# Effect of acid production by Penicillium oxalicum on physicochemical properties of bauxite residue

**by** Zhang, Y., Xue, R., He, X., Cheng, Q., Hartley, W. and Xue, S

# Effect of acid production by *Penicillium oxalicum* on physicochemical properties of bauxite residue

Yifan Zhang[a], Rui Xue[b], Xuan He[a], Qingyu Cheng, William Hartley[c], Shengguo Xue[a,*]

[a]*School of Metallurgy and Environment, Central South University, 932 Lushan South Road, Changsha, Hunan, 410083, PR China*

[b]*College of Environmental Sciences, Sichuan Agricultural University, Chengdu 611130, P.R. China*

[c]*Crop and Environment Sciences Department, Harper Adams University, Newport, Shropshire, TF10 8NB, United Kingdom*

#Address correspondence to Shengguo Xue; E-mail address:sgxue@csu.edu.cn.

*Present address: School of Metallurgy and Environment, Central South University (CSU), Changsha 410083, PR China

**ABSTRACT** Large-scale discharge of bauxite residue, an alkaline, saline and nutrient-deficient waste material produced in the process of alumina production, has created extreme environments that are challenging to restore. Microbial pathways are found to play a critical role in the rehabilitation of these residues. In this study, *Penicillium oxalicum*, an alkali-resistant acid-producing fungus screened from bauxite residue disposal sites, was used to examine its effectiveness for restoration of bauxite residue. By comparing different biomass pretreatment methods, steam explosion pretreatment biomass was added to the medium to enhance microbial metabolism, through production of organic acids and various enzymes. *P. oxalicum* mainly secreted oxalic acid, formic acid and acetic acid. Addition of pretreatment biomass and microbes significantly lowered bauxite residue pH, whilst increasing EC and enzyme activity. Furthermore, the metabolic process of this fungus may promote the release of basic ions dominated by $Na^+$ and increase soluble cations. This study provides an experimental demonstration of bioremediation in bauxite residue, and enables future large-scale simulation of vegetation establishment on bauxite residue disposal areas.

**KEYWORDS** Bauxite residue; *Penicillium oxalicum*; Biomass pretreatment; Organic acid; bioremediation

## Introduction

Extreme harsh environments are characterized by combinations of variables, such as high or low temperature, high pressure, drying, high alkalinity or salinity, high acidity, high-intensity ultraviolet (UV) radiation, and poor nutrients (Kaur et al., 2019; Yin et al., 2019). Microorganisms surviving optimally under extreme conditions are considered to be extremophiles, first proposed by Macelroy (1974), whereas those that have special physiological functions are described as functional microorganisms. They form a specific metabolic mechanism in the process of adapting to extreme environments (Sayed et al., 2020). The study of functional microbes is of great significance to understand the ecological functions and biogeochemical cycles of elements in extreme conditions (Chen et al., 2018).

Bauxite residue is a waste material generated from the Bayer process used for alumina refining. It has extremely high alkalinity, salinity, exchangeable sodium percentage (ESP), and fine particle size (Santini et al., 2015). Bauxite residue occupies large land resources and forms an extreme harsh environment, where its pH is likely to cause alkalization of groundwater at the disposal site or the surrounding areas (Jones and Haynes, 2011). The effective restoration of this waste material is therefore of great importance. Previous studies have reported that controlling alkalinity of bauxite residue is key to improving its physical and chemical properties and thus converting it to arable land (Gräfe and Klauber, 2011). A number of methods can be used for bauxite residue restoration, including inorganic acid neutralization (Xue et al., 2016), carbonization,

gypsum modification, seawater neutralization, and bioremediation. Borra et al. (2015) applied seawater and mineral acids (e.g., HCl, $HNO_3$ or $H_2SO_4$) to neutralize the residue, but this was not considered in the eco-engineering scope due to the high costs. In combination with $CaSO_4$, organic matter (OM) (i.e., sugarcane mulch, Lucerne hay) significantly lowered porewater pH from 11.4 to 9.0 in the bauxite residue (You et al., 2019). Microbial activity contributed to pH neutralization in acidic waste material and this is also likely to be the case for alkaline bauxite residue (Santini et al., 2015). Among these neutralization methods, bioremediation through functional microorganisms has received much attention. Microbial restoration may be accomplished by metabolic processes, such as organic and inorganic acids and carbon dioxide production. However, limited research has been reported about the action of functional microorganisms on bauxite residue restoration. Specifically, elucidation of the mechanisms of microbial action on alkaline regulation of bauxite residue which is urgently needed.

