

## Article

# A Newly Acidophilic Bacterium *Acidomyces acidothermus* Was Isolated to Efficiently Bioleach Copper from Waste Printed Circuit Boards (WPCBs)

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**Abstract:** An acidophilic metal-resistant bacterial strain, *Acidomyces acidothermus* (*A. acidothermus*), was isolated and identified by morphology, physiology, biochemistry, and 16S rDNA. *A. acidothermus* culture conditions were optimized by response surface methodology (RSM). Results showed that with a temperature of 40 °C, a pH of 3 in a 9k medium, and a rotation speed of 140 r/min, the copper leaching rate reached the highest value of 39.8%. Furthermore, SEM images and a heatmap of differential metabolites indicated that *A. acidothermus* adsorbed on the surface of WPCBs and extracellular polymeric substances (EPS), mainly D-glucuronic acid, were secreted, suggesting the highly efficient mechanism of copper recovery from WPCBs.

**Keywords:** *Acidomyces acidothermus*; culture optimization; bioleaching mechanism



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## 1. Introduction

With the rapid development of information technology, large amounts of waste electrical and electronic equipment (WEEE) are being produced. According to the 2020 Global Electronic Waste Monitoring report released by the United Nations, the global electronic waste output reached 53.6 million tons in 2019, of which China was the largest producer at 10.129 million tons. Printed circuit boards have been found in almost all WEEE. Waste printed circuit boards (WPCBs) composed of polymers, inert oxides, and metals, which have a high resource value, are the most difficult type of electronic waste to dispose of. The metal content is up to 40% (20–30% copper, 5–6% zinc, 3–4% lead, 1–2% nickel, 0.5–0.9% silver, gold 0.04–0.07%, etc.) [1,2]. Obviously, improper treatment of WPCBs would cause serious harm to the environment.

At present, the commonly used methods for extracting metals from WPCBs include mechanical and physical methods [3], fire methods [4], and hydrometallurgy [5]. Mechanical and physical methods have a high evaluation of the effects of renewable resources, environmental friendliness, and industrial application rate, but the equipment used has a high investment and high energy consumption, and it is difficult to separate various metals in the products, so the economic feasibility is relatively poor [6,7]. The fire method has the advantages of simplicity, convenience, and a high recovery rate, but it also has many disadvantages: it produces a large amount of harmful gases (such as dioxins, etc.), the recovery rate of lead, tin, and other nonferrous metals is low, and a large number of nonmetallic components are lost in the incineration process [8,9]. The wet process has the advantages of less exhaust gas emissions and a simple process but requires a strong acid to dissolve, so the leaching solution and residue are highly toxic [10,11].

The biological treatment of recycled WPCB metals has the advantages of low energy consumption and less environmental pollution, but it is still in the experimental stage. Biological treatment of WPCBs began in the 1980s, and metals could be recovered by oxidation, reduction, dissolution, adsorption, and other methods through the interaction of microorganisms or their metabolites with metals [12]. At present, the microorganisms used to treat WPCBs include *Acidithiobacillus ferrooxidans* (*A. ferrooxidans*), *Acidithiobacillus thiooxidans* (*A. thiooxidans*), *Aspergillus niger*, *Penicillium*, cyanogen bacteria, etc. [13]. *A. ferrooxidans* and *A. thiooxidans* have been widely used in bioleaching technology [14]. However, as *A. ferrooxidans* and *A. thiooxidans* competed with each other for nutrient substrates, strict conditions (low pH and high reduction potential) needed to be maintained to promote balanced growth of the bacterium, thus enhancing metal leaching by WPCBs [15]. Most fungal strains, such as *Aspergillus niger* [16] or *Penicillium chrysogenum* [17], with the ability to yield high amounts of organic acids, were also applied in the bioleaching of WPCBs. Faraji et al. revealed that *Aspergillus niger* was capable of the simultaneous leaching of 100% of Zn, 80.39% of Ni, and 85.88% of Cu in 30 days [16]. Nevertheless, long reaction times and unclear leaching mechanisms limit the commercial application of bioleaching. To improve the metal leaching efficiency, the screening and separation of acidophilic metal-resistant bacteria is the key to metal recovery by biological methods.

Accordingly, the objective of this study was to screen for acidophilic metal-resistant bacteria and optimize culture conditions, to explore the leaching of copper from WPCBs under optimal growth conditions. The exploration of the bioleaching mechanism would provide a theoretical basis for improving the efficiency of metal recovery.

## 2. Material and methods

### 2.1. Experimental Materials

The main reagent used in the experiment, such as LB medium,  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{K}_2\text{HPO}_4$ ,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , KCl,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{Ca}(\text{NO}_3)_2$ , sulfur,  $\text{H}_2\text{SO}_4$ , NaOH, Agar powder, were purchased from Sinopharm Chemical Reagent Co., LTD. (Shanghai China)

WPCBs, without any physical or mechanical separation, were collected from an electronic waste recycling company in Changzhou. For experimental use, WPCBs were further ground by a grinder (YXD-100, Qingdao Yaixin Intelligent Technology Co., Ltd., Qingdao, China) and sieved to obtain a mesh size of 100–200  $\mu\text{m}$ .

The activated sludge (~3 g/L) was obtained from a municipal sewage treatment plant in Changzhou.

### 2.2. Microbial Screening and Isolation

Screening and acclimation of bacterial strains: A total of 110 mL of sludge and 1 g of waste circuit board were placed into 90 mL of LB liquid medium (pH = 3) and incubated at 30 °C at 140 r/min in an oscillatory incubator (ZHLY-300S, Shanghai Zhichu Instrument Co., Ltd., Shanghai, China). After five days, the inoculum was inoculated into fresh liquid medium with a volume ratio of 10%, and the culture was repeated four times. Enrichment of acidophilic metal-resistant bacteria liquid was obtained.

Strain isolation: after repeated domestication and culture, the bacterial liquids was diluted and inoculated on the acidic LB solid medium plate (pH = 3) at 30 °C in a constant temperature incubator (DHP, Shanghai a constant scientific instrument co., LTD, Shanghai, China). After 48 h of training, single colonies of different shapes and colors were selected and incubated on fresh acidic LB solid medium plates. After seven days of culture at 30 °C in a 100 r/min shaking incubator, 5 mL of culture medium was collected and cultured in fresh LB solid medium. The above separation operation was repeated six times.

For strain purification, took the above culture solution and dilute it in a gradient of  $10^{-1}$  to  $10^{-8}$ , coating on acid LB solid medium (pH = 3), and inverted culture for 1–7 days. The growth of colonies was observed every day, and pure single colonies were obtained. The purified strain was stored in a refrigerator at  $-81^\circ\text{C}$  for later use.

### 2.3. Optimization of Culture Conditions

Firstly, a single-factor test was conducted. The pH (0, 0.5, 1.5, 2.0, 3.0, and 5.0), temperature (30, 35, and 40 °C), rotational speed (0, 100, 140, and 180 r/min), and medium (9K medium, LB medium) were changed, respectively, to investigate the growth of the purified strain and determine the corresponding optimum culture conditions.

On the basis of a single-factor test, a response surface test was designed according to Box-Behnken. By establishing a multiple quadratic regression model equation, the relationship between each factor and index in this experiment was approximated by a polynomial equation, and the optimal culture conditions were obtained by response surface graph analysis of the interaction of each factor.

### 2.4. Bioleaching of WPCBs

The purified strain was inoculated into an acid-liquid 9k medium and cultured until the OD<sub>600</sub> value reached 0.8. 1 g of WPCBs was added to different volumes of the cultured bacterial solution, stirred at a speed of 140 r/min. The sample liquid was centrifuged. The supernatant, after high-speed and low-temperature centrifugation, was filtered to obtain the required clear and transparent leaching solution. The concentration of copper ions in waste circuit board wastewater when different amounts of bacteria were added was measured by an inductively coupled plasma mass spectrometer (ICP TQ, Thermo Fisher Scientific Co, Bremen, Germany) after 250 times dilution of the leaching solution.

### 2.5. Analytical Methods

The cell morphology of the purified strain was observed by optical microscope (BX51, Shanghai Xiniu Optical Technology Co., LTD, Shanghai, China). The purified strain was sent to Shanghai Shengong Bioengineering Co., Ltd. (Shanghai, China), for 16S rDNA sequencing to determine the species [18]. The growth of bacteria was expressed as a value of OD<sub>600</sub>. A microplate reader (Infinite 200PRO, Beijing Longyue Biotechnology Development Co., Ltd., Beijing, China) was used to measure the OD<sub>600</sub> value. Scanning electron microscopy (SEM, SU1510, Hitachi, Tokyo, Japan) was used to observe the surface morphology in the residue of WPCBs. The metal content in the residue was observed by X-ray diffraction (XRD, X'PERT POWDER, Panaco, Amsterdam, The Netherlands). The metabolomics were used to analyze the metabolites in the bioleaching process [19].

