–200 μL RNase-free water.

The nucleic acid concentration was adjusted to the optimised concentration, and the optimised amount of salt was added to the nucleic acid and mixed well. RNA was first collected by centrifugation at 12 000× *g* for 30 min at 4 °C. After the removal of RNA, the DNA in the supernatant was

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precipitated overnight with 0.6 volume isopropanol and collected by centrifugation at 12 000× *g* for 30 min at room temperature. Both the isolated DNA and RNA were purified with 70% ethanol and collected by centrifugation at 12 000× *g* for 30 min at room temperature. The DNA and RNA pellets were air dried for 10–20 min and resuspended with 100–200 μL RNase-free water.

2.4 Optimisation of nucleic acid extraction and separation protocol

2.4.1 Optimisation of PIPES extraction buffer composition and conditions. PIPES extraction buffer was used to extract total nucleic acid. The extraction conditions such as incubation time, extraction buffer pH and other controllable factors that could influence the extent of RNA damage were optimised with a pure culture of *A. f* cells based on previously described DNA extraction and purification methods.[38,39] The concentration of *A. f* cells was approximately 10^7 cells per mL, and we combined all cultured cells together and separated them equally to ensure that the biomass was same in each condition optimisation experiment. The pH of the PIPES extraction buffer was changed from 6.0 to 7.5 in increments of 0.5. The effect of proteinase K concentration on the extraction was examined using concentrations of 0, 10, 50 and 100 mg mL^{-1}. The sensitivity of this method was tested using different *A. f* biomasses ranging from 1×10^9 cells to 4×10^9 cells.

2.4.2 Optimisation of separation conditions. The nucleic acid of the pure culture *A. f* was used to optimise the separation conditions including type of salt, salt concentration, initial nucleic acid concentration, incubation temperature and time, and other controllable factors that may influence the efficiency of the separation of RNA from DNA. A total of three kinds of salt solutions including one third volume of NaCl [concentrations of 1, 2, 3, 4, 5 M and saturated], an equal volume of $CaCl_2$ [concentrations of 0.5, 2, 4, 6, 8 and 10 M] and a quarter volume of LiCl [concentrations of 10, 12.5, 15, 17.5 M and saturated] were selected for evaluating RNA precipitation. These salt solutions were added to the nucleic acid, mixed well and then incubated for times ranging from 0.5–1.5 h (intervals of 0.5 h) at −20 °C. Precipitated RNA was collected by centrifugation at 12 000× *g* for 30 min at 4 °C. DNA in the supernatant was precipitated with 0.6 volume isopropanol and then collected by centrifugation at 12 000× *g* for 20 min at room temperature. Both DNA and RNA were purified twice with 70% ethanol, centrifuged, air dried and resuspended with 100–200 μL RNase-free water. The universality of the extraction and separation method was tested using another four pure cultures and three acidic environmental samples.

2.5 Separation and purification of RNA and DNA from total nucleic acid using a QIAGEN All Prep DNA/RNA Mini Kit

Total crude nucleic acid containing DNA and RNA can be also separated using the QIAGEN All Prep DNA/RNA Mini Kit (Gene Company Limited, Shanghai, China) according to the manufacturer's protocols. Due to the low $A_{260/230}$ value of the DNA and RNA separated by the QIAGEN kit, further purification was needed. First, 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volume of cold absolute ethanol were added sequentially to precipitate the resultant RNA and DNA. The mixture was then centrifuged at 12 000× *g* for 30 min at room temperature to obtain the nucleic acid pellet. Third, the nucleic acid pellet was washed twice with 70% ethanol, centrifuged, air dried and resuspended with 100–200 μL RNase-free water.

2.6 Quantification of DNA quality and quantity

Generally, two methods were used to measure the quality and quantity of the extracted or/and purified DNA samples: (i) gel electrophoresis with 1% agarose gel stained with ethidium bromide and (ii) measurement of the absorbance ratios of $A_{260/280}$ and $A_{260/230}$ with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). DNA with A_{260}/A_{280} and A_{260}/A_{230} ratios of >1.7 and >1.8, respectively, was considered to good quality and used for further analysis. DNA and RNA quality was evaluated by further subsequent molecular analysis such as polymerase chain reaction (PCR) and reverse transcription PCR.