Courtney et al., used organic matter to enhance microbial activity in bauxite residue, with its alkalinity decreasing from 13.0 to 7.0 (Courtney et al., 2013). Microorganisms may metabolize acidic substances through various pathways to dissolve a large amount of binding alkali, thereby reducing its alkalinity and salinity. Santini et al. investigated the influence of microorganisms on the alkalinity of bauxite residue and found that microorganisms can secrete organic acids through glucose metabolism, thereby reducing the pH from 9.5 to 6.5 (Santini et al., 2016). Bulk storage of bauxite residue provides an anaerobic environment for microorganisms, enabling

them to metabolize organic acid. Organic acid is a common product after decomposition of organic matter and is due to the ability of some microorganisms to decompose simple organic nutrients and natural polymers, such as cellulose, lignin, and humus (Mesbah et al., 2012); fungal genera such as *trichoderma, fusarium, penicillium, aspergillus, mucor,* and *botrytis* have all been shown to decompose cellulose (Adsul et al., 2007). Organic wastes like hay or wood chips have also been used to replace glucose for microorganisms to produce organic acids, thereby neutralizing bauxite residue pH (Salomskiene et al., 2019).

*Penicillium oxalicum* has been screened from bauxite residue, revealing both alkali-tolerance and acid production (Liao et al., 2018). *P. oxalicum* has been observed to decrease pH to 3.6 after 5 days of culture, producing approximately 4000 mg/L of organic acids (Li et al., 2016). *P. oxalicum* was also shown to enhance the degradation efficiency of lignocellulosic materials (Du et al., 2017). *P. oxalicum* has a relatively complete cellulase production enzyme system and can degrade cellulose into small molecules of sugar for its own growth and metabolism, while producing energy. However, the effect of this functional fungus on bauxite residue restoration remains unclear and requires further investigation.

In addition, the remediation process of bauxite residue be closely correlated with the change of enzyme activity. The soil enzymes are one of the most active organic components in soil, and promote the metabolic process of soil (Whiffin et al., 2007).

Cellulase, urease, protease, phosphatase and other enzymes are widely found in soil. Therefore, the assessment of soil enzyme should be taken into account for remediation of bauxite residue.

Giving that *P. oxalicum* can secrete organic acids and decompose biomass, it was used in this study for the alkaline regulation of bauxite residue. Organic wastes (bagasse and bran) was used as a carbon source from the practical operation point of view. The difference in acid production by microorganism under different biomass pretreatment methods was examined. The results provide a new approach for the restoration of bauxite residue and converting it to arable land.

## Materials and methods

### *Collection of samples*

In July 2016, fresh bauxite residue samples (<3 years) were collected from a bauxite residue disposal site in Guangxi province (108°18′~107°53′E, 23°12′~23°54′N), while biomass (bagasse and bran) samples were obtained from a local sugar refinery. The average annual rainfall and temperature in the sample collection region were 1359 ± 50 mm and 11–17 °C, respectively, which is typically subtropical. The collected samples were air dried and ground, and passed through a 2.0 mm sieve. The samples were then placed in sealed bags for later use. Physicochemical properties were as follows; pH 10.43, EC 2.11 mS/ cm, ESP 58.89%, CEC 339.49 cmol/kg, and exchangeable Na concentration $2.07 \times 10^3$ g/kg, respectively (Tian et al., 2019).

