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METHODS OF EXTRACTING PLATINUM GROUP METALS

Technical Field

- 5 The present invention relates, in general terms, to a method of extracting platinum group metals (PGM) from a material, the material further comprising Cu, Zn, Fe, Ti or a combination thereof.

Background

- 10 Amongst precious metals, platinum group metals [PGMs; platinum (Pt), palladium (Pd), and rhodium (Rh)] are widely used in the manufacture of space materials, catalysts, hydrogen fuel cells, and in the chemical and biomedical industries. Automotive catalytic converter (ACC) is a major consumer in the global production of PGM. PGM are
- 15 extensively used in ACC due to their high stability, activity, and selectivity. They act as active catalysts that convert toxic exhaust gases into less harmful products. Since the mid-1970s, ACC has been used increasingly to convert carbon monoxide and hydrocarbons to carbon dioxide and water (H₂O). Two-way (oxidation) catalytic converters are replaced with three-way (oxidation-reduction) catalytic converters
- 20 because of their ability to purify nitrous oxide, which is an important greenhouse gas. The catalytic converter is a honeycomb monolith structure, with a refractory oxide support composed of cordierite (Mg₂Al₄Si₅O₁₈) incorporated into stainless steel container or cerianite (CeO₂) or different proprietary base metal. In most cases, the inner surface of the monolith structure is coated with 90% γ - Al₂O₃ and a mixture of
- 25 metal oxides additives, such as cerium (Ce), zirconium (Zr), nickel (Ni), and iron (Fe). The catalysts containing PGM and other metals are then fixed onto the coated surface in reduced metallic form, and detoxify the exhaust gases. The metal composition of ACC varies significantly with age, origin, and manufacturer. For example, in ACC, platinum (Pt) content ranges between 300-1000 ug g⁻¹, while palladium (Pd) and rhodium (Rh)
- 30 content range between 200-800 ug g⁻¹ and 50-100 ug g⁻¹, respectively. However, the total concentration of PGM in ACC is always lower than 0.1%.

- An autocatalyst contains two or more precious metals in a very low amount (0.1-0.3 wt. % of the monolith). A major portion of global production of PGM, about 50% of produced
- 35 platinum, 80% of rhodium, and 80% of palladium are used in ACC. The high demand

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and price and decreasing concentration of PGM in natural ores make it necessary to harvest PGM from secondary sources. Indeed, these secondary sources contain a higher concentration of PGM than natural ores. The recovery of PGM from spent automotive catalysts (SAC) not only conserves natural primary ores to meet future demands but
5 also supports sustainable development. Moreover, it also minimizes waste disposal, limits power consumption, and reduces environmental pollution. For example, 1kg of platinum from primary ores is obtained after processing 150Mg (tones) of ores and generation of 400Mg waste, whereas the same amount of platinum can be recovered from the recycling of 2Mg of SAC. Compared to mining from ores, the recovery of these
10 metals from SAC is more economical and environmentally benign. Although conventional techniques such as hydrometallurgical and pyrometallurgical processing yield high recovery of these metals, the use of additional solvents, the generation of hazardous liquid waste and gaseous emissions, the high operating cost, and energy requirements are major limitations.

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It would be desirable to overcome or ameliorate at least one of the above-described problems.

Summary

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The present invention provides a method of extracting platinum group metals (PGM) from a material, the method comprising:

- a) surface oxidising the material;
- b) ultrasonicated the surface oxidised material of step a) in the presence of an
25 acid; and
- c) bioleaching the ultrasonicated material of step b) in the presence of cyanide in order to form a leachate comprising PGM, the cyanide being formed from a cyanide producing microbe in the presence of a cyanide precursor.

30 In some embodiments, the surface oxidation is performed at about 700 °C to about 900 °C, or preferably at about 850 °C.

In some embodiments, the surface oxidised material of step a) is ultrasonicated at a frequency of about 30 kHz to about 80 kHz, or preferably about 37 kHz to about 80 kHz.

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In some embodiments, the surface oxidised material of step a) is ultrasonicated at a power of about 30W to about 120W.

5 In some embodiments, the surface oxidised material of step a) is ultrasonicated for a duration of about 10 min to about 150 min, or preferably for about 70 min to about 80 min.

10 In some embodiments, the surface oxidised material of step a) is ultrasonicated at a temperature of about 30 °C to about 80 °C, or preferably about 70 °C.

In some embodiments, the acid is selected from nitric acid, hydrochloric acid, sulphuric acid, or a combination thereof.