2.7 PCR and reverse transcription PCR (RT-PCR)

RNA was reverse transcribed using the primer *gln* A-F (5′-CATCCGCATTCCCTTCGTCAAC-3′) for the glutamine synthetase gene with an RT reagent kit (TAKARA BIOTECHNOLOGY CO., LTD, Dalian, China). Aliquots (1 μL) of the reverse transcription products from a total volume of 20 μL were used for PCR amplification in PCR buffer containing 12.5 μL 2× PCR mix (TIANGEN Biotech Co., Ltd., Beijing, China), 1 μL of forward (*gln* A-F) or reverse primer (*gln* A-R; 5′-GGCAGGTCGTAAA GATTCTTGTCC-3′) and 9.5 μL ddH_2O and generated a 174-bp fragment. The PCR amplification included denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1.5 min for 30 cycles followed by a final extension at 72 °C for 7 min (Bio-Rad Laboratories, Inc., Shanghai, China). The quality of DNA was examined by PCR amplification of the 16S rRNA gene using the bacterial universal primers 27F (5′-AGAGTTT GATCMTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTAC GACTT-3′).[40] PCR amplification was conducted in reaction mixtures containing 100 ng of DNA template, 1× PCR buffer [10 mM Tris–HCl (pH 8.3), 50 mM KCl and 2 mM $MgCl_2$], 2 mM dNTPs, 5 pM of each of the forward and reverse primers, 2.5 U Taq polymerase (Invitrogen, Shanghai, China), and deionised water to a final volume of 50 μL. The thermal cycling protocol included an initial denaturation at 94 °C for 5 min followed by 35 cycles at 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 90 s. A final extension step of 72 °C for 7 min was also used. Negative controls with ddH_2O as the template were performed for all PCR reactions.

2.8 Statistical analysis

All experiments were performed in triplicate and repeated at least three times to check the reproducibility of the results. Statistical analysis was carried out using the Microsoft EXCEL 5.0 statistical package for calculating mean and standard error.

3. Results

3.1 Optimisation of PIPES extraction buffer pH and extraction conditions

PIPES buffer can be used as a total nucleic acid extraction buffer at a suitable pH.[38] Therefore, the DNA and RNA extraction conditions were optimised with *A. f* under different conditions. The results showed that different amounts of DNA and RNA were recovered from the same amount of biomass for different pH values of the extraction buffer (Table 1 and Fig. S1†). This indicated that the pH of the PIPES extraction buffer affected the recovered amount of DNA and RNA. A total of 42 ± 2 μg DNA and 54 ± 8 μg RNA could be extracted from 400 mL *A. f* at a concentration of 10^7 cells per mL when using PIPES buffer at pH 7.0. The amount of DNA and RNA extracted by the pH 7.0 PIPES buffer was significantly higher than the amounts extracted at the other three pH conditions ($n = 3$; $P < 0.1$). No difference was found between 1 h and 2 h of incubation at 65 °C (data not shown). A total of 42 μg DNA and 63 μg RNA were extracted when no proteinase K was added; however, the DNA and RNA amounts decreased with increasing amount of proteinase K (Table 2 and Fig. S2†). Thus, it seems that proteinase K might cause DNA and RNA loss. These results indicated that a pH of 7.0, incubation at 65 °C for 1 h, and no proteinase K were the best extraction conditions for the simultaneous extraction of DNA and RNA from *A. f* (Fig. 1).

3.2 The sensitivity of the developed extraction method

To test the sensitivity of the PIPES extraction buffer, samples containing different amounts of *A. f* biomass were used to extract DNA and RNA. The results showed that 6 ± 2 μg DNA and 14 ± 2 μg RNA could be simultaneously extracted from approximately 1×10^9 cells (Table 3). The amount of obtained DNA was significantly linearly dependent on the biomass ($R = 0.99$, $P = 0.0074$); however, there was no linear relationship between RNA amount and biomass (Fig. S3†). These results indicated that this method could extract large amounts of DNA and RNA from limited cells, and the extracted DNA is significantly correlated with the amount of biomass.

