Bioleaching of trace metals from coal ash using local isolate from coal ash ponds

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Abstract. Bioleaching of chromium, copper, manganese and zinc from coal ash were investigated using isolates from coal ash ponds particularly *Pseudomonas spp.* Six (6) different coal ash ponds were examined however, after initial screening *Pseudomonas spp.* were only present in three (3) coal ash ponds. Among the three coal ash ponds, results showed that eight (8) putative *Pseudomonas spp.* isolates were present that were identified using the Polymerase Chain Reaction (PCR). Using the eight putative *Pseudomonas spp.* for bioleaching at optimum conditions and 15 days, the pH value ranges from 8.26 to 8.84 which was basic in nature. Moreover, the maximum metal leached were 8.04% Cr, 12.05% Cu, 4.34% Mn and 10.63% Zn.

1 Introduction

Coal is the most widely used and cheap source of fossil resource on earth. The global consumption of coal has increased from 4.92 billion tons in 2002 to 7.43 billion tons in 2012. Coal has been used as a solid fuel to produce heat and electricity via combustion in power plants [1-3]. Upon combustion of coal, 80% is composed of fly ash and 20% is composed of bottom ash. In a global perspective, 40 to 45% of the electric power generated came from thermos power plants amounting to 600 million tons of coal ash are produce annually [2-4]

As of 2016, Philippine's coal consumption is 20.296 million metric tons [5] and more than 47% of the Philippine's power generation came from coal [6]. With the rapid increase in power demand, high quantities of coal ash are being discharge daily. Majority of the coal ash are being disposed in ash ponds and lagoons that can pose environmental and health risks [4, 7-9]. Due to massive amount of coal ash generated, it is considered as secondary material for different applications such as brick production, mine filling, drainage media, replacement for conventional soil and gravel for road side embankment, road construction, structural fill and various cement-based composites including concrete and mortar [10-12].

In the recent years, green technologies are significant in addressing issues on environmental pollution such as biological leaching or bioleaching [10,13]. Bioleaching is an ecological friendly process of using microorganism to extract soluble material from secondary resources via the production of organic or inorganic acids [13-15].

This research used indigenous microorganism from coal ash pond particularly *Pseudomonas spp.* *Pseudomonas spp.* is the second most used bioleaching

microorganism due to its production of organic acid such as gluconic, oxalic and citric acid [16-17].

The main objective of this research to determine the maximum percent metal leached from coal ash using an indigenous microorganism from coal ash pond under optimum conditions. Moreover, the identified microorganism was *Pseudomonas spp.*, heterotrophic bacterium that produces organic acid. The metals under investigation are chromium, copper, manganese and zinc

2 Materials And Methods

2.1 Coal ash from coal ash pond

A mixture of fly and bottom ash was used in the experiment collected from the ash pond. The coal ash was provided by the private coal fired power plant that uses local and imported coal. Moreover, the power plant uses Circulating Fluidized Bed (CFB) Boiler. The coal ash was dried at 105°C for 24 hours, then sieved passing through particle size 0.075 microns (#200 mesh). The particle size was constant throughout the experiment. The ash is alkali in nature with pH of 10-11.5.

2.2 Determination of Microbial Population and Isolation of the Microorganism from Coal Ash Pond

2.2.1 Coal Ash sample from the Philippines

The ash samples were collected from the different coal power plant in the Philippines namely : PP1 from the partner power plant, PP 2 and PP3 from Central Luzon, PP 4 and PP 5 from Southern Luzon and PP 6 from Visayas. All samples were collected from the oldest part

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of the ash pond, one (1) meter below. And stored in a sterile container.

The screening and identification of the microbial population was performed by the Microbiology Department of University of the Philippines – Los Baños (UPLB). Moreover, the

Also, the preservation of the isolated microorganism was done through lyophilization by UPLB National Institute of Molecular Biology and Biotechnology.

2.2.2 Determination of the Microbial Population of the Coal Ash

The initial screening of total heterotrophic bacteria, yeast and molds and detection of *Pseudomonas spp.* was conducted. Initially all coal ash samples were basic in nature with a pH value of 9 to 11. The following procedures were done: (1) heterotrophic bacterial count: plate count agar with 1,000 units/ml nystatin; (2) molds and yeast count: potato dextrose agar with 100 ppm streptomycin and 50 ppm rose bengal, adjusted to pH 9.0 with 5.0 M NaOH and (3) detection of *Pseudomonas spp.* using most probable number (MPN) method on Arginine broth. Heterotrophic bacteria and *Pseudomonas spp.* were counted after 3 days of incubation while yeast and molds were counted after 5 days.

2.2.3 Isolation and Purification of the Microorganism

Based on literature, *Pseudomonas spp.* is the widely used heterotrophic bacteria for bioleaching. This microorganism was isolated in the coal ash samples. The isolated *Pseudomonas spp.* were preserved using lyophilization at UPLB National Institute of Molecular Biology and Biotechnology.