122 ***Biomass pretreatment***

123 Steam explosion pretreatment, acid pretreatment, alkaline pretreatment and hydrogen
124 peroxide pretreatment were adopted for biomass (bagasse:bran = 2:1) pretreatment. The
125 pretreatment process was carried out according to Kumar et al., (2009). Before
126 pretreatment, bagasse and bran were crushed by a pulverizer, and then passed through
127 a 0.25 mm nylon sieve. During the steam explosion pretreatment, biomass was
128 autoclaved at 180 °C, after which the pressure was released suddenly. For acid
129 pretreatment, 1% (w/w) $H_2SO_4$ solution was mixed with biomass then treated with high-
130 pressure steam at 121°C for 1 hour. For alkali pretreatment, each gram of biomass was
131 mixed with 0.075 g calcium hydroxide, and the mixture was heated for 4 h at 120 °C.
132 During hydrogen peroxide pretreatment, 5% (w/w) $H_2O_2$ solution was mixed with
133 biomass and treated at 30 °C for 24 h in a water bath shaker. The solid-to-liquid ratio
134 of each method was 1:10.

135 Biomass medium was prepared by mixing the treated biomass with distilled water.
136 A freeze-preserved strain of *Penicillium oxalicum*, EEEL01, was inoculated into the
137 medium and a blank control was used without inoculation. The pH which revealed acid-
138 production capacity, was determined after 9 days of incubation in a shaker at 28 °C at
139 160 r/min. The acid-producing effects of *P. oxalicum* under different methods were
140 compared to select an optimal biomass pretreatment method.

141

142 ***Analysis of organic acids by HPLC***

143 *P. oxalicum* was inoculated into a glucose medium (glucose 1.0 g, peptone 0.5 g, NaCl
144 concentration 0.8%, initial pH 9.0, distilled water 50 mL, abbreviated as GM) and a
145 biomass medium (bagasse 1.0 g, bran 0.5 g, distilled water 50 mL, abbreviated as BM),
146 respectively. The medium was cultivated in a shaker at 28 °C for 7 days at 160 r/min,
147 and the filtrate of the medium was then analyzed by high performance liquid
148 chromatography (HPLC).

149 Standards and samples were analysed using a Hypersil C18 chromatographic
150 column (250 mm × 4.6 mm id, 5 m), and 0.2% $H_3PO_4$ buffer (pH = 2.6) as the mobile
151 phase. The injection volume was set to 10 μl and the column oven temperature to 25 °C.
152 The UV detection wavelength and gradient elution flow rate were 210 nm and 0.5
153 mL/min, respectively. The standard single organic acid solution (2 mg/mL) and
154 standard mixed organic acid solution (including oxalic acid, formic acid, malic acid,
155 acetic acid, citric acid, propionic acid, butyric acid and pentanoic acid) were filtered by
156 a 0.22 m filtration membrane, and the peak sequence and time of elution of the various
157 organic acids was determined by HPLC.

158 The fermentation broth of the glucose medium (GM) and biomass medium (BM)
159 were collected. For preliminary filtration, 80 μm filter paper was used to remove the
160 bacteria. An appropriate amount of filtrate was taken and centrifuged at 10000 rpm for
161 10 min. The supernatant was mixed with acetonitrile solution at a ratio of 1:3 and placed
162 in the sample bottle. Organic acids in the samples were analysed by HPLC. The

concentration of samples was calculated to determine the content of organic acids:

$$Cx = Cr \times (Ax/Ar) \times 100\% \quad (1)$$

Where Cx is the sample solution concentration, Cr is the standard solution concentration, Ax is the sample solution peak area, and Ar is the standard solution peak area.

***Neutralization of bauxite residue***

The experiment was replicated three times and included three groups, 1) 500 g of bauxite residue and 50 g of biomass (bagasse:bran = 2:1) mixed in a plastic container and inoculated with *P. oxalicum* regularly (once every 5 days, abbreviated as RF, 2) 500 g of bauxite residue and inoculated with *P. oxalicum* regularly, named as BF, 3) 500 g of bauxite residue without any treatment, abbreviated as CK. Bauxite residue was air-dried to a constant weight after the 6th, 12th, 18th, 24th, 30th and 36th day. Bauxite residue (5 g) was mixed with 50 mL of distilled water, and then filtered to obtain the supernatant for determiing pH, EC and soluble $Na^+$, $K^+$, $Ca^{2+}$ and $Mg^{2+}$ concentrations (ICP-AES, Optima 5300DV, American Perkin Elmer company) every 6 days. The urease content in bauxite residue was determined by Qin et al., (2010), and the cellulase content was determined by 3, 5-dinitrosalicylic acid assay (Nannipieri et al., 2012).