15 In some embodiments, the acid has a concentration of about 2 M to about 16 M, or preferably about 8 M to about 9 M.

20 In some embodiments, the surface oxidised material of step a) is ultrasonicated at a frequency of about 37kHz, a temperature of about 70 °C, a power of about 100W, a duration of about 80 min, and the acid is nitric acid at about 8 M to about 8.5 M.

In some embodiments, the method further comprises a step after step b) of reducing the ultrasonicated material of step b) in the presence of a reducing agent.

25 In some embodiments, the reducing agent selected from formic acid, ascorbic acid, glycolic acid, malonic acid, or a combination thereof.

In some embodiments, the reducing agent has a concentration of about 1 % v/v to about 15 % v/v, or preferably about 5 % v/v to about 10 % v/v.

30 In some embodiments, the ultrasonicated material of step b) is reduced for a duration of about 30 min to about 120 minutes, or preferably of about 60 min to about 90 min.

35 In some embodiments, the ultrasonicated material of step b) is reduced at a temperature of about 50 °C to about 90 °C, or preferably of about 50 °C to about 80 °C.

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In some embodiments, the ultrasonicated material of step b) is reduced at a reducing agent concentration of about 5 vol%, duration of about 90 min, and temperature of about 80°C.

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In some embodiments, the cyanide precursor has a concentration of about 0.5 g/L to about 20 g/L, or preferably about 10 g/L.

10 In some embodiments, the ultrasonicated material of step b) is bioleached at a pulp density of about 0.1% w/v to about 12% w/v, or about 0.5% w/v.

In some embodiments, the ultrasonicated material of step b) is bioleached at a pH of about 7 to about 11, or preferably about pH 9 to about 10.5.

15 In some embodiments, the ultrasonicated material of step b) is bioleached at a temperature of about 22 °C to 38 °C, or preferably about 30 °C.

20 In some embodiments, the ultrasonicated material of step b) is bioleached at a cyanide precursor concentration of about 10 g/L, a pulp density of about 0.5 % w/v, a pH of about 9 to about 10.5, and temperature of about 30 °C.

25 In some embodiments, the ultrasonicated material of step b) is bioleached in the presence of the cyanide producing microbe and cyanide precursor under aerobic conditions, the cyanide producing microbe characterised by a HCN synthase operon (hcnA, hcnB, and hcnC) in its genome.

In some embodiments, the bioleaching step comprises:

- 30 i) pre-incubating the cyanide producing microbe and the cyanide precursor under aerobic conditions in order to produce the cyanide; and
- ii) mixing the cyanide producing microbe and the cyanide precursor of step i) with the ultrasonicated material of step b);
- wherein the cyanide producing microbe characterised by a HCN synthase operon (hcnA, hcnB, and hcnC) in its genome.

35 In some embodiments, the bioleaching step comprises:

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i) pre-incubating the cyanide producing microbe and the cyanide precursor under aerobic conditions in order to produce the cyanide; and

ii) isolating the cyanide from the cyanide producing microbe in order to form a cell-free medium and mixing the cell-free medium with the ultrasonicated material of step

5 b);

wherein the cyanide producing microbe characterised by a HCN synthase operon (hcnA, hcnB, and hcnC) in its genome.

In some embodiments, the aerobic condition is an O₂ % saturation of about 30%.

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In some embodiments, the cyanide producing microbe is selected from *Chromobacterium violaceum*, *Pseudomonas fluorescens*, *Bacillus megaterium*, or a combination thereof.

15 In some embodiments, the step of pre-incubating the microbe comprises incubating the microbe at a pH of about 7.5, followed by incubating the microbe at a pH of about 9.

In some embodiments, the ultrasonicated material of step b) is bioleached in the presence of the microbe and H₂O₂, wherein H₂O₂ is at a concentration of about 0.02 %

20 v/v to about 0.16 % v/v, or preferably about 0.08 % v/v.

In some embodiments, the ultrasonicated material of step b) is bioleached in the presence of a ROS scavenger and/or a dispersant.

25 In some embodiments, the ROS scavenger is added at least 10 h after mixing the reduced material with the microbe.

In some embodiments, the ROS scavenger has a concentration of about 0.2 g/L to about 2 g/L, or preferably about 0.6 g/L.

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In some embodiments, the dispersant has a concentration of about 0.2 g/L to about 1 g/L, or preferably about 0.4 g/L.

In some embodiments, the cell-free medium has a pH of about 10.5.