2.3 Polymerase Chain Reaction (PCR) amplification of DNA and DNA Sequencing

The identification of the specific *Pseudomonas* specie was done using TaKaRa PCR thermal Cyclor Dice TP600 at the Nakasaki Laboratory of Tokyo Institute of Technology (Japan). The extracted DNAs were amplified using the primers 16SF (5'AGAGTTTGATCCTGGCTCAGGA3') and 16SR (5'GGTTACCTTGTACG3'). The detected genome sequence was matched using Basic Location Alignment Search Tool (BLAST) database provided by the National Center of Biotechnology and Information (NCBI).

2.4 Bioleaching Experiment

2.4.1 Reviving the *Pseudomonas spp*

The ampules of the lyophilized *Pseudomonas spp* were opened using a metal file. Then, 1 ml of sterile water was added to the ampule and was swirled and gently tapped for 5 to 10 seconds. The liquid was transferred in

a 100 ml fresh nutrient broth media. The *Pseudomonas spp* was incubated at 37°C at 90 rpm for 7 days [18-20].

2.4.2 Growth of *Pseudomonas spp*

The incubated *Pseudomonas spp* were streaked in a nutrient agar plates. The nutrient agar plates were placed in an incubator at 37°C for 3 days. After which, one colony was place in an inoculating looped then transferred to 100 ml fresh nutrient broth media. *Pseudomonas spp.* was cultured in a Nutrient broth medium containing of 1 g/L yeast, 2 g/L peptone and 5 g/L glucose. 8 grams of nutrient broth medium was dissolved in 1 liter of deionized water. The solution was autoclaved at 121°C for 15 minutes [21]. The resulting solution has a pH of 7.2. Then the inoculum was placed in a water bath shaker (Visio VS-1205SW1) at 90 rpm and 37°C. *Pseudomonas spp* were incubated for 5 days prior to bioleaching [18-20].

2.5 Bioleaching experiment using *Pseudomonas spp*

The bioleaching experiments were conducted in a 250 mL Erlenmeyer flask with the following conditions: 1% pulp density (1 g of ash per 100 ml medium), 90 rpm and 37°C. The solution were autoclaved at 121°C for 15 minutes and cooled. 5 ml of the inoculum was placed in a 100 ml fresh medium with the ash. The bioleaching experiment was for 15 days [22]. The extracted solution was filtered using a 0.45 micrometer cellulous acetate filter.

2.6 Analytical Methods

X-ray Diffraction (XRD) analysis was performed using Bruker D8 Advance diffractometer equipped with copper radiation Sol-X detector (CuK α , λ = 0.15406 nm). The data were recorded with a 1s step time and 0.02° scan step between 2° to 80°. On the other hand, X-ray Fluorescence (XRF) analysis was conducted using Pananalytical Xflourescence spectrometer equipped with 150 eV (Mn Ka) resolution Energy Dispersive Minipal 4 tube (Rh X Ray tube – 30 kV –9W). All samples were dried at room temperature.

Also, metal analysis was done using Shimadzu AA-6300 AAS analysis. Different wavelength were used namely: 267 nm for Cr, 324 nm for Cu, 257 nm for Mn and 213 nm for Zn were used to detect the concentration of the trace metals in the solution, reported as mg/kg or ppm. To preserve the sample, few drops of concentrated nitric acid was added.

3 Results and Discussion

3.1 Mineralogical composition: XRD

Fig. 1 shows the mineral composition of the coal ash namely: quartz-SiO₂, lime-CaO, anhydrite, tricalcium aluminate, periclase, melilite, mullite and rutile. These

minerals were also identified by the different researchers [23-29].

Fig. 1. XRD Analysis for Coal Ash (a: Quartz-SiO₂, b: Melilite, c: Anhydrite, d: Tricalcium aluminate, f: Lime-CaO, g: Periclase, m: Mullite, r: Rutile).

3.2 Elemental Composition: XRF

The chemical analyses of the ash samples were performed to determine the composition of the material. Table 1 shows the major and trace elemental composition.

Table 1. XRF analysis of ash samples.

Element	% by weight
Calcium	39.46
Silicon	25.70
Aluminum	10.62
Iron	6.20
Sulfur	5.98
Sodium	3.99
Chlorine	2.11
Potassium	1.83
Magnesium	1.67
Titanium	1.51
Strontium	0.43
Barium	0.22
Zirconium	0.05
Tin	0.035
Vanadium	0.034
Chromium	0.033
Nickel	0.025
Manganese	0.023
Tellurium	0.021
Copper	0.020
Zinc	0.011
Bromine	0.011
Yttrium	0.007

The highest element is calcium which may be originated on the limestone that was being added during the combustion process [24,28,30]. The minerals identified by XRD coincide with the chemical results of the main composition obtained by XRF analysis (Table

2). Moreover, the total metal contents in the ash are 0.96 mg/kg Zn, 4.13 mg/kg Mn, 0.81 mg/kg Cr and 2.22 mg/kg Cu.