### *Data analysis*

SPSS 19.0 was used for data analysis, and Origin 8.0 for data fitting and image processing. ANOVA was used to analyze changes in bauxite residue properties under different treatments. Near-side X-ray absorption spectroscopy (NEXAFS) analysis was performed on a BL08U1A beamline at the Shanghai Synchrotron Radiation Facility (SSRF, Shanghai, China).

## Results and discussion

### *Optimum biomass pretreatment*

Figure 1 reveals pH changes after 9 days of inoculation with *P. oxalicum* in the medium. In comparison with CK treatments, pH decreased significantly after steam explosion pretreatment and acid treatment, while no significant changes could be found for pH in BM following alkaline or hydrogen peroxide pretreatments. This indicated that *P. oxalicum* had a relatively strong capacity to produce acid by the first two pretreatments which enabled them to use bagasse and bran for spontaneous growth and metabolism. However, in the acid pretreatment, toxicity, corrosiveness of equipment, recovery of acid, and the production of fermentation inhibitors such as furan-type inhibitors, prevented widespread application of this method. By contrast, steam explosion pretreatment hydrolyzed hemicellulose, transformed lignin at high temperature, and

improved the efficiency of microbial metabolism using biomass (Haghighi Mood et al., 2013). Therefore, steam explosion pretreatment was used as an optimal method to pretreat biomass in the following experiments.

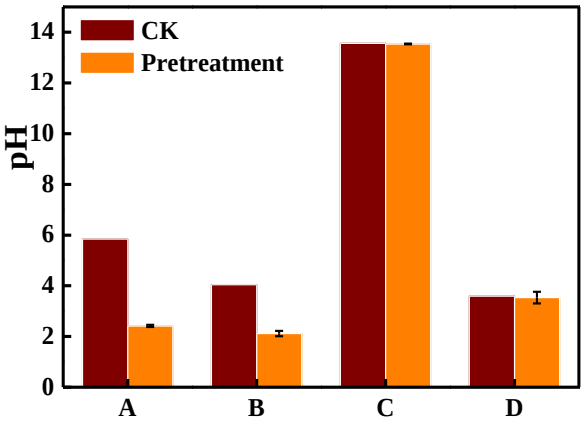



**Figure 1** Comparison of pH in biomass medium with different pretreatments (A: Steam explosion pretreatment; B: Acid pretreatment; C: Alkaline pretreatment; D: Hydrogen peroxide pretreatment)

### *Acid production by fermentation*

Pure compounds of different acids were used as standards to identify components. By injecting standard solutions and comparing retention times, it was possible to identify organic acids types. The type of organic acid used as standard was selected based on the acids reported in *P. oxalicum*, therefore, formic acid, malic acid, acetic acid, citric acid, propionic acid, oxalic acid, butyric acid and valeric acid were selected. Table 1

describes the specifications obtained from the HPLC specimen of standard acids. The retention time for oxalic acid, formic acid, malic acid, acetic acid, citric acid, propionic acid, butyric acid and valeric acid were approximately 3.522 min, 3.849 min, 4.191 min, 4.330 min, 4.885 min, 14.10 min, 19.62 min, and 25.60 min respectively, which is consistent with the peak sequence of organic acids reported in other studies (Krishna et al., 2005).