3.3 Optimisation of DNA and RNA separation conditions

Considering the cost and treatment capacity, a chemical method was developed as an alternative to the QIAGEN All Prep

Table 1 DNA and RNA recovered using PIPES buffer at different pH values

	DNA			RNA		
Buffer pH	Amount/μg	$A_{260/280}$	$A_{260/230}$	Amount/μg	$A_{260/280}$	$A_{260/230}$
pH 6	19 ± 4	1.90 ± 0.01	2.15 ± 0.03	22 ± 5	1.94 ± 0.01	2.48 ± 0.01
pH 6.5	16 ± 0.9	1.89 ± 0.01	2.17 ± 0.02	22 ± 3	2.02 ± 0.01	2.43 ± 0.04
pH 7	42 ± 2	1.89 ± 0.01	2.21 ± 0.01	54 ± 8	2.02 ± 0.03	2.36 ± 0.02
pH 7.5	27 ± 18	1.84 ± 0.07	2.1 ± 0.1	20 ± 13	1.9 ± 0.1	2.38 ± 0.07

Table 2 The effects of proteinase K on DNA and RNA recovery

	DNA			RNA		
Proteinase K mg mL^{-1}	Amount/μg	$A_{260/280}$	$A_{260/230}$	Amount/μg	$A_{260/280}$	$A_{260/230}$
0	42 ± 8	1.62 ± 0.01	2.24 ± 0.02	63 ± 20	1.81 ± 0.02	2.30 ± 0.02
10	14 ± 13	1.7 ± 0.2	1.5 ± 0.4	49 ± 34	1.74 ± 0.04	1.8 ± 0.4
50	31 ± 14	1.62 ± 0.05	1.9 ± 0.3	66 ± 32	1.80 ± 0.02	2.1 ± 0.3
100	24 ± 11	1.65 ± 0.06	2.0 ± 0.2	51 ± 24	1.82 ± 0.03	2.0 ± 0.3

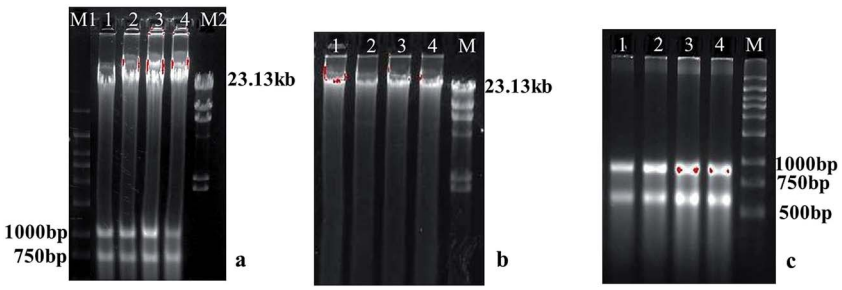

Fig. 1 Simultaneous DNA and RNA extraction from *A. f* using the optimised method. (a) Total nucleic acid extracted from *A. f*; lane M1: 1 kb ladder, lane M2: *Hind* III-cut lambda molecular size marker, lanes 1–4: biological replicates. (b) DNA precipitated by isopropanol. (c) RNA precipitated by LiCl.

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Table 3 DNA and RNA extracted from different amounts of biomass

	DNA			RNA		
Biomass/cells	Amount/μg	$A_{260/280}$	$A_{260/230}$	Amount/μg	$A_{260/280}$	$A_{260/230}$
1×10^9	6 ± 2	1.7 ± 0.1	1.8 ± 0.1	14 ± 2	1.97 ± 0.03	2.29 ± 0.03
2×10^9	21 ± 6	1.85 ± 0.02	2.13 ± 0.04	17 ± 2	1.92 ± 0.03	2.31 ± 0.02
3×10^9	34 ± 8	1.87 ± 0.01	2.20 ± 0.01	20 ± 3	2.07 ± 0.07	2.28 ± 0.09
4×10^9	56 ± 23	1.83 ± 0.05	2.0 ± 0.2	90 ± 2	2.1 ± 0.1	1.9 ± 0.5