## 3. Results and Discussion

### 3.1. Biological Characteristics and Identification Results of the Strain

The colonies of the obtained strain were grey-black, round, raised in the middle, hard and difficult to pick, and presented filamentous colonies under the microscope (Figure 1).

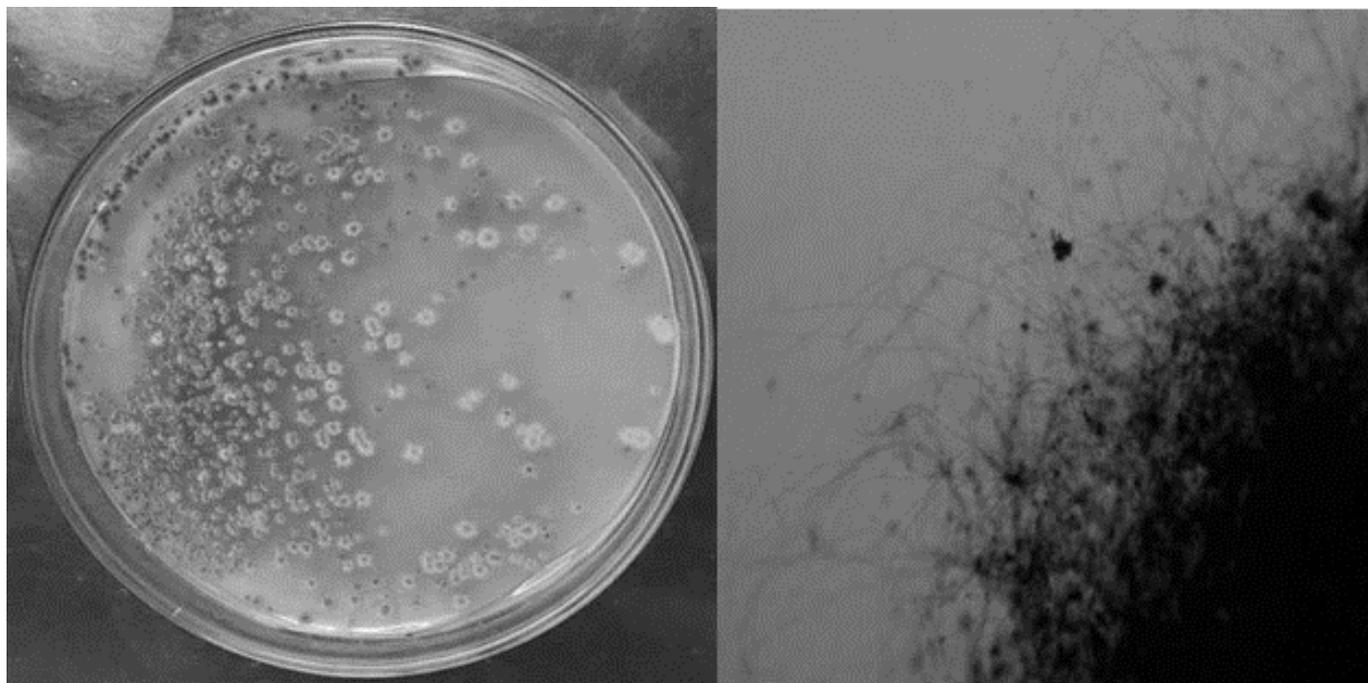
The 16S rDNA gene sequences of the strain were found to have the highest sequence similarity to *Acidomyces acidothermus* (*A. acidothermus*) at 100% in the ribosome database <http://rdp.cme.msu.edu/index.jsp> (accessed 10 May 2021). The *A. acidothermus* has been deposited in China General Microbiological Culture Collection Center with the deposit number of CGMCC No. 22431.

### 3.2. Optimization of Culture Conditions

Rapid cultivation of massive, highly active microorganisms under optimal conditions is important for efficient bioleaching of metals. The growth and reproduction of microorganisms are significantly sensitive to changes in the environment (e.g., nutrients, pH, temperature, dissolved oxygen, etc.).

The medium is the place where microorganisms are supplied with energy and multiply. Different mediums can affect the growth and multiplication of microorganisms due to differences in nutrients or nutrient ratios. The *A. acidothermus* was inoculated into 100 mL of 9k liquid medium and LB liquid medium, respectively, at a volume ratio of 5% and incubated for 48 h at 30 °C and 100 r/min with shaking. As shown in Figure 2A, from 0 h to 12 h, the OD<sub>600</sub> values of the bacterial solution in 9k medium kept increasing, and after

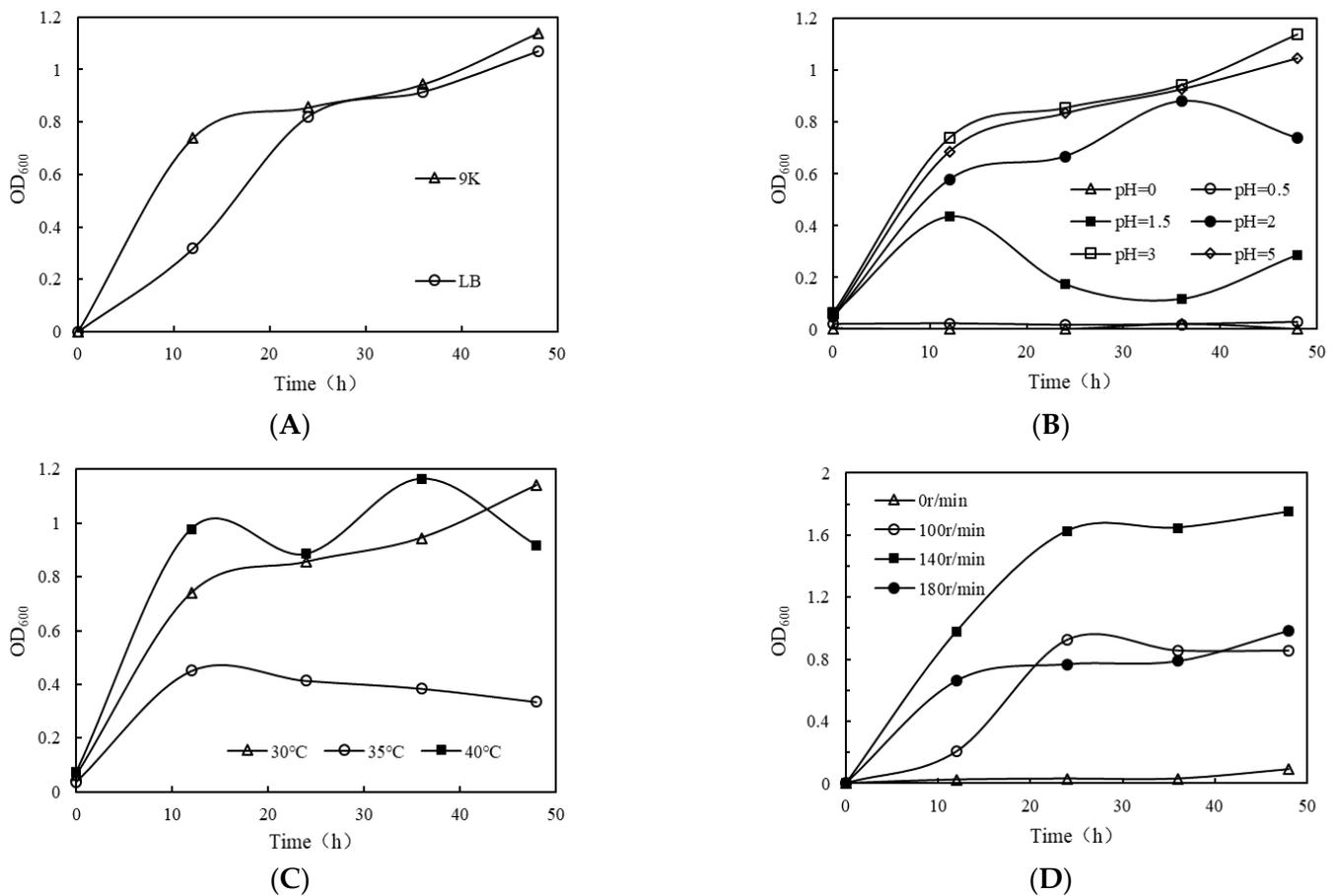
12 h, the OD<sub>600</sub> values tended to be flat. From 0 h to 24 h, the OD<sub>600</sub> values of the bacterial solution in LB medium kept increasing, and after 12h, the OD<sub>600</sub> values tended to be flat. Significantly, the OD<sub>600</sub> value of bacterial solution at 9k was always higher than that in LB. Therefore, the suitable medium is 9k medium.