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In some embodiments, the material is a spent catalytic converter or a spent automotive catalyst.

In some embodiments, the material further comprises Cu, Zn, Fe, Ti or a combination thereof.

In some embodiments, the method is characterised by a volume of at least 1L.

In some embodiments, the method further comprises a step of bioreducing the leachate of step c) in order to form nanoparticles.

In some embodiments, the leachate of step c) is bioreduced using *Cupriavidus metallidurans*.

In some embodiments, *Cupriavidus metallidurans* is pre-incubated.

In some embodiments, the leachate of step c) is bioreduced at a pH of about 4 to about 8, preferably about 6.

In some embodiments, the nanoparticles are characterised by an average size of about 10 nm to about 80 nm.

In some embodiments, the nanoparticles are characterised by a hydrodynamic diameter of about 80 nm to about 110 nm.

In some embodiments, the nanoparticles are characterised by polydispersity index of about 0.2 to about 0.3.

The present invention also provides a method of extracting platinum group metals (PGM) from a material, comprising:

bioleaching the material in the presence of cyanide in order to form a leachate comprising PGM, the cyanide being formed from a cyanide producing microbe in the presence of a cyanide precursor, and additionally in the presence of a ROS scavenger and/or a dispersant.

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The present invention also provides a material comprising platinum group metals (PGM), the method comprising:
ultrasonicing the material in the presence of an acid;
wherein at least a surface of the material is oxidised.

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The present invention also provides a method of pretreating a material comprising platinum group metals (PGM), the method comprising:
reducing the material in the presence of a reducing agent;
wherein at least a surface of the material is oxidised.

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In some embodiments, the method further comprises a step before the reduction step of ultrasonicing the material in the presence of an acid.

Brief description of the drawings

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Embodiments of the present invention will now be described, by way of non-limiting example, with reference to the drawings in which:

Figure 1 shows the effect of process variables on Cu extraction. (a) Ultrasound duration (min), (b) Ultrasound power (%), (c) Ultrasound frequency (kHz), (d) Nitric acid concentration (M), and (e) Temperature (°C).

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Figure 2 shows the effect of process variables on Zn extraction. (a) Ultrasound duration (min), (b) Ultrasound power (%), (c) Ultrasound frequency (kHz), (d) Nitric acid concentration (M), and (e) Temperature (°C).

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Figure 3 shows the effect of process variables on Fe extraction. (a) Ultrasound duration (min), (b) Ultrasound power (%), (c) Ultrasound frequency (kHz), (d) Nitric acid concentration (M), and (e) Temperature (°C).

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Figure 4 shows the effect of process variables on Ti extraction. (a) Ultrasound duration (min), (b) Ultrasound power (%), (c) Ultrasound frequency (kHz), (d) Nitric acid concentration (M), and (e) Temperature (°C).

Figure 5 shows the response surface plots for the interactive effect of process variables on copper extraction (%). The 3D surface plots between (a) ultrasound frequency and temperature, (b) ultrasound frequency and nitric acid concentration, (c) ultrasound frequency and ultrasound power, and (d) ultrasound frequency and ultrasound duration.

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The interaction plot between (e) ultrasound frequency and temperature.

Figure 6 shows the response surface plots for the interactive effect of process variables on zinc extraction (%). The 3D surface plots between (a) ultrasound frequency and temperature, (b) ultrasound frequency and nitric acid concentration, (c) ultrasound frequency and ultrasound power, and (d) ultrasound frequency and ultrasound duration.

5 The interaction plot between (e) ultrasound frequency and temperature.

Figure 7 shows the response surface plots for the interactive effect of process variables on iron extraction (%). The 3D surface plots between (a) ultrasound frequency and temperature, (b) ultrasound frequency and nitric acid concentration, (c) ultrasound frequency and ultrasound power, and (d) ultrasound frequency and ultrasound duration.

10 The interaction plot between (e) ultrasound frequency and temperature.

Figure 8 shows the response surface plots for the interactive effect of process variables on titanium extraction (%). The 3D surface plots between (a) ultrasound frequency and temperature, (b) ultrasound frequency and nitric acid conc., (c) ultrasound frequency and ultrasound power, and (d) ultrasound frequency and ultrasound duration. The interaction plots between (e) ultrasound power and ultrasound frequency, and (f) ultrasound power and nitric acid concentration.