DNA/RNA Mini Kit for the separation of DNA and RNA. Several DNA and RNA separation conditions including type of salt, salt concentration, initial nucleic acid concentration and precipitation temperature and time were optimised. The purity and intactness were considered as important factors when choosing separation conditions. The results showed that RNA could not be precipitated by NaCl, and DNA was co-precipitated with RNA by $CaCl_2$ (data not shown). For an operational volume of 100 μL, an initial total nucleic acid concentration of 400 ng μL^{-1} and saturated LiCl solution showed the best separation efficiency (Table S1†). No significant difference was found for DNA and RNA precipitation among incubation temperatures of 4 °C, −20 °C and −80 °C (Fig. S4†). No significant difference was found for DNA precipitation using 0.6 volume or 1 volume isopropanol (data not shown). In order to optimise the initial nucleic acid concentration for the downstream DNA and RNA separation, 100, 200, 400, 600, 800 and 1000 ng μL^{-1} initial nucleic acid was first used for RNA precipitation by 1/4 volume saturated LiCl, and DNA was then precipitated by 0.6 volume isopropanol. The results showed that separation efficiency decreased, RNA was degraded when the initial nucleic acid concentration exceeded 400 ng μL^{-1} (Table S2†). In contrast, nucleic acid was easy to lose when the concentration of initial nucleic acid was less than 400 ng μL^{-1}. Therefore, an initial nucleic acid concentration of 400 ng μL^{-1} was regarded as the best concentration for an operational volume of 100 μL.

In short, the nucleic acid could be separated (Fig. 2a–c) by the following optimised separation procedure. First, RNA was precipitated by saturated LiCl from an initial total nucleic acid concentration of 400 ng μL^{-1} after incubation at −20 °C for 30 min. DNA was then precipitated by 0.6 volume isopropanol after incubation at −20 °C for 30 min. It is better to incubate the

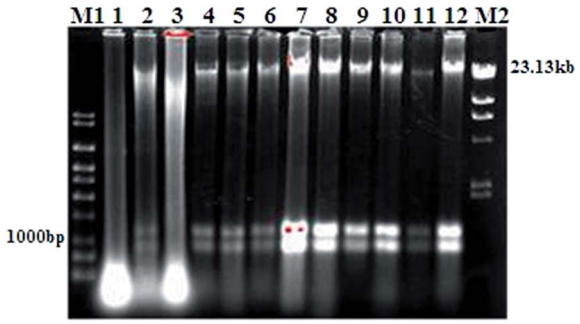

Fig. 2 Simultaneous extraction of DNA and RNA from four pure cultures. Note: lane M1 is 1 kb marker, lanes 1–3 are *A. c* biological replicates, lanes 4–6 are *A. a* biological replicates, lanes 7–9 are *F. t* biological replicates, lanes 10–12 are *L. f* biological replicates, and lane M2 is *Hind* III-cut bacteriophage λ marker.

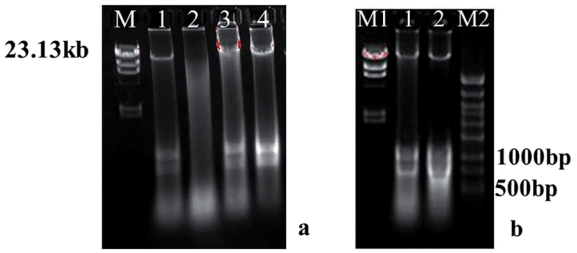

Fig. 3 Simultaneous extraction of DNA and RNA from acidic environmental samples. (a) Extracted DNA and RNA of the Xxi water sample (lane 1), the Daye sediment sample (lane 2) and the Daye water samples (lanes 3 and 4) and (b) extracted DNA and RNA of the Axi water sample (lane 1) and sediment sample (lane 2). Lanes M and M1 are *Hind* III-cut bacteriophage λ marker, and lane M2 is 1 kb marker.

Table 4 DNA and RNA extracted from different pure cultures and diverse environmental samples

	DNA			RNA		
Sample	Amount (μg)	$A_{260/280}$	$A_{260/230}$	Amount (μg)	$A_{260/280}$	$A_{260/230}$
A. c	7 ± 3	1.94 ± 0.03	1.9 ± 0.2	24 ± 13	1.85 ± 0.03	1.9 ± 0.2
A. a	2 ± 1	1.8 ± 0.1	1.2 ± 0.1	5 ± 3	1.75 ± 0.04	2.00 ± 0.07
F. t	19 ± 1	1.98 ± 0.01	2.29 ± 0.02	116 ± 6	1.98 ± 0.03	2.48 ± 0.06
L. f	7 ± 0.4	1.88 ± 0.03	1.88 ± 0.04	24 ± 2	1.93 ± 0.02	2.35 ± 0.02
Daye I	2 ± 0.2	1.84 ± 0.04	2.18 ± 0.2	1 ± 0.3	1.66 ± 0.03	2.58 ± 0.5
Daye II	16 ± 2	1.94 ± 0.00	2.37 ± 0.03	24 ± 2	2.00 ± 0.01	2.45 ± 0.03
Axi I	12 ± 4	1.72 ± 0.01	2.51 ± 0.01	11 ± 0.3	1.77 ± 0.01	2.58 ± 0.01
Axi II	18 ± 1	1.59 ± 0.01	1.72 ± 0.01	10 ± 0.2	1.67 ± 0.01	2.03 ± 0.01
Xxi	5 ± 0.1	1.19 ± 0.01	1.46 ± 0.01	—	—	—