**Figure 1.** Morphology and cell morphology of the purified strain.

Changes in pH affect not only the activity of the microbial metabolic enzymes but also the absorption of nutrients from the environment. The *A. acidothermus* was inoculated into 100 mL of 9k liquid medium at a 5% volume ratio, with initial pH values of 0, 0.5, 1.5, 2.0, 3.0, and 5.0 and incubated at 30 °C and 100 r/min for 48 h in shock culture. Figure 2B showed that after 12 h of culture, the OD<sub>600</sub> values of the *A. acidothermus* bacterial solution with a pH of 1.5–5 increased rapidly. Moreover, during the whole culture cycle, the OD<sub>600</sub> values of the *A. acidothermus* bacterial solution with a pH of 3–5 always increased, while the OD<sub>600</sub> values of the bacterial solution with a pH of 0–0.5 were always lower than 0.03. It indicated that the *A. acidothermus* was not suitable to grow at a pH less than 0.5. In comparison, the OD<sub>600</sub> value of *A. acidothermus* at a pH of 3 was always higher than that at other pH values, so the optimal initial pH was 3.

Changes in temperature affect the biochemical reactions in microorganisms, thus affecting their metabolic activity. The *A. acidothermus* was inoculated into 100 mL of 9k liquid medium (pH = 3) at a 5% volume ratio, and the initial temperatures were 30 °C, 35 °C, and 40 °C, respectively. The bacterial solution was incubated for 48 h at 100 r/min with shaking. As shown in Figure 2C, from 0 h to 12 h, the OD<sub>600</sub> value of *A. acidothermus* at temperatures ranging from 30 °C to 40 °C continued to rise. At 24 h, the OD<sub>600</sub> value of *A. acidothermus* at 30 °C increased compared with that at 12 h, and the OD<sub>600</sub> value of *A. acidothermus* at 35 °C to 40 °C decreased compared with that at 12 h. However, the OD<sub>600</sub> value of the strain at 40 °C was still higher than that at other temperatures, indicating that the strain grew well under this condition. In comparison, *A. acidothermus* entered the rapid growth phase earlier at 40 °C, so the optimal initial temperature was 40 °C.



**Figure 2.** Growth of *A. acidothermus* under different conditions. (A) Growth of strain in different media. (B) Growth of strain in different pH. (C) Growth of strain in different temperature. (D) Growth of strain in different rotation speed.

Changes in the dissolved oxygen affect the respiration rate of microorganisms and therefore the efficiency of the reaction. The speed of the shaker controls the dissolved oxygen content in the culture medium to a certain extent. The *A. acidothermus* was inoculated into 100 mL of 9k liquid medium (pH = 3) at a 5% volume ratio, and the initial rotation speeds were 0, 100 r/min, 140 r/min, and 180 r/min, respectively. The bacterial solution was incubated for 48 h at 40 °C with shaking. As shown in Figure 2D, the OD<sub>600</sub> value of *A. acidothermus* at a rotation speed of 100–180 r/min increased from 0 h to 12 h. At 100 r/min and 180 r/min, the OD<sub>600</sub> values of *A. acidothermus* were approximately 0.2 and 0.6, respectively, while at 140 r/min, the OD<sub>600</sub> value of *A. acidothermus* reached approximately 0.9. At 24 h, the OD<sub>600</sub> value of *A. acidothermus* increased at all speeds, and the OD<sub>600</sub> value of bacteria increased steadily at 140 r/min. At 36 h and 48 h, the OD<sub>600</sub> value of *A. acidothermus* at 140 r/min was always higher than that at other speeds, indicating that the strain grew well under this condition. In comparison, *A. acidothermus* entered the rapid growth phase earlier under the condition of 140 r/min, so the optimal speed was 140 r/min.

Design-Expert 8.0.6 software was used to optimize the experimental design and analysis of the culture conditions of *A. acidothermus*. The response surface analysis and variance analysis were shown in Tables 1–3, respectively. As shown in Table 1, OD<sub>600</sub> was the response value, and pH (A), temperature (B), and rotational speed (C) were used as independent variables to optimize the culture conditions of the purified strain.

**Table 1.** Response surface test factor levels.

The Level	pH (A)	Temperature (B)/(°C)	Rotational Speed (C)/(r/min)
1	2	30	100
2	3	35	140
3	5	40	180

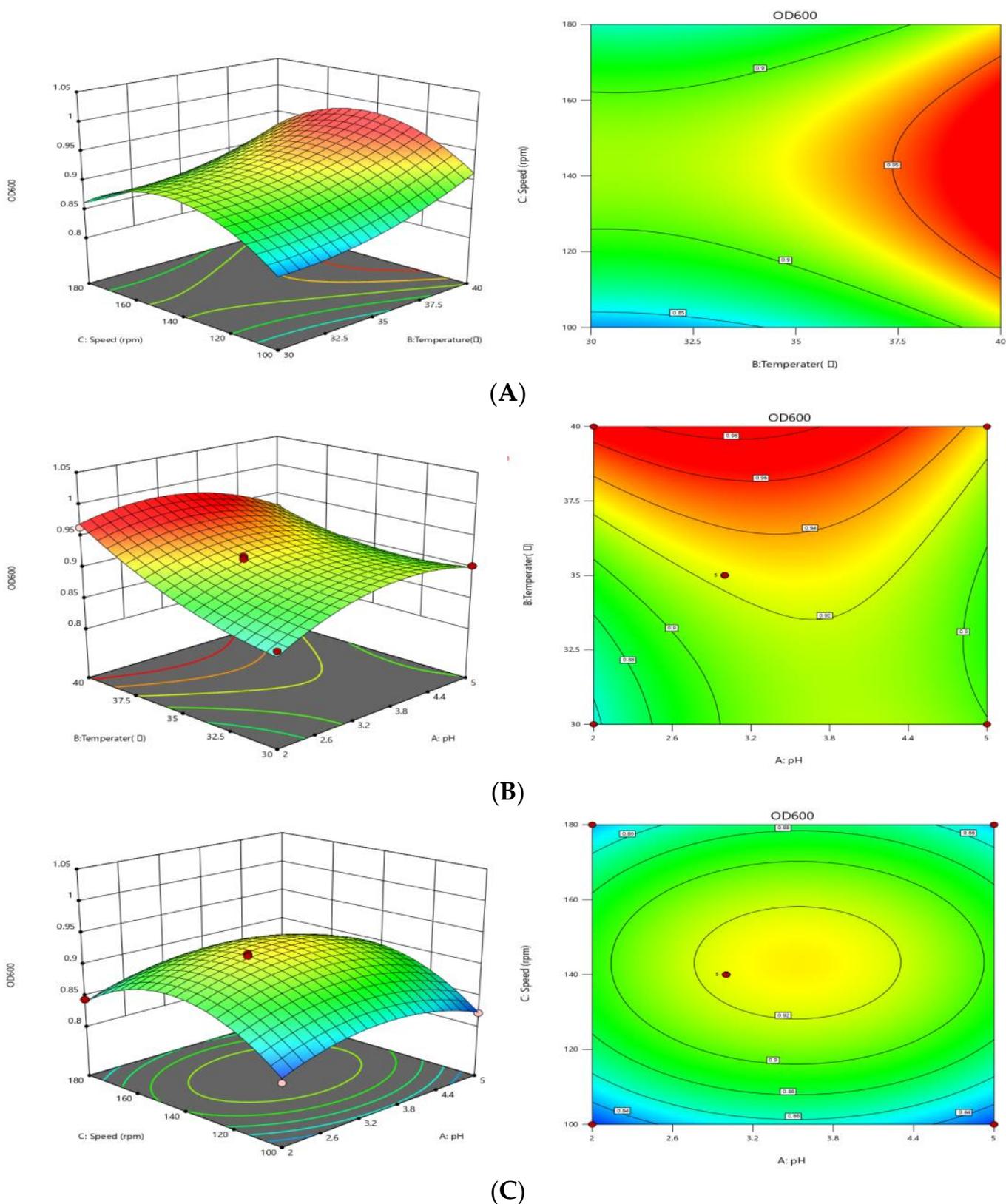
**Table 2.** Sample results of response surface analysis.