3.3 Microbial Population Determination in Coal Ash Samples

Microbial populations were analyzed to identify the possible microorganism that can be used for bioleaching of coal ash. Microbial count was determined by Microbiology Department of UPLB. Based on literature, other than *Acidithiobacillus spp.*, *Pseudomonas spp.* was considered as a possible microorganism for bioleaching [31-35]. Table 2 shows bacterial and molds count and putative *Pseudomonas spp.* present in the coal ash sample.

Table 2. Microbial Count Summary

Power Plant	Bacteria	Molds	Putative <i>Pseudomonas</i> species	Number of <i>Pseudomonas</i> isolate
PP 1	< 2500 cfu/g	-----	< 1 MPN/g	No isolate
PP 2	3.0×10^4 cfu/g	2.0×10^4 cfu/g	< 1 MPN/g	No isolate
PP 3	4.9×10^5 cfu/g	3.3×10^3 cfu/g	233 MPN/g	4 isolate
PP 4	4.9×10^6 cfu/g	1.3×10^5 cfu/g	33 MPN/g	4 isolate
PP 5	2.2×10^3 cfu/g	2.4×10^4 cfu/g	< 1 MPN/g	No isolate
PP 6	2.1×10^5 cfu/g	4.5×10^2 cfu/g	4 MPN/g	1 isolate

cfu: colony forming unit; MPN: Most Probable Number

Table 2 shows that 9 possible *Pseudomonas spp.* isolate were identified. There are 4 isolates from PP 3 and PP4 and 1 isolate from PP 6. On the other hand, no isolate were detected on PP 1, PP 2 and PP 5.

Table 3. PCR result of *Pseudomonas spp*

Samples	Most closely related species (Identity: 100%)
PP 3-1	<i>P. aeruginosa</i> , <i>P. alcaligenes</i> , <i>P. citronellolis</i> , <i>P. delhiensis</i> , <i>P. denitrificans</i> , <i>P. guezennnei</i> , <i>P. knackmussii</i> , <i>P. multiresinivorans</i> , <i>P. nitroreducens</i> , <i>P. resinovorans</i> , <i>P. stutzeri</i>
PP 3-2	<i>P. putida</i> , <i>P. indologxydans</i> , <i>P. plecoglossicida</i> , <i>P. pseudoalcaligenes</i>
PP 3-3	<i>P. aeruginosa</i> , <i>P. alcaligenes</i> , <i>P. citronellolis</i> , <i>P. denitrificans</i> , <i>P. knackmussii</i> , <i>P. resinovorans</i> , <i>P. stutzeri</i>
PP 6-1	<i>P. aeruginosa</i> , <i>P. alcaligenes</i> , <i>P. citronellolis</i> , <i>P. delhiensis</i> , <i>P. denitrificans</i> , <i>P. guezennnei</i> , <i>P. knackmussii</i> , <i>P. multiresinivorans</i> , <i>P. nitroreducens</i> , <i>P. resinovorans</i> , <i>P. stutzeri</i>
PP 4-1	<i>P. aeruginosa</i> , <i>P. putida</i> , <i>P. alcaligenes</i> , <i>P. brassicacearum</i> , <i>P. denitrificans</i> , <i>P. entomophila</i> , <i>P. monteilii</i> , <i>P. helmanticensis</i> , <i>P. knackmussii</i> , <i>P. lini</i> , <i>P. poae</i> , <i>P. resinovorans</i> , <i>P. stutzeri</i> , <i>Azotobacter vinelandii</i>
PP 4-2	<i>P. aeruginosa</i> , <i>P. alcaligenes</i> , <i>P. citronellolis</i> , <i>P. delhiensis</i> , <i>P. denitrificans</i> , <i>P. guezennnei</i> , <i>P. knackmussii</i> , <i>P. multiresinivorans</i> , <i>P. nitroreducens</i> , <i>P. resinovorans</i> , <i>P. stutzeri</i>
PP 4-3	<i>P. aeruginosa</i> , <i>P. alcaligenes</i> , <i>P. citronellolis</i> , <i>P. delhiensis</i> , <i>P. denitrificans</i> , <i>P. guezennnei</i> , <i>P. knackmussii</i> , <i>P. multiresinivorans</i> , <i>P. nitroreducens</i> , <i>P. resinovorans</i> , <i>P. stutzeri</i>
PP 4-4	<i>P. aeruginosa</i> , <i>P. alcaligenes</i> , <i>P. citronellolis</i> , <i>P. delhiensis</i> , <i>P. denitrificans</i> , <i>P. guezennnei</i> , <i>P. knackmussii</i> , <i>P. multiresinivorans</i> , <i>P. nitroreducens</i> , <i>P. resinovorans</i> , <i>P. stutzeri</i>

P: *Pseudomonas*

Pseudomonas spp. were identified using Polymerase Chain Reaction (PCR). Table 3 shows the putative *Pseudomonas spp.* present in the different coal ash.