mixture of LiCl and total nucleic acid at 4 °C for 6–8 h after incubation at −20 °C for 30 min.

3.4 DNA and RNA extracted from the pure culture and environmental samples

To evaluate the versatility of our optimised methods, several pure cultures (*A. c*, *A. a*, *L. f* and *F. t*) and environmental samples (Daye I, Daye II, Xxi, Axi I and Axi II) were used for the simultaneous extraction of total nucleic acid, and RNA and DNA were then separated from total nucleic acid. The results showed that total nucleic acid could be simultaneously extracted from all the tested samples (Fig. 2 and 3). A total of 2–19 μg DNA and 1–116 μg RNA could be obtained from these samples with good quality, with the exception of the Xxi sample (Table 4). The poor quality of nucleic acid in the Xxi sample is mainly explained by the low biomass in this sample. All these results indicated that our optimised methods are suitable for the simultaneous recovery of DNA and RNA from pure cultures and environmental samples.

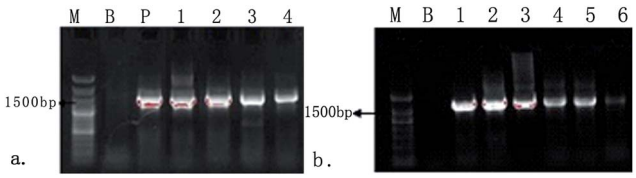

Fig. 4 16S rRNA gene PCR for pure culture and environmental samples. (a) Lane M, 100 bp marker; lane B, negative control for amplification; lane P, positive control for amplification (*A. f*); lanes 1–4, DNA separated from pure culture *A. c*, *A. t*, *F. t*, *L. f*. (b) Lanes 1–6, DNA separated from Daye I, Daye II, Daye slurry I, Xxi, Axi and Axi slurry samples.

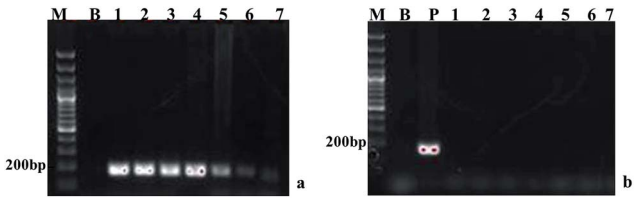

Fig. 5 The RT-PCR for pure cultures and environmental samples. Lane M, 100 bp marker; lane B, negative control for amplification; lane P, positive control for amplification (*A. f*); (a) lanes 1–7, RT-PCR for *A. f*, *A. c*, *A. t* DayeI, DayeII, Xxi and Axi. (b) Lanes 1–7, PCR amplification using RNA template extracted from *A. f*, *A. c*, *A. t* DayeI, DayeII, Xxi and Axi. Note: B means negative control; P means positive control.

3.5 PCR and RT-PCR of DNA and RNA isolated from pure cultures and environmental samples

To evaluate the quality of DNA and RNA isolated from pure cultures and environmental samples, PCR and RT-PCR were performed. The results showed that DNA and RNA separated by LiCl were of high quality and suitable for downstream analyses such as PCR and RT-PCR (Fig. 4 and 5); no DNA contamination was found in RNA (Fig. 5b).

3.6 Evaluation of QIAGEN All Prep DNA/RNA Mini Kit and LiCl separation methods

To evaluate the efficacy of the QIAGEN All Prep DNA/RNA Mini Kit and LiCl separation methods, the four pure cultures mentioned above were tested using both methods. The results showed that both methods could successfully separate DNA and RNA from the four strains; however, the amount of DNA and RNA separated by the QIAGEN kit was less than that separated by the LiCl separation method (Table 5). There was no significant difference in the ratio of $A_{260/280}$, while the $A_{260/230}$ ratio of DNA and RNA separated by the QIAGEN kit was lower than that obtained by the LiCl separation method. Therefore, further purification using sodium acetate precipitation was necessary.