Test No.	A	B	C	OD <sub>600</sub>
1	5	30	140	0.9032
2	2	40	140	0.9643
3	3	30	180	0.8413
4	2	35	100	0.8143
5	3	30	100	0.8243
6	5	35	100	0.8216
7	3	40	180	0.9316
8	3	35	140	0.9319
9	3	35	140	0.9274
10	3	35	140	0.9094
11	3	35	140	0.9235
12	3	35	140	0.9291
13	5	35	180	0.8482
14	5	40	140	0.9258
15	3	40	100	0.9263
16	2	35	180	0.8439
17	2	30	140	0.8649

**Table 3.** The variance analysis.

Source	Sum of Squares	DOF	MS	The F Value	p > F	Significance
Model	0.0371	9	0.0041	40.56	<0.0001	significant
A-pH	0.0000	1	0.0000	0.1599	0.7012	
B-Temperature	0.0097	1	0.0097	95.82	<0.0001	
C-r/min	0.0007	1	0.0007	7.36	0.0301	
AB	0.0019	1	0.0019	18.26	0.0037	
AC	$1.663 \times 10^{-6}$	1	$1.663 \times 10^{-6}$	0.0164	0.9018	
BC	0.0000	1	0.0000	0.3368	0.5799	
A squared	0.0035	1	0.0035	34.69	0.0006	
B squared	0.0016	1	0.0016	15.89	0.0053	
C squared	0.0167	1	0.0167	164.29	<0.0001	
Residual	0.0007	7	0.0001			
Lack of Fit	0.0004	3	0.0001	1.70	0.3045	not significant
Pure Error	0.0003	4	0.0001			
Cor Total	0.0378	16				

As seen from Table 3, the Model  $p < 0.0001$  indicates that the model is highly significant, and the misfit term  $p = 0.3045 > 0.05$  indicates that the difference is not significant. The calculation of  $R^2$  was 0.9812, which indicates that the model is well correlated. From the analysis results of the F value, it can be seen that the order of influence of each factor on the OD<sub>600</sub> value is temperature, rotational speed, and pH. The interaction between the optimal conditions and parameters can be clearly seen from the response surface analysis diagram. The interaction between the elliptic shape is the best, and the circular shape is the worst [20]. Figure 3 showed that the interaction between pH and rotational speed was the strongest, while the interaction between pH and temperature was the weakest.



**Figure 3.** Response surface curves. (A) Response surface and contour of temperature and rotation speed. (B) Response surface and contour of temperature and pH. (C) Response surface and contour of pH and rotation speed.

After fitting the data, the multiple quadratic regression equation was obtained:

$$OD_{600} = 0.9285 + 0.0014 + 0.0358 + 0.0099 C \text{ to } 0.0210 AB + B 0.0006 AC - 0.0029 - 0.0335 - A BC^2 + 0.0196 B^2 - 0.0630 - C^2.$$

According to the model prediction, when the pH is 3.33374, the temperature is 39.8628 °C, the rotation speed is 147.857 r/min, and the OD<sub>600</sub> value reaches the maximum value of 0.9824. The optimized conditions of the model are similar to those of the single-factor experiment. Considering the practical reasons, a pH of 3, a temperature of 40 °C, and a rotation speed of 140 r/min were selected to verify the predicted value. The results of the predicted value and the actual value were essentially the same. This shows that the regression equation can compare the real reaction of the influence of various factors on OD<sub>600</sub> [21].

### 3.3. Bioleaching of WPCBs

Under optimized culture conditions, 1g of WPCBs was put into different volumes of *A. acidothermus* solution (10, 20, 30, 40, 50, and 100 mL), respectively. The leaching effect of the volume of the *A. acidothermus* solution on copper in WPCBs is shown in Table 4.

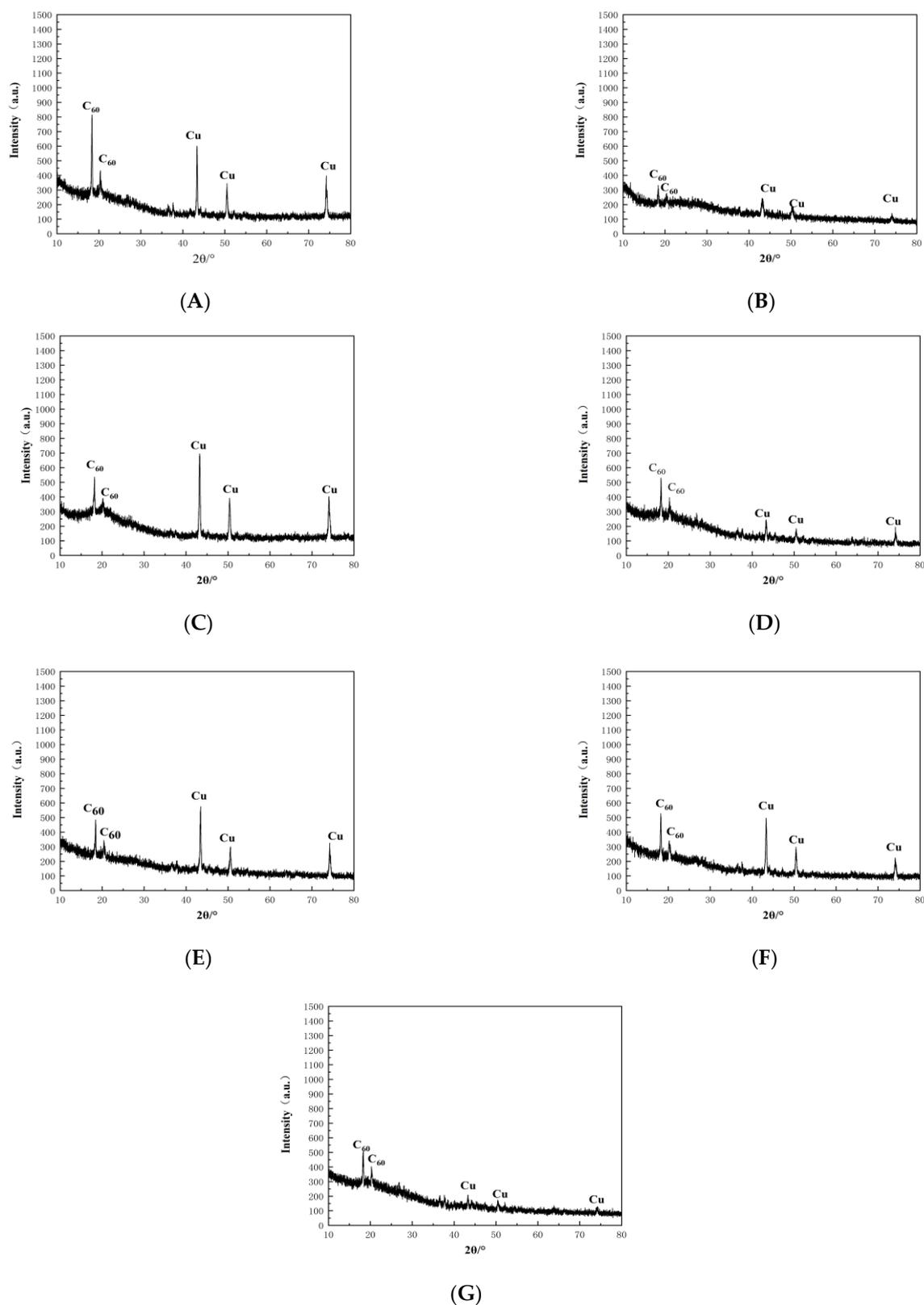
**Table 4.** Copper leaching rate.

Time/(h)	Copper Leaching Rate (%)					
	10 mL	20 mL	30 mL	40 mL	50 mL	100 mL
1	0.6 ± 0.2	1.6 ± 0.1	3.5 ± 0.2	3.7 ± 0.1	7.5 ± 0.1	6.4 ± 0.2
2	1.8 ± 0.1	0.9 ± 0.1	1.2 ± 0.1	3.2 ± 0.2	3.2 ± 0.1	5.2 ± 0.1
4	7.1 ± 0.1	17 ± 0.2	37.9 ± 0.1	29.6 ± 0.1	19.6 ± 0.2	39.8 ± 0.1
8	2.8 ± 0.1	5.6 ± 0.2	11.1 ± 0.1	12.2 ± 0.2	17.3 ± 0.1	32.9 ± 0.2