**Table 1** HPLC data for organic acids

| Types of acid | Molecular formula | Retention time (min) | Peak area (mAU) | Peak height | Solution concentration（mg/mL） |
|---|---|---|---|---|---|
| oxalic acid | $C_2H_2O_4$ | 3.522 | 8011809 | 514182 | 8.88 |
| formic acid | $CH_2O_2$ | 3.849 | 1841116 | 233993 | 2.10 |
| malic acid | $C_4H_6O_5$ | 4.191 | 276017 | 26464 | 10.01 |
| acetic acid | $C_2H_4O_2$ | 4.330 | 973983 | 183686 | 1.93 |
| citric acid | $C_6H_8O_7$ | 4.885 | 532011 | 82843 | 10.02 |
| propionic acid | $C_3H_6O_2$ | 14.10 | 2267036 | 129292 | 1.99 |
| butyric acid | $C_4H_8O_2$ | 19.62 | 1501711 | 137017 | 2.05 |
| valeric acid | $C_5H_{10}O_2$ | 25.60 | 919513 | 80843 | 1.85 |

After 7 days of culture, chromatographic analysis of the organic acids in GM and BM were obtained (Figure 2). Components with retention times of 3.51 min, 3.83 min, 4.30 min are the main compositions in the medium due to height and surface area. By comparing the data presented in Table 1 for pure compounds with the results presented

227 in Figure 2, under both culture conditions, *P. oxalicum* could secrete different
228 concentrations of oxalic acid, formic acid and acetic acid, accompanied by the
229 production of some secondary metabolites. Studies have shown that this fungus can
230 metabolize organic acids dominated by oxalic acid, and the types and content of acid
231 are strongly related to carbon, nitrogen and phosphorus sources (Peng et al., 2017). The
232 carbon and nitrogen source have much more effect on the secretion of organic acids by
233 *P. oxalicum* than phosphorus. Gong and co-workers found that the nitrogen source
234 could directly affect the pathway of acid production of *P. oxalicum*, and it mainly
235 secreted malic acid, acetic acid, propionic acid, citric acid and succinic acid when
236 ammonium nitrogen was provided (Gong et al., 2014).

237 When using pretreatment biomass as a carbon source for the growth and
238 metabolism of *P. oxalicum*, oxalic acid, formic acid and acetic acid secreted were 0.12
239 mg/mL, 0.51 mg/mL and 0.31 mg/mL, respectively. The production of organic acids is
240 directly related to the reduction of the medium pH and provides a basis for its
241 subsequent use in neutralizing alkali in bauxite residue. In this study, the content of
242 acetic acid produced by *P. oxalicum* in either GM or BM was relatively high. These
243 results indicate that this functional fungus has the capacity to metabolize acid by using
244 pretreated biomass as a carbon source. In addition, pretreated biomass instead of
245 glucose for the growth of *P. oxalicum* can greatly reduce the practical application cost.
246 Its acid production capacity is related to the molecular structure of carbon and nitrogen
247 sources. In this study, the molecular structure of bagasse and bran is relatively complex.

The microorganism needs to metabolize and produce cellulase to degrade biomass into small molecular substances so as to provide for its own growth and acid production (Roberts et al., 2015).

Some components were exposed after the time of 15 min but were not found in the standards. It can be assumed that *P. oxalicum* can also metabolize other acidic substances. This functional fungus can produce secondary metabolites (such as secalonic acid A), and the unknown components in Figure 2 may be secondary metabolites (Wang et al., 2013). They were not components of interest, and further studies are required to identify these compounds that are beyond the scope of this study.