Table 5 Comparison of DNA and RNA separated using the QIAGEN kit and LiCl separation methods

		A. c	*A. a*	*F. t*	*L. f*
Total nucleic acid[a]	Amount (μg)	101 ± 55	37 ± 13	56 ± 9	33 ± 4
	$A_{260/280}$	2.04 ± 0.02	2.06 ± 0.01	2.01 ± 0.01	2.08 ± 0.01
	$A_{260/230}$	1.49 ± 0.04	2.07 ± 0.01	2.19 ± 0.01	1.78 ± 0.01
DNA[b]	Amount (μg)	10 ± 0.6	5 ± 0.5	8 ± 2	4 ± 0.8
	$A_{260/280}$	1.86 ± 0.01	1.79 ± 0.03	1.79 ± 0.03	1.75 ± 0.06
	$A_{260/230}$	0.65 ± 0.01	0.5 ± 0.1	0.6 ± 0.2	0.26 ± 0.04
RNA[b]	Amount (μg)	36 ± 13	20 ± 2	38 ± 8	18 ± 4
	$A_{260/280}$	2.04 ± 0.01	2.08 ± 0.01	2.09 ± 0.02	2.09 ± 0.01
	$A_{260/230}$	2.00 ± 0.01	1.1 ± 0.2	1.9 ± 0.1	1.2 ± 0.3
DNA[c]	Amount (μg)	14 ± 0.4	16 ± 0.2	29 ± 2	7 ± 0.5
	$A_{260/280}$	1.73 ± 0.01	1.75 ± 0.01	1.7 ± 0.01	1.73 ± 0.01
	$A_{260/230}$	2.81 ± 0.05	2.43 ± 0.01	2.56 ± 0.02	2.32 ± 0.02
RNA[c]	Amount (μg)	39 ± 2	22 ± 0.1	28 ± 0.6	28 ± 2
	$A_{260/280}$	1.84 ± 0.02	1.82 ± 0.01	1.81 ± 0.02	1.78 ± 0.02
	$A_{260/230}$	2.30 ± 0.04	2.39 ± 0.01	2.56 ± 0.01	2.35 ± 0.01

[a] Original nucleic acid extracted by optimised protocol. [b] DNA and RNA separated by QIAGEN All Prep DNA/RNA Mini Kit. [c] DNA and RNA separated by LiCl separation methods.

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4. Discussion

Nucleic acid extraction is a key issue in microbial community analysis. However, the process is often disturbed by impurities such as various metal ions and humic acid or other organic acid. For AMD, the low pH and high concentrations of heavy metal ions not only shear the nucleic acid, but also inhibit or influence enzyme activity and stability during the extraction and subsequent downstream molecular analysis. Much effort had been made to improve the extraction methods, especially for soil samples. However, only a few reports have focused on the extraction of DNA and RNA from AMD samples. Herein, by adapting a previously reported method,[38,39] we developed an optimised method that could simultaneously extract DNA and RNA from bioleaching pure cultures and AMD samples. The results showed that the quantity and quality of extracted DNA and RNA was good enough for the following downstream molecular analysis.

In this study, a widely used efficient DNA extraction method[39] was optimised for the simultaneous extraction of crude nucleic acid (including DNA and RNA) from acidic habitat samples, and an LiCl-based DNA and RNA separation method was developed for DNA and RNA isolation from total nucleic acid. The pH of the PIPES buffer ranged from 6.1 to 7.5. Based on our results, the PIPES extraction buffer at pH 7.0 was considered suitable for buffering the acidic sample and stabilising the structure of nucleic acid. Higher buffer pH such as 7.5 had a negative influence on total nucleic acid recovery in our study. In general, cell lysis using alkali is the first step of the nucleic acid extraction. Once the cytoplasmic inclusion is released, the nucleic acid is easy to degrade and difficult to conglomerate under such conditions due to their single strand form. In contrast, the nucleic acid can be hydrolysed under acidic conditions. Thus, a buffer pH of 7.0 is best for the extraction of DNA and RNA, mainly because the pH 7.0 buffer could guarantee the integrity of the nucleic acid and avoid the influence from other ions.