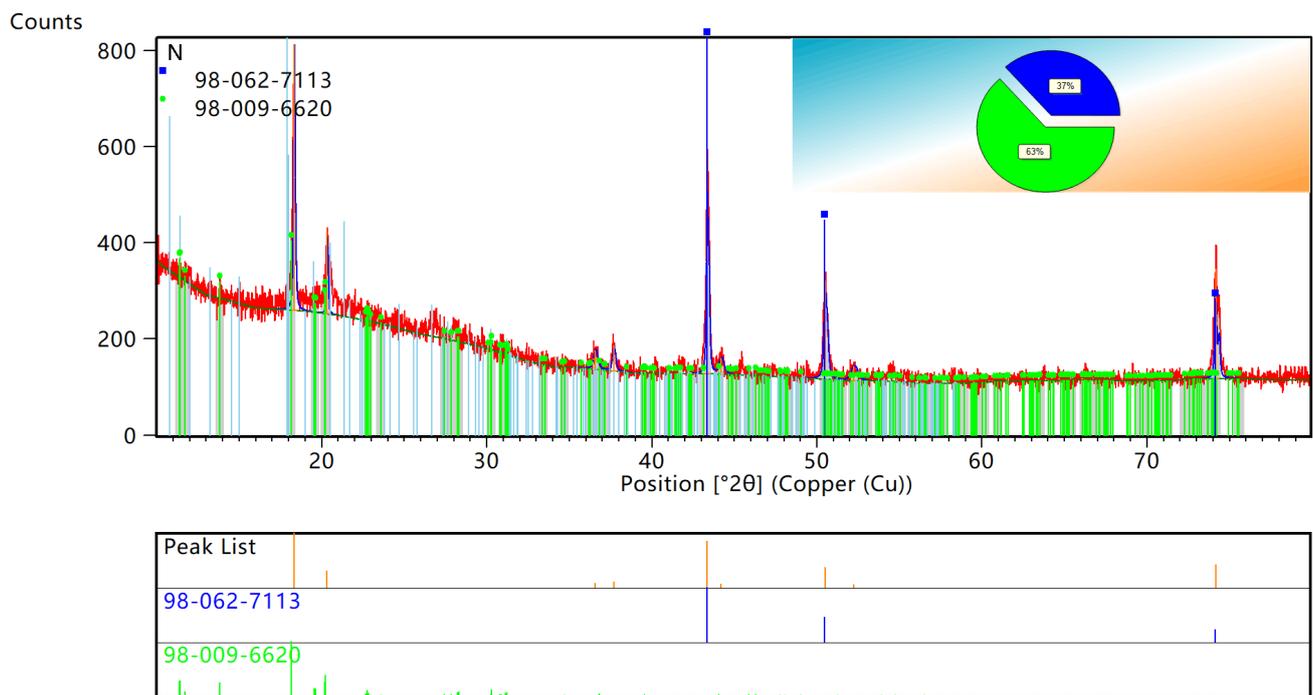
Table 4 exhibited that the initial bacterial liquid product for 10, 20, 30, 40, 50, and 100 mL, under the condition of acid and heavy metal, the copper leaching rate of *A. acidothermus* over time increased first, then decreased, and at the age of 4 h, the leaching rate was highest. This may be that the microbes in the logarithmic growth phase into environment containing waste circuit board, when the strains thrive in the first 4 h. The strain grew vigorously during the first 4 h, and the leaching rate was high. After 4 h, the leaching rate decreased. This might be because secondary precipitation occurs during the decomposition of WPCBs, and the released elements might be immobilised by the precipitation and enter the solution in lower amounts. [22]. Moreover, Rogers et al. pointed out that the WPCBs might contain nutrients needed for bacterial metabolism [23]. It could be inferred that part of the copper ions leached from WPCBs were absorbed by *A. acidothermus* for growth and reproduction. As the concentration of the *A. acidothermus* bacterial solution increased, the leaching rate increased. The leaching rate of 100 mL of bacterial solution was the highest, up to 39.8 ± 0.1%. Due to the high concentration of bacterial solution, the number of microorganisms and secreted metabolites increased, and the adsorption of microorganisms on the surface of WPCBs was promoted to improve the leaching rate [24].

After 4 h of leaching of *A. acidothermus* WPCBs, the residue in the bacterial solution was removed, baked in an oven at 70 °C for 24 h, and ground into a powder. The XRD was scanned continuously from 10° to 80° with a step distance of 0.00657° to obtain the XRD pattern (Figure 4).

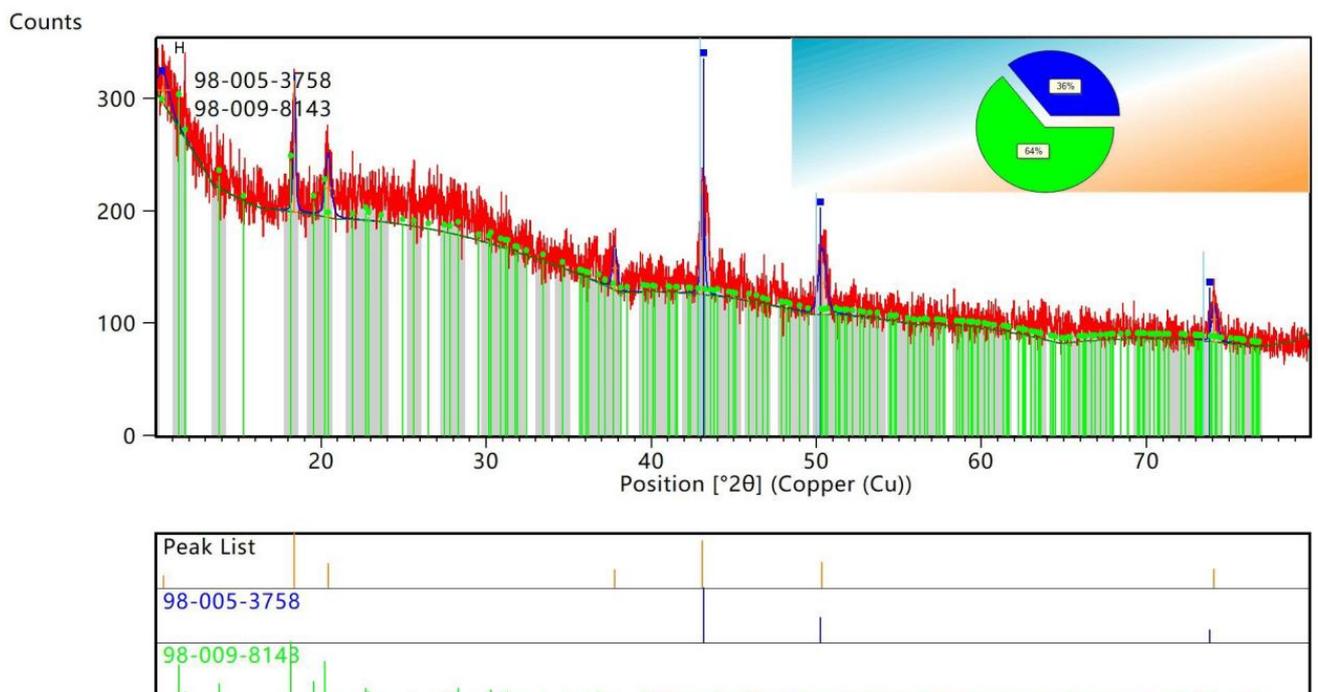
XRD patterns showed that the characteristic peaks of elemental copper and C<sub>60</sub> retrieved from PDF0178–2076 by computer were consistent. Using HighScore for semiquantitative analysis of XRD data, the results are shown in Figure 5. As seen in Figure 5G, the copper accounted for 37% and the C<sub>60</sub> accounted for 63% of the original WPCBs, while the copper accounted for 9% and the C<sub>60</sub> accounted for 91% of the WPCB residues. Therefore, it could be concluded that the leaching rate of copper in the 100 mL bacterial solution was the highest.



**Figure 4.** XRD patterns of WPCBs residues. (A) original WPCBs. (B) WPCBs residues after 10 mL bacterial solution leaching. (C) WPCBs residues after 20 mL bacterial solution leaching. (D) WPCBs residues after 30 mL bacterial solution leaching. (E) WPCBs residues after 40 mL bacterial solution leaching. (F) WPCBs residues after 50 mL bacterial solution leaching. (G) WPCBs residues after 100 mL bacterial solution leaching.