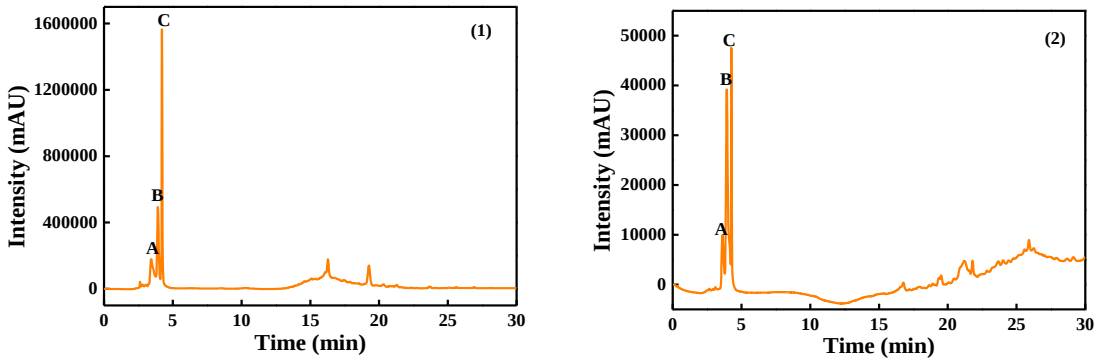
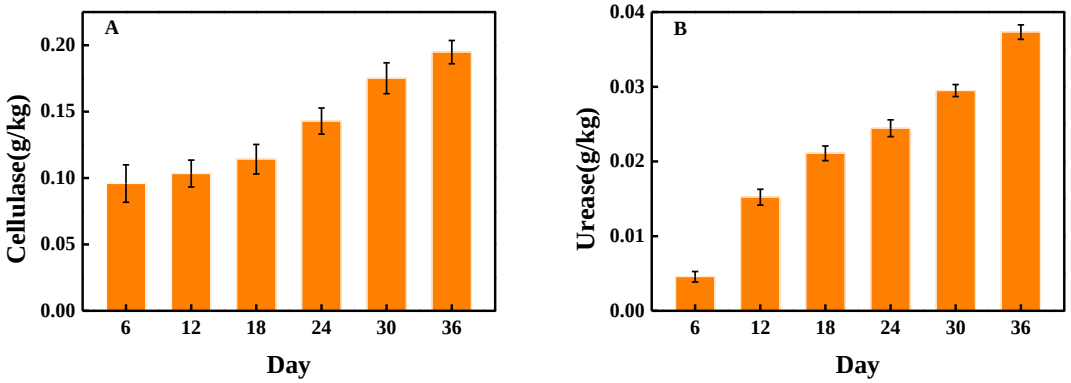



**Figure 2** Organic acid secretion by *Penicillium oxalicum,* (1)GM, (2) BM (A-oxalic acid, B-formic acid, C-acetic acid) as determined by HPLC.

### *Cellulase and urease activity*

Inoculation of *P. oxalicum* had little effect on enzyme activity in bauxite residue, while enzyme activity increased with extension of culture time under the addition of biomass. This illustrates that biomaterial addition leads to the increase of enzyme activity, which is consistent with former experimental results in our laboratory (Liao et al., 2019). Urease activity and cellulose enzymes were below detection limits of RF and CK and not shown in Figure 3. Cellulase activity in bauxite residue significantly increased after 24 days, and reached the maximum value (0.19g /kg) after 36 days of culture (Figure 3). Urease activity determined the conversion efficiency of organic nitrogen to available nitrogen and the supply level of inorganic nitrogen in the soil, which mainly comes from microorganisms and plants (Roscoe et al., 2000). Urease activity in bauxite residue increases with the extension of culture time, but the activity was not high.

*P. oxalicum* can degrade biomaterial and promote carbon cycles because of its high cellulase production ability. Cellulase production from sugarcane bagasse pretreatments and pure synthetic substrates has been studied showing that optimal cellulase enzyme production was at pH 4.9 and between 52 to 58 °C (de Castro et al., 2010). It has also been shown that CMC enzyme activity may reach 31.12 IU/mL under optimal enzyme production conditions (Tao et al., 2011). However, the high alkaline environment of bauxite residue in this study restricts the growth and metabolism of microorganisms, therefore having an effect on enzyme activity.




281

282 **Figure 3** Cellulase (A) and urease (B) activities in bauxite residue.

283

284 ***pH, EC and soluble cations***

285 After 36 days, the pH of bauxite residue in RF decreased from 10.9 to 7.2, which was
286 significantly lower than BF (Figure 4). This indicates that the combined action of *P.*
287 *oxalicum* and pretreatment biomass can significantly reduce bauxite residue alkalinity.
288 During 6 to 12 days of culture, bauxite residue pH declined significantly. This may be
289 due to acid production in the early stages of culture being neutralized by free alkali. In
290 the latter period, alkali was slightly released, maintaining the acid-base balance in the
291 whole culture system, so pH decreased slowly. Khaitan believed that the alkaline
292 dissolution process of bauxite residue required at least 50 days to reach the chemical
293 equilibrium under laboratory conditions (Khaitan et al., 2009). Although the pH of
294 bauxite residue may be reduced by microorganisms, the addition of biomass can lower

the pH further. It can be assumed that biomass can promote the activity of *P. oxalicum* for it to continuously produce organic acids, thereby neutralizing more alkaline substances in the residue. It has also been revealed that microorganisms can metabolize acid and reduce the pH at the initial stage of growth in alkaline and saline environments (bauxite residue), but the pH was shown to rise when the microorganisms entered their decline phase (Qu et al., 2013).