15 **Figure 9** shows normal probability vs internally studentized residuals and actual vs predicted values. (a) Normal probability plot and (b) actual vs predicted values of Cu; (c) Normal probability plot and (d) actual vs predicted values of Zn; (e) Normal probability plot and (f) actual vs predicted values of Fe; and (g) Normal probability plot and (h) actual vs predicted values of Ti.

Figure 10 shows the effect of process variables on Pt recovery. (a) Glycine concentration (g/L), (b) Pulp density (% w/v), (c) pH, (d) H₂O₂ concentration (% v/v), and (e) Temperature (°C).

25 **Figure 11** shows the effect of process variables on Pd recovery. (a) Glycine concentration (g/L), (b) Pulp density (% w/v), (c) pH, (d) H₂O₂ concentration (% v/v), and (e) Temperature (°C).

Figure 12 shows the effect of process variables on Rh recovery. (a) Glycine concentration (g/L), (b) Pulp density (% w/v), (c) pH, (d) H₂O₂ concentration (% v/v), and (e) Temperature (°C).

30 **Figure 13** shows the effect of pulp density on PGM recovery in two-step bioleaching at pH 9 and 9.5 (glycine concentration 10 g/L; H₂O₂ concentration 0.08 %v/v; temperature 30°C) (a) Pt, (b) Pd, and (c) Rh. Error bars are the standard deviations (n=3). *P* value for (a-c) >.05.

Figure 14 shows the effect of pH on PGM recovery in two-step bioleaching at pulp densities 0.5 %w/v and 1 %w/v (glycine concentration 10 g/L; H₂O₂ concentration 0.08 %v/v; temperature 30°C) (a) Pt, (b) Pd, and (c) Rh. Error bars are the standard deviations (n=3). *P* value for (a-c) >.05.

- 5 **Figure 15** shows the effect of H₂O₂ on PGM recovery in two-step bioleaching at pH 9, 9.5, and 10 (pulp density 1 %w/v; glycine concentration 10 g/L; H₂O₂ concentration 0.08 %v/v; temperature 30°C) (a) Pt, (b) Pd, and (c) Rh. Error bars are the standard deviations (n=3). *P* value for (a-c) >.05.

- 10 **Figure 16** shows the response surface and perturbation plots for the interactive effect of process variables on Pt recovery (%). The 3D surface plots between (a) glycine concentration and pulp density, (b) pulp density and pH, (c) pulp density and H₂O₂, and (d) pulp density and temperature. (e) Perturbation plot for Pt recovery.

- 15 **Figure 17** shows the response surface and perturbation plots for the interactive effect of process variables on Pd recovery (%). The 3D surface plots between (a) glycine concentration and pulp density, (b) pulp density and pH, (c) pulp density and H₂O₂, and (d) pulp density and temperature. (e) Perturbation plot for Pd recovery.

- 20 **Figure 18** shows the response surface and perturbation plots for the interactive effect of process variables on Rh recovery (%). The 3D surface plots between (a) glycine concentration and pulp density, (b) pulp density and pH, (c) pulp density and H₂O₂, and (d) pulp density and temperature. (e) Perturbation plot for Rh recovery.

Figure 19 shows normal probability vs internally studentized residuals and actual vs predicted values. (a) Normal probability plot and (b) actual vs predicted values of Pt; (c) Normal probability plot and (d) actual vs predicted values of Pd; and (e) Normal probability plot and (f) actual vs predicted values of Rh.

- 25 **Figure 20** shows bacterial growth and cyanide concentration during two-step bioleaching at pH 9.4 (glycine concentration 10 g/L; pulp density 1 %w/v; temperature 30°C). (a) cell counts in batch mode, (b) free cyanide concentration in batch mode, (c) cell counts in fed-batch mode, and (d) free cyanide concentration in fed-batch mode. Fresh medium was added after 2 days (dashed line) to stimulate bacterial growth and cyanide production. Error bars are the standard deviations (n=2).

Figure 21 shows a schematic diagram of experimental design for pretreatment of SAC prior to leaching.

- 35 **Figure 22** shows PGM recovery from reduced SAC under spent medium leaching. Experimental conditions for SAC reduction (a) reducing agent concentration 1-15 %vol, reduction time 60 min, reduction temperature 70°C; (b) reduction time 30-120 min,

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reducing agent concentration 5 and 10 %vol for formic acid and ascorbic acid, respectively, reduction temperature 70°C; and (c) reduction temperature 50-90 °C, reducing agent concentration 5 and 10 %vol for formic acid and ascorbic acid, respectively, reduction time 90 and 60 min for formic acid and ascorbic acid, respectively. Error bars are the standard deviations (n=3). *P* value for (a-c) <.05.