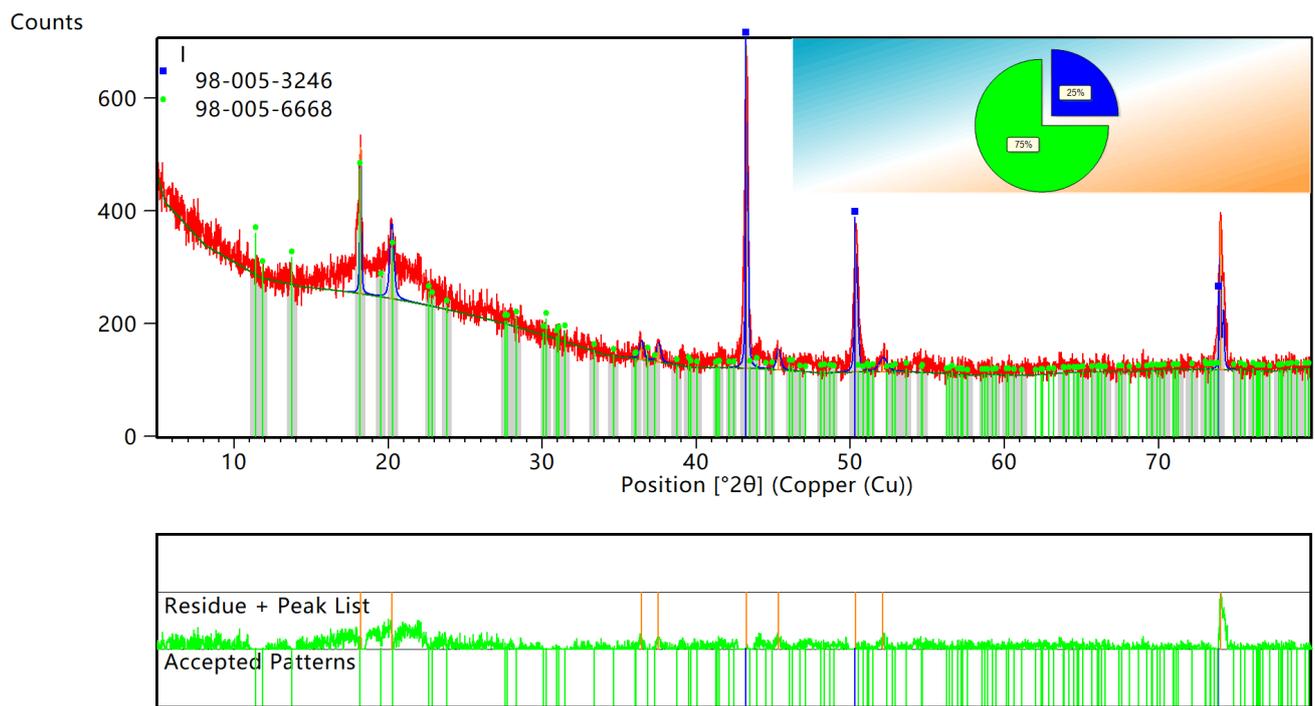


(A)

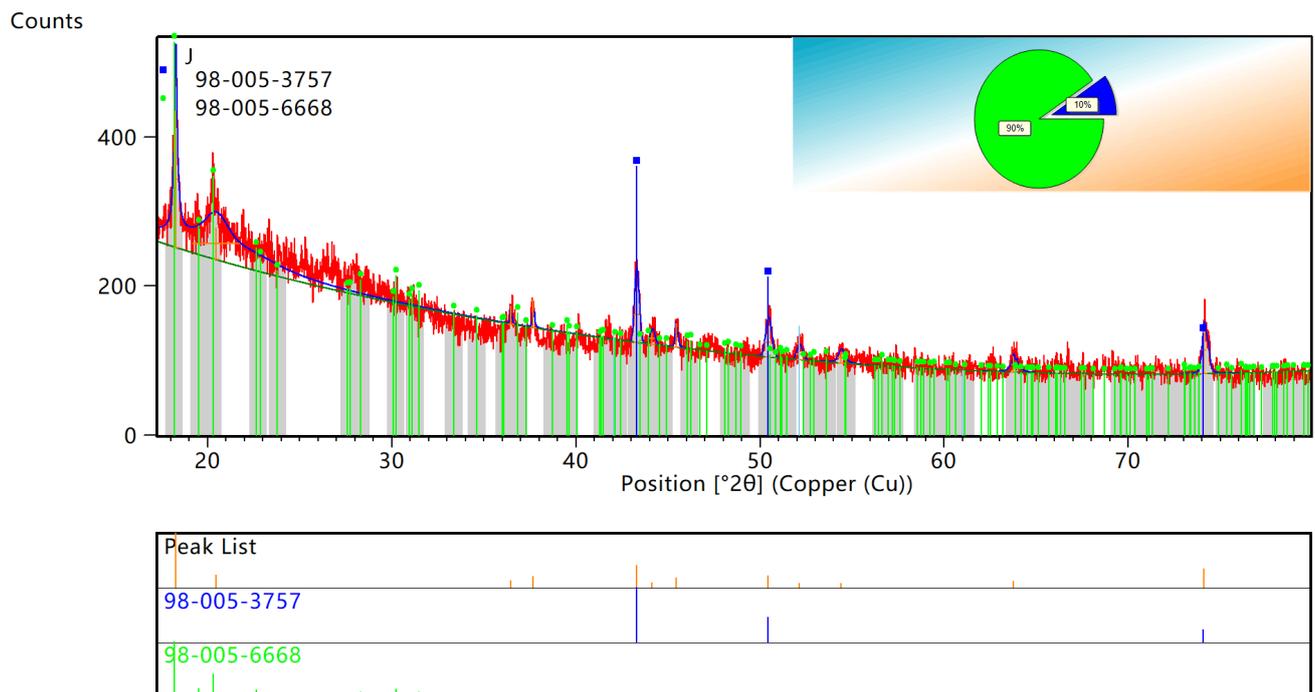


(B)

Figure 5. Cont.

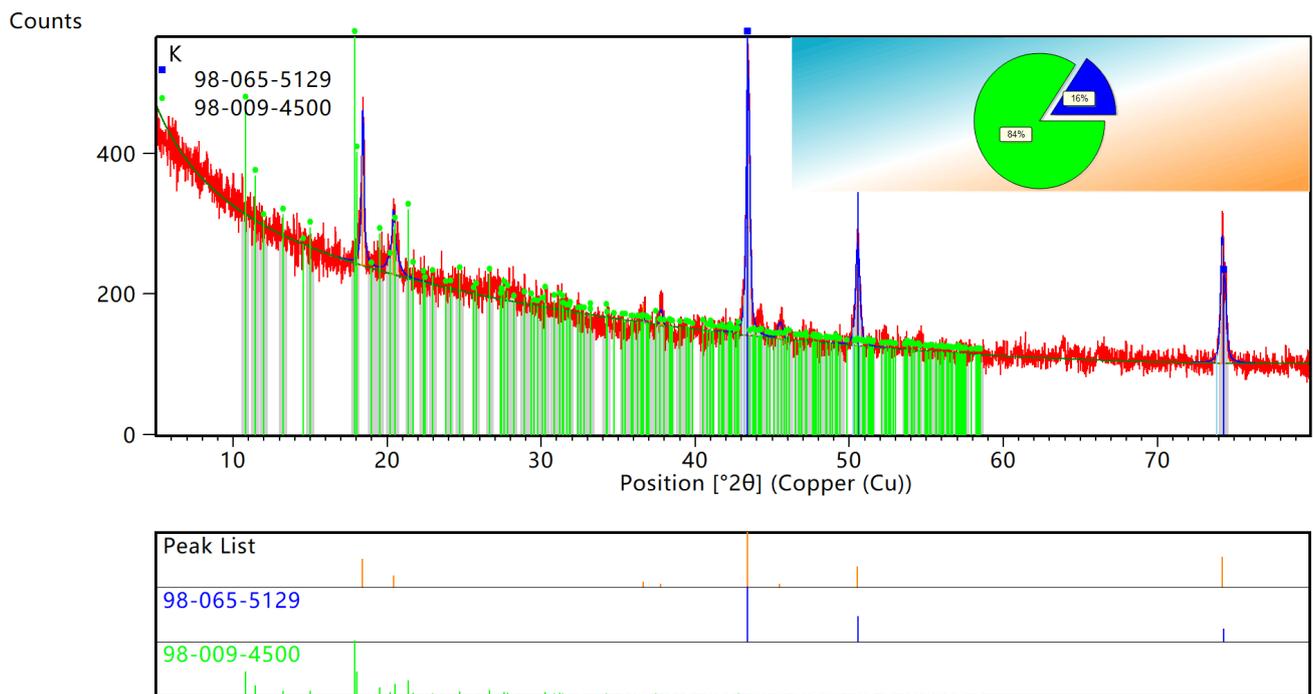


(C)