To lyse the cells and minimise the DNA and RNA degradation from nuclease, we ground the cells three times with sterilised silica sand at −196 °C using liquid nitrogen. The cells–sand mixture was then incubated at 65 °C for 1 h with 20% SDS so that the cells in the samples could lyse sufficiently. Meanwhile, we tested proteinase K by adding different quantities. A previous study showed that proteinase K could protect RNA to some extent.[28] However, our results showed that this kind of protection was not obvious. Actually, the high molecular weight bands of the nucleic acid extracted without proteinase K did not show less integrity than those extracted with proteinase K. In contrast, proteinase K may have led to an excess release of protein as the observed thick and sticky middle layer between the supernatant liquid layer and the underlying organic layer formed. As a result, these released proteins likely entangled with nucleic acid and caused loss.

The purity and intactness were considered important factors when selecting separation conditions. Halons or sulphate have been previously used to precipitate nucleic acid.[35,41] On the one hand, alkali metal ions can compete with bound-water for nucleic acid, improving its aggregation and sedimentation. On the other hand, the positive charge of the ions can neutralise the negative charge of nucleic acid, changing the configuration of nucleic acid and finally weakening the repulsive interactions between nucleic acid molecules. In addition, the metal ions can combine with multiple nucleic acids as a bridge to precipitate the nucleic acids. By using different concentrations of NaCl as a precipitator, DNA and RNA almost simultaneously disappeared from solution, which was not desired.[42] It is well known that 0.14 M NaCl is usually used for DNA separation; however, in our study, there was no difference among different amounts of NaCl.

To purify plasmid, $CaCl_2$ has been used previously for polysaccharides.[43] $CaCl_2$ has also been used for the recovery of RNA.[44] Therefore, in this study, we designed a $CaCl_2$ concentration gradient to verify its function. Although $CaCl_2$ (ref. 43) could sufficiently precipitate RNA, a lot of DNA was co-precipitated. Our results confirmed the above conclusion, and we also found that a low concentration of $CaCl_2$ may be helpful for the DNA separation process. However, higher concentrations of $CaCl_2$ might cause serious DNA loss.

Previous studies showed that LiCl could precipitate nucleic acid with different sizes of nucleic acid fragments under different extraction conditions.[35,45] Therefore, it is necessary to test LiCl for the separation of nucleic acid extracted from AMD samples simultaneously. First, several low temperatures for RNA precipitation were compared based on the literature. For RNA, a precipitation temperature lower than 4 °C might cause the co-precipitation of trace DNA with RNA. In our results, RNA precipitated at 4 °C for 30 min tended to redissolve in solution if RNA was collected using the centrifugation at room temperature. However, the results from both gel electrophoresis and the NanoDrop ND1000 showed that precipitation at 4 °C and centrifugation is the best choice. It seems that at 4 °C, both DNA and RNA dynamically changed between dissolving and precipitating, as demonstrated by the reported 4 °C centrifugation temperature.[46] Therefore, it is better to keep the operation at 4 °C, not only to obtain a higher recovery rate, but also to minimise the activity of nuclease. Our results also showed that the initial concentration of nucleic acid could influence the separation of DNA and RNA. When the initial nucleic acid concentration was low (corresponding to the low total amount based on the equal volume), it was difficult to find the centrifugation pellet, and the pellet was easy to lose. On the other hand, RNA and DNA intertwined due to their analogous structures and could be precipitated together when the tested nucleic acid solution concentration was high. Therefore, controlling the initial nucleic acid concentration is very important to separate DNA and RNA. In our experiment, we considered an initial concentration of 400 ng μL^{-1} to be optimal at an operational volume of 100 μL for the separation of DNA and RNA.