Nevertheless, electrical conductivity (EC) of bauxite residue increased with time. After 18 days of cultivation with biomass, EC was stable at approximately 2.18 ms /cm, which was greater than that of microbe action alone in the whole culture process; increase in EC has been shown to be related to the dissolution of basic ions in bauxite residue (Kong et al., 2017). Other investigations have shown that EC increased with time, probably due to accelerated hydrolysis of sodium-rich minerals in the residue.

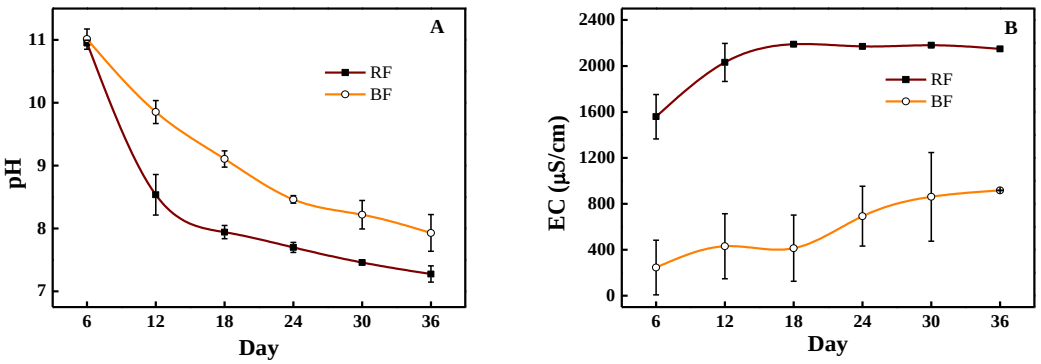



**Figure 4** Comparison of pH (A) and EC (B) in bauxite residue with different treatments

309

310 Soluble $Na^+$ content in bauxite residue increased gradually with time, leading to
311 an increase in EC (Figure 5). Studies had shown that soluble $Na^+$ content represented
312 the concentration of Na buffer substances in bauxite residue (Kong et al., 2017). The
313 metabolic process of *P. oxalicum* may promote the release of metal ions dominated by
314 $Na^+$ and increase the content of soluble cations. With the addition of biomass and fungus,
315 the content of $Ca^{2+}$ in bauxite residue increased slowly after 24 days of cultivation,
316 while $K^+$ and $Mg^{2+}$ showed no significant changes. Soluble $Na^+$ increased when *P.*
317 *oxalicum* acted alone in bauxite residue, but the amendment effect on $K^+$, $Ca^{2+}$ and
318 $Mg^{2+}$ was not obvious during the whole culture process. It was observed that the
319 addition of biomass in bauxite residue significantly improved the soluble cation content,
320 and $Na^+$ content in the supernatant of bauxite residue increased by one-fold (Kong et
321 al., 2018).