According to the PCR result, putative *Pseudomonas spp.* isolates are the same for PP 3-1, PP 3-3, PP 4-2 to PP 4-4 and PP 6-1. However, PP 3-2 and PP 4-1 has a different consortium of *Pseudomonas spp.* *Pseudomonas aeruginosa* is present in 7 samples (PP 3-1, PP 3-3, PP 4-1 to PP 4-4 and PP 6-1) while *Pseudomonas putida* is only present in PP 3-3. In previous researches, these microorganisms were used in bioleaching for extracting metals from secondary resources. These different *Pseudomonas spp.* isolate were used in the bioleaching coal ash. Figure 2 shows the streak plate of the 8 isolate samples.

Fig. 2. Different *Pseudomonas spp.* isolates (From L to R and T to B: PP 3-1, PP 3-2, PP 3-3, PP 6-1, PP 4-1 to PP 4-4)

3.4 Bioleaching using *Pseudomonas spp.*

After 15 days of bioleaching experiment, Table 4 shows the percent metal leached for chromium, copper, manganese and zinc for each *Pseudomonas spp.* isolates. Moreover, Figure 3 illustrate the different pH profile for the different *Pseudomonas spp.* isolates.

Table 4. % Metal Leached using *Pseudomonas spp.* isolates

Isolate	% Zn	% Cr	% Mn	% Cu
PP 3-1	10.63	7.52	4.34	10.10
PP 3-2	10.39	8.04	2.75	12.05
PP 3-3	9.19	8.03	3.90	8.87
PP 6-1	9.61	5.83	3.19	9.47
PP 4-1	9.03	5.99	2.35	9.38
PP 4-2	8.34	7.27	4.14	8.98
PP 4-3	10.41	6.63	3.70	10.24
PP 4-4	9.52	7.90	3.94	11.09

Table 4 shows that the maximum metal leached for chromium is 8.04% and copper is 12.05% from PP 3-2 and 4.34% for manganese and 10.63% for zinc from PP 3-1. Based on the PCR result, PP 3-2 contains *Pseudomonas aeruginosa* and PP 3-1 contains *Pseudomonas putida* in the consortium.

Fig. 3. pH values against the *Pseudomonas spp.* isolate

Figure 3 shows the pH values of the *Pseudomonas spp.* isolate after the bioleaching experiment. The pH value of the solution ranges from 8.26 to 8.84 which is basic. However, taking into consideration the pH of the ash which is 11, the decrease in the pH value is an indirect indication of possible formation of organic acid such as oxalic, gluconic or citric acid by the *Pseudomonas spp.* [18-19, 35-36]

Based on literature, bioleaching using *Acidithiobacillus spp.* extracted more metal as compared to *Pseudomonas spp.* The microbial activity of *Pseudomonas spp.* is in the circumneutral pH value of 7.8, because of this the efficiency of metal extraction is lower compared to low pH value. It should be noted that metal extraction is favorable under acidic conditions. Most metals like chromium, copper, manganese and zinc favor metal extraction at lower pH. On the other hand, production of extracellular polymeric substance (ESP) enhances metal solubilization using *Pseudomonas spp.* [31, 37].

4. Conclusion

Bioleaching of coal ash using *Pseudomonas spp.* isolate from coal ash pond was investigated. Based on the result, the total metal content of the coal ash are 0.81 mg/kg Cr, 2.22 mg/kg Cu, 4.13 mg/kg Mn and 0.96 mg/kg Zn. Moreover, the minerals identified were quartz-SiO₂, lime-CaO, tricalcium aluminate and the major elements were calcium, silicon and aluminum. Also, high calcium content results in the alkalinity of the ash sample.

From the initial screening and identification 8 putative *Pseudomonas spp.* were obtained. PCR result shows that 6 isolate has the same *Pseudomonas spp.* consortium namely: PP 3-1, PP 3-3, PP 4-2 to PP 4-4 and PP 6-1. On the other hand, PP 3-2 and PP 4-1 have different consortium. *Pseudomonas aeruginosa* is present in 7 samples namely (PP 3-1, PP 3-3, PP 6-1 and PP 4-1 to PP 4-4) and *Pseudomonas putida* in PP 4-2.

After 15 days of bioleaching experiment, the maximum metal extracted were 12.05% Cu and 8.04% Cr from PP 3-2 isolate and 10.63% Zn and 4.34% Mn from PP 3-1 isolate

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