In addition, the effect of the LiCl concentration was non-ignorable. Different initial concentrations with various doses have been reported, and one quarter volume with 10 M LiCl has been commonly used.[47–49] However, in this study, adding 1/4 volume of saturated LiCl solution was better than other

concentrations. This was consistent with the reported 8 M LiCl with equal volume added.[43,50] It was reported that overnight sedimentation may be a challenge for RNA integrity.[48,50] Thus, we aimed to identify a shorter time interval with the least influence based on RNA quality and quantity, and 6 h at 4 °C was recommended according to our results. The main advantages of using a kit are the time-saving and high efficacy when dealing with small sample amounts. Compared with the QIAGEN All Prep DNA/RNA Mini Kit, the maximum load for each column was less than 100 μg, while a "saturated load" was not observed when using LiCl as precipitator. DNA and RNA can be separated even more efficaciously compared to using the kit. Furthermore, repeated centrifugation was necessary when the volume was larger than 700 μL when using the kit. Additionally, when the initial nucleic acid solution concentration was high, insufficient separation was commonly observed, and the recovery rate was relatively low. Interestingly LiCl was far more efficient than the kit for the recovery of DNA from *F. t*, whose G + C% was about 34% (as opposed to 50–61.4% for the other samples).

The advantages of our optimised method are as follows (1) DNA and RNA can be simultaneously recovered as much as possible from one sample. Time consuming and complex processes for the separate extraction of DNA and RNA is avoided, and the DNA and RNA extraction time interval is also avoided. No RNase or DNase usage means no nucleic acid loss after extraction. (2) The method is low-cost. Saturated LiCl solution was used to separate DNA and RNA instead of a high-cost kit and special equipment such as the equipment for CsCl-based ultracentrifugation procedures, decreasing the cost of the whole process. (3) The method exhibits high efficiency and quality. Our optimised method is based on a widely used, highly efficient DNA extraction method and resulted in satisfactory amount of DNA and RNA. The obtained DNA was greater than 23 kb. 16S rRNA and 23S rRNA were obviously observed in the gel electrophoresis. The DNA and RNA could be directly used for PCR and RT-PCR amplification, respectively, indicating that downstream molecular analysis can be satisfied. (4) The method is universally applicable; it can be effectively applied to Gram-negative bacteria (*A. f*, *A. c*, *A. a*, *L. f* and *E. coli*), Gram-positive bacteria (*S. t*) as well as archaea (*F. t*) and fungus in wastewater biofilm. This method could also be successfully applied to environmental samples.

All in all, our optimised protocol is as follows (Fig. S5†):

(1) The collected cell/sediment samples were mixed with 2 g sterilised sand and then ground three times from freezing to thawing using liquid nitrogen.

(2) The mixture was transferred into a RNase-free tube containing 16.5 mL PIPES extraction buffer and 1.83 mL 20% SDS, mixed well and incubated at 65 °C for 1 h with gentle inversion every 15 min.

(3) After incubation, the mixture was centrifuged at 6000× *g* for 10 min, and the upper supernatant liquid layer was transferred to a new centrifuge tube and extracted with chloroform–isoamylol (v/v = 24 : 1) for 10 min. The mixture was then centrifuged at 6000× *g* for 10 min, and the supernatant liquid containing DNA and RNA was transferred to a new centrifuge tube.

(4) The RNA in crude nucleic acid was first precipitated with 1/4 volume of saturated LiCl for 30 min at −20 °C and collected by centrifugation at 12 000× *g* for 30 min at 4 °C.

(5) After the removal of RNA, DNA in the supernatant was then precipitated with 0.6 volume isopropanol for 30 min at −20 °C and then collected by centrifugation at 12 000× *g* for 30 min at room temperature.

(6) Both isolated DNA and RNA were purified with 70% ethanol and collected by centrifugation at 12 000× *g* for 30 min at room temperature.

(7) The DNA and RNA pellets were air dried for 10–20 min and resuspended with 100–200 μL RNase-free water.

5. Conclusion

Nucleic acid isolation is a key procedure in the molecular analysis of the environmental microbial community. Herein, we reported an optimised method that could simultaneously extract total nucleic acid using a high-salt PIPES buffer (pH 7.0) and separated RNA and DNA by saturated LiCl solution from diverse bioleaching pure cultures (including *A. f*, *A. c*, *A. a*, *L. f* and *F. t*) and AMD samples (including AMD samples taken from the Daye copper mine, Xiangxi gold mine and Axi gold mine). The obtained DNA and RNA could be amplified and also showed high quality ($A_{260/280}$ was around 1.8–2.0, and $A_{260/230}$ was around 1.8–2.2). This method could be widely used in the extraction and separation of DNA and RNA from bioleaching bacteria, providing a solid foundation for AMD microbial molecular analysis and the study of environmental microbial ecology.

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