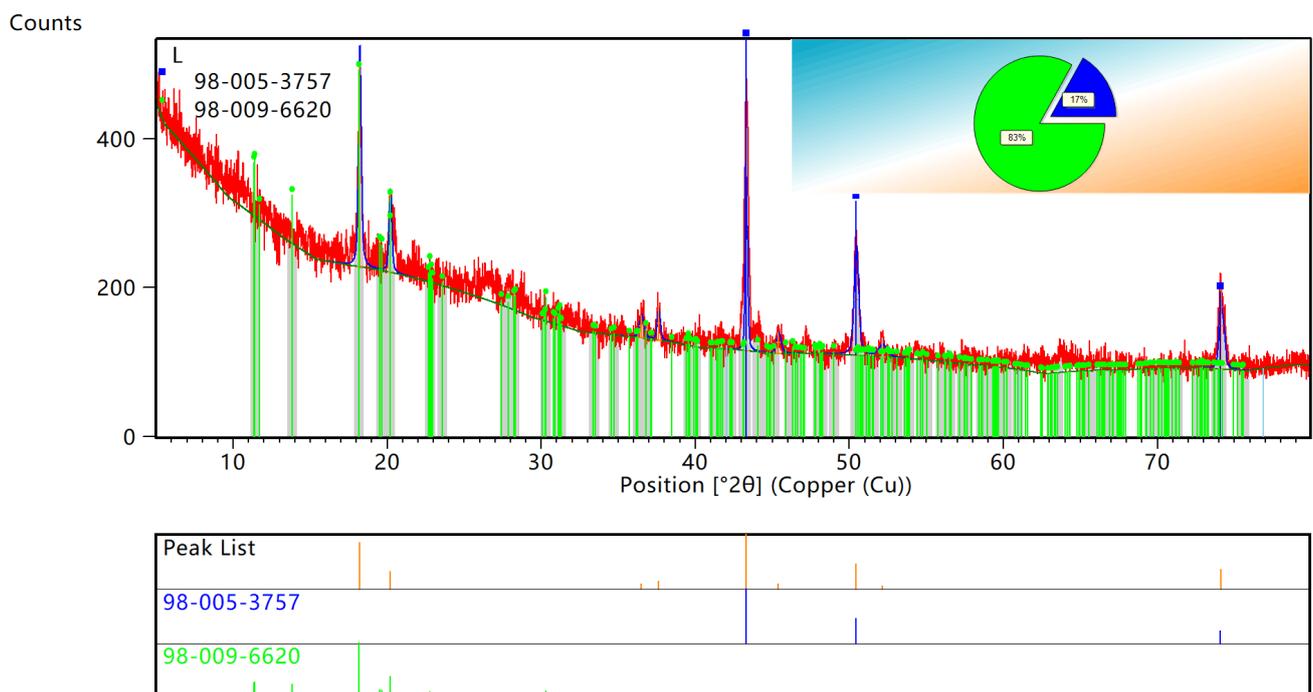


(D)

Figure 5. Cont.

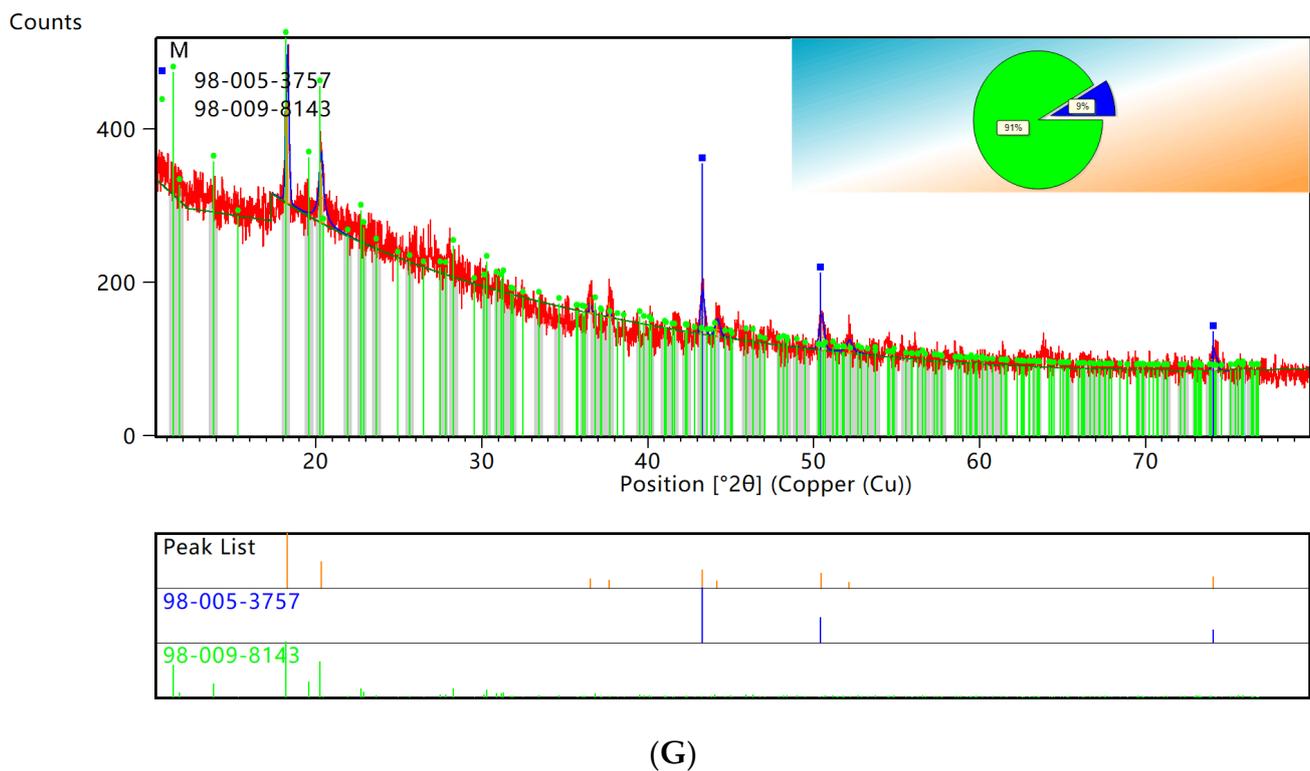


(E)



(F)

Figure 5. Cont.



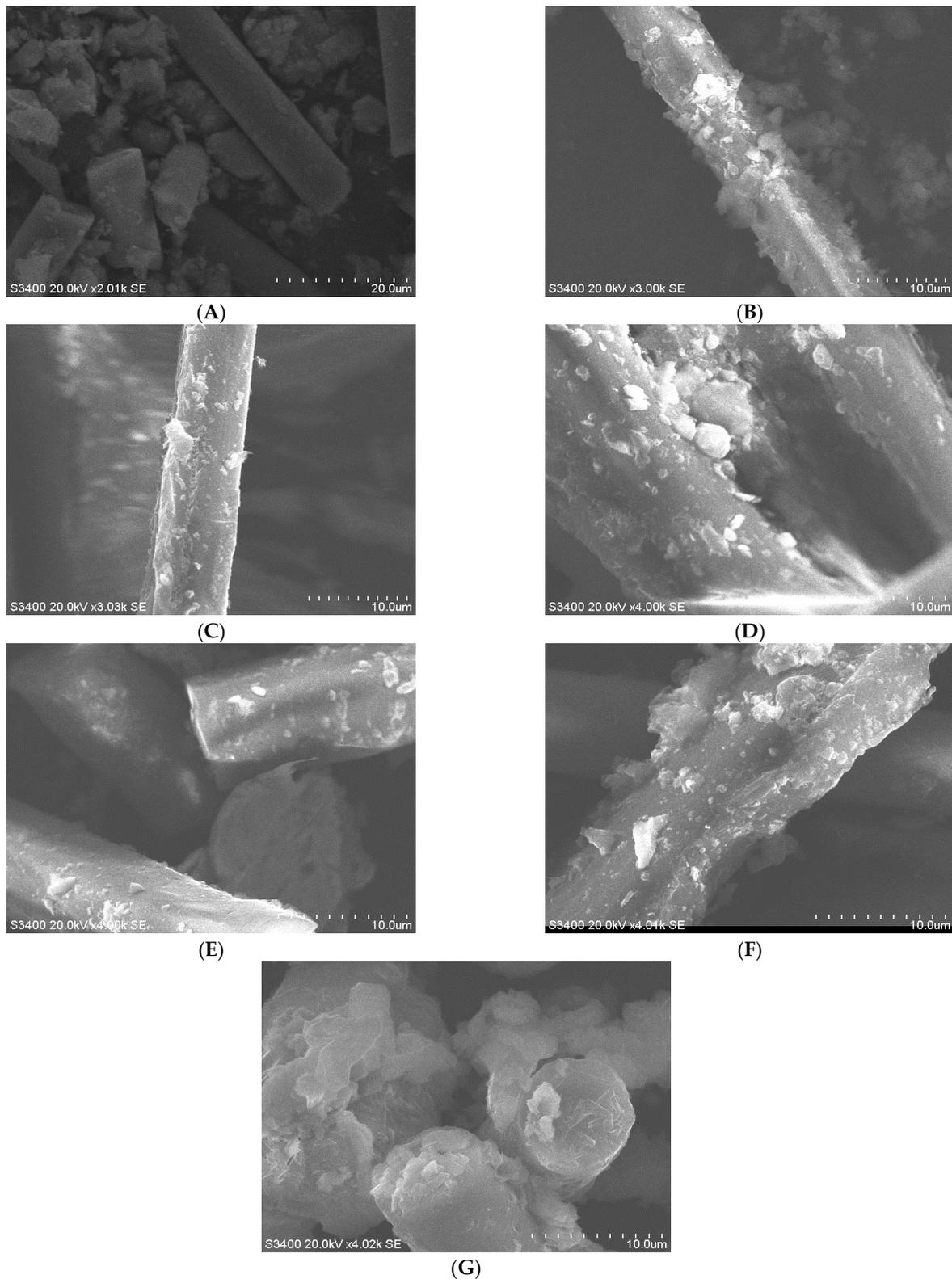
**Figure 5.** XRD patterns semiquantitative analysis. (A) original WPCBs. (B) WPCBs residues after 10 mL bacterial solution leaching. (C) WPCBs residues after 20 mL bacterial solution leaching. (D) WPCBs residues after 30 mL bacterial solution leaching. (E) WPCBs residues after 40 mL bacterial solution leaching. (F) WPCBs residues after 50 mL bacterial solution leaching. (G) WPCBs residues after 100 mL bacterial solution leaching.