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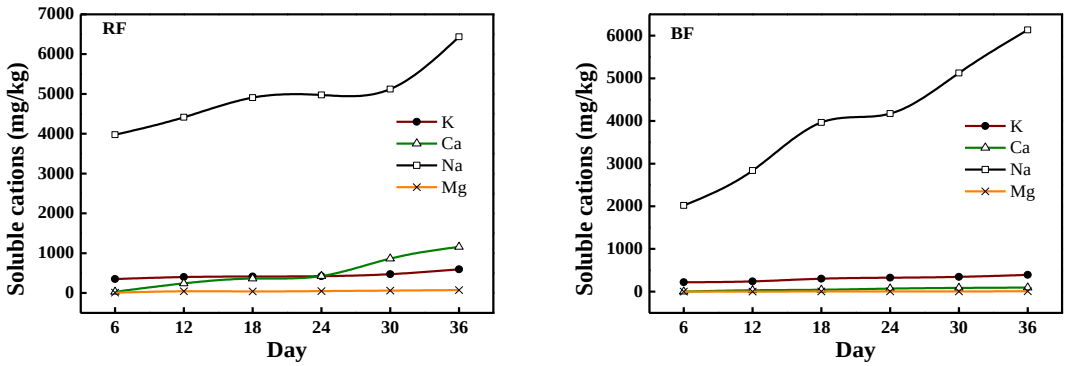
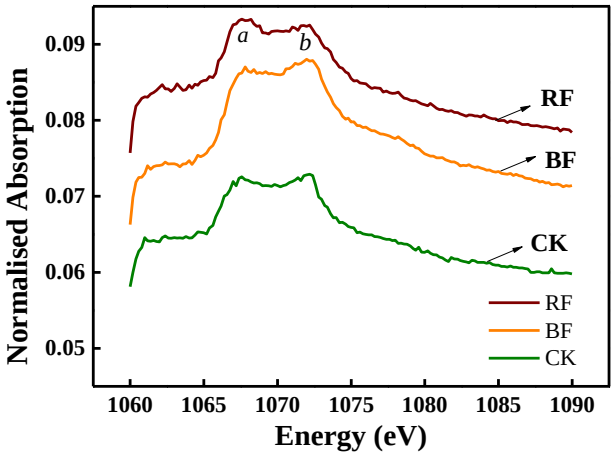


**Figure 5** Soluble cation concentrations in bauxite residue with different treatments

## *Morphology characteristics*

Na K-edge X-ray absorption near edge structure (XANES) spectra of bauxite residue has two prominent absorption peaks *a* and *b*, near 1068.2 and 1072.0 eV (quoted to ±0.2 eV) (Figure 6), and the peak positions determine the coordination structure of Na in bauxite residue. XANES analysis of Na K-edge indicated that two prominent absorption peaks *a* and *b* from RF and BF, were almost uniform and similar to CK. In this study, the local ordering around Na and the chemical morphology of calcium nephrite ($Na_8Al_6Si_6O_{24}(CO_3)(H_2O)_2$), and sodium quadrate ($Na_8Al_6Si_6O_{24}Cl_2$), did not change in the residues . The effect of *P. oxalicum* combined with pretreatment biomass widened the main spectral peaks in the residue, and the displacement of peak *a* to the high energy position was about 0.1-0.3eV. Absorption peaks of RF and BF in residues was consistent with CK, proving that the two treatments did not transform its chemical speciation. The normalized strength of bauxite residue under the action of *P. oxalicum* was higher than that of the original bauxite residue, which is similar to the result of Kong directly treating bauxite residue with organic acids (Kong et al., 2017).

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340 **Figure 6** Normalized Na K-edge XANES spectra collected from bauxite residue, transformed residues

341 by different methods

342

343 ## Conclusions

344 This work presents evidence for the bioremediation of bauxite residue using

345 pretreatment biomass as a carbon source, in order to reduce its pH and EC, increase

346 cellulase and urease activity, and attempt to change the physicochemical properties of

347 bauxite residue. The biomass after steam explosion pretreatment was found to

348 significantly promote a decline in medium pH owing to acid production by *P. oxalicum*.

349 Organic acids metabolized by microbes in the biomass medium was consistent with the

350 glucose medium, including oxalic acid, formic acid and acetic acid. *P. oxalicum* may

351 also stimulate cellulase and urease activity in bauxite residue. In addition, inoculation

352 of the functional fungus with the addition of biomass significantly reduced the

353 alkalinity of bauxite residue, while EC increased with time, probably due to the

dissolution of basic ions. It was also found that the metabolic process of the fungus could increase $Na^+$. Absorption peaks of different treatments in residues was almost uniform and similar, proving that the microbe and biomass did not transform its chemical speciation. *P. oxalicum* is an important fungus in neutralizing alkalinity of bauxite residue by producing organic acids, and bioremediation may be considered as a promising way forward for the effective restoration on bauxite residue disposal areas.

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