### 3.4. Bioleaching Mechanisms

In general, bioleaching mechanisms included direct action mechanisms, indirect action mechanisms, and combined action mechanisms. WPCBs were amorphous, non-crystalline substances that were better suited to acid attacks [25]. 9k medium, with a large amount of  $\text{Fe}^{2+}$ , was highly acidic. Moreover, *A. acidothermus*, which secretes acidic enzymes, contributed significantly to the leaching of metals [26].

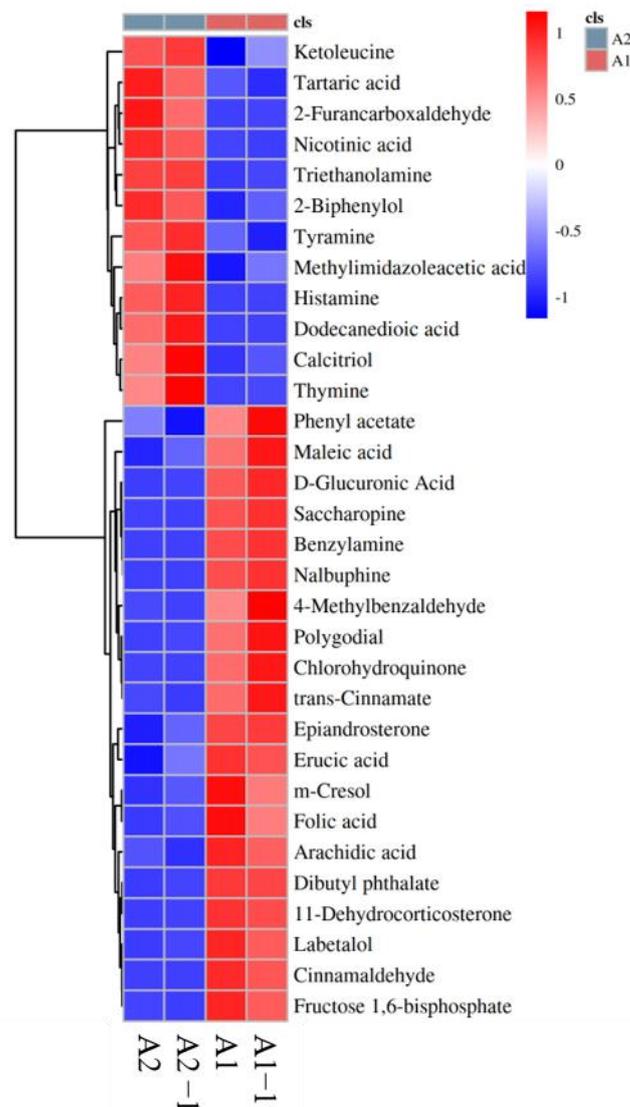
To illustrate the direct effect of bacterial leaching, the adsorption of bacteria on the WPCBs' surface should be demonstrated first. After leaching out the WPCBs, the residue in the bacterial solution was removed, baked in an oven at 70 °C for 24 h, and ground into a powder. SEM was used to observe the surface morphology of the residue (Figure 6).

Figure 6A displays the morphology of the original WPCBs. It can be seen from the figure that the WPCBs are composed of rod-shaped and globular polymers, and some rod-shaped surfaces are smooth. Figure 6B was the SEM image of the leaching residue of 10 mL of *A. acidothermus* bacterial solution, the surface of which was no longer smooth, and there were flocculent materials and lumps attached to the WPCBs. Figure 6C was the SEM image of the leaching residue of 20 mL of *A. acidothermus* bacterial solution, and the WPCBs had tiny protrusions and clumps attached to them. Figure 6D was the SEM image of the leaching residue of 30 mL of *A. acidothermus* bacterial solution, and the WPCBs were attached to large and small lumps and flocculents, whose surfaces were unevenly eroded by microorganisms. Figure 6E was the SEM image of the leaching residue of 40 mL of *A. acidothermus* bacterial solution, and there were small lumps on the surface of the WPCBs. Figure 6F was the SEM image of the leaching residue of 50 mL of *A. acidothermus* bacterial solution. The surface of the WPCBs, eroded by microorganisms, was uneven, with lumps and tiny protrusions. Figure 6G was the SEM image of the leaching residue of 100 mL of *A. acidothermus* bacterial solution. The end of the powder rod of the WPCBs was no longer smooth and became bumpy after being eroded by microorganisms, and a large number of lumps were attached to it.



**Figure 6.** SEM images of WPCBs residues. (A) original WPCBs. (B) WPCBs residues after 10 mL bacterial solution leaching. (C) WPCBs residues after 20 mL bacterial solution leaching. (D) WPCBs residues after 30 mL bacterial solution leaching. (E) WPCBs residues after 40 mL bacterial solution leaching. (F) WPCBs residues after 50 mL bacterial solution leaching. (G) WPCBs residues after 100 mL bacterial solution leaching.

Figure 6 showed bacteria tended to adhere to the WPCB's surface, which is similar to the research results of Roberts et al. [24,27]. With the extension of reaction times, microorganisms grew on the surface of WPCBs and secreted extracellular polymers to form biofilms. It had been illustrated that the microorganisms adsorbed on the surface of WPCBs and secreted extracellular polymeric substances (EPS) [28]. The heatmap of differential metabolites (Figure 7) revealed that in the process of microbial leaching, metabolites such as ketoleucine, 2-Furancarboxaldehyde, thymine, etc. constituted EPS. The EPS matrix also contained D-glucuronic acid, which during bioleaching was able to form stable complexes with iron ions, thus giving the cells a positive net charge and thus allowing them to adsorb onto the negatively charged WPCB's surface [29]. On the one hand, iron-D-glucuronic complex could strengthen the EPS matrix of biofilm. On the other hand, the complexes were able to enhance bioleaching. EPS forms a biofilm leaching area, which created conditions for  $H^+$  to go deep into the interior of WPCBs for acid dissolution. EPS matrix enabled microbial biofilm to capture nutrients from the aqueous phase or WPCB's surface, and EPS matrix acted as a common external digestive system. In addition, EPS could ensure the activity of enzymes and promote the bioleaching reaction. In conclusion, biofilm could make microorganisms attach more firmly to the mineral surface, and it was also the micro-environment with the most active material exchange and chemical reaction between biological and WPCBs, becoming the external force for the continuous decomposition of WPCBs [30].



**Figure 7.** The heatmap of differential metabolites ((A1): Before bioleaching reaction, (A2): After bioleaching reaction).

Although bacteria can directly decompose WPCBs,  $\text{Fe}^{3+}$ , the most important oxidising agent, produced by bacterial oxidation is also the key to copper bioleaching [31].  $\text{Fe}^{2+}$  could be oxidized to  $\text{Fe}^{3+}$  by floating *A. acidothermus* with  $\text{O}_2$  in existence [32], and then  $\text{Fe}^{3+}$  oxidized copper into copper ions while  $\text{Fe}^{2+}$  was reduced to  $\text{Fe}^{3+}$ . Afterwards, the next cycle of reactions would begin [33].

In addition, organic acids and inorganic acids secreted through bacterial metabolism can also dissolve WPCBs and release metal ions. Figure 7 also showed that in the process of bioleaching, metabolites included tartaric acid, nicotinic acid, methylimidazoleacetic acid, dodecanedioic acid, maleic acid, D-glucuronic acid, erueic acid, folic acid, arachidic acid, etc. It could be inferred that these organic acids were conducive to the leaching of metals.

#### 4. Conclusions

In this study, one strain was screened and isolated from the sludge. According to the physiological, biochemical, and 16rDNA characteristics, the obtained strain was identified as *Acidomyces acidothermus*. The optimal culture conditions for *A. acidothermus* were as follows: temperature: 40 °C, pH: 3, rotational speed: 140 r/min by single-factor experiment and response surface experiment. The interaction between pH and rotational speed was the strongest, while the interaction between pH and temperature was the weakest. Under the optimal conditions, 1 g of WPCBs (mesh size of 100–200  $\mu\text{m}$ ) were added to 100 mL of *A. acidothermus* bacterial solution and shaken for 4 h, and the highest copper leaching rate of 39.8% was achieved. *A. acidothermus* adsorbed on the surface of WPCBs and secreted EPS and organic acids, which promoted copper leaching. The use of the bioleaching method to leach copper ions from WPCBs is a technical concept that follows the current trend of green and sustainable development.

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