Figure 23 shows (a) Pt, (b) Pd, and (c) Rh recovery from non-reduced and reduced SAC under spent medium leaching under optimised conditions. Error bars are the standard deviations (n=3). *P* value for (a-c) <.05.

Figure 24 shows the effect of pulp density on PGM recovery during two-step bioleaching (pH 9.4; glycine concentration 10 g/L; H₂O₂ concentration 0.08 %v/v; temperature 30°C) (a) Pt, (b) Pd, and (c) Rh. Error bars are the standard deviations (n=3). *P* value for (a-c) >.05.

Figure 25 shows (a) Free cyanide concentration and (b) cell counts during two-step bioleaching (pH 9.4; glycine concentration 10 g/L; H₂O₂ concentration 0.08 %v/v; temperature 30°C). Error bars are the standard deviations (n=3). *P* value for (a-b) >.05.

Figure 26 shows relative fluorescence units during two-step bioleaching at different pulp densities (pH 9.4; glycine concentration 10 g/L; H₂O₂ concentration 0.08 %v/v; temperature 30°C) (a) DHR-123 RFU, (b) DCFH-DA RFU, and (c) DHE RFU. Error bars are the standard deviations (n=3). *P* value for (a) >.05, (b) <.05, and (c) >.05.

Figure 27 shows (a) Cell counts and free cyanide concentration and (b) Metal recovery during two-step bioleaching at different pulp densities (pH 9.4; glycine concentration 10 g/L; H₂O₂ concentration 0.08 %v/v; temperature 30°C). Error bars are the standard deviations (n=3). *P* value for (a-b) >.05.

Figure 28 shows relative fluorescence units during two-step bioleaching at pulp density 4 %w/v and at different GSH concentrations (g/L) (pH 9.4; glycine concentration 10 g/L; H₂O₂ concentration 0.08 %v/v; temperature 30°C) (a) DHR-123 RFU, (b) DCFH-DA RFU, and (c) DHE RFU. Error bars are the standard deviations (n=3). *P* value for (a-c) <.05.

Figure 29 shows cell counts during two-step bioleaching at pulp density 4 %w/v and at different GSH concentrations (g/L) (pH 9.4; glycine concentration 10 g/L; H₂O₂ concentration 0.08 %v/v; temperature 30°C). Error bars are the standard deviations (n=3). *P* value >.05.

Figure 30 shows PGM recovery during two-step bioleaching at pulp density 4 %w/v and at different GSH concentrations (g/L) (pH 9.4; glycine concentration 10 g/L; H₂O₂

concentration 0.08 %v/v; temperature 30°C) (a) Pt, (b) Pd, and (c) Rh. Error bars are the standard deviations (n=3). *P* value for (a-c) >.05.

Figure 31 shows the distribution of the SAC particles in the bacteria solutions. (a-c) The adsorption of the SAC particles on bacteria in the absence of PVP, (d) The agglomeration of the SAC particles in the absence of PVP, and (e-f) The dispersed SAC particles after the addition of PVP (0.4 g/L).

Figure 32 shows cell counts and free cyanide concentration during two-step bioleaching at pulp density 4 %w/v, GSH concentration 0.6 g/L, and PVP concentration 0.4 g/L (pH 9.4; glycine concentration 10 g/L; H₂O₂ concentration 0.08 %v/v; temperature 30°C). Error bars are the standard deviations (n=3). *P* value >.05.

Figure 33 shows PGM recovery during two-step bioleaching at pulp density 4 %w/v in the absence (control) and presence of GSH (0.6 g/L) and PVP (0.4 g/L) (pH 9.4; glycine concentration 10 g/L; H₂O₂ concentration 0.08 %v/v; temperature 30°C). Error bars are the standard deviations (n=3). *P* value <.05.

Figure 34 shows cell counts and free cyanide concentration during two-step bioleaching at different pulp densities in the absence (control) and presence of GSH (0.6 g/L) and PVP (0.4 g/L) (pH 9.4; glycine concentration 10 g/L; H₂O₂ concentration 0.08 %v/v; temperature 30°C) (a) cell counts and (b) free cyanide concentration. Error bars are the standard deviations (n=3). *P* value for (a-b) >.05.

Figure 35 shows PGM recovery during two-step bioleaching at different pulp densities in the absence (control) and presence of GSH (0.6 g/L) and PVP (0.4 g/L) (pH 9.4; glycine concentration 10 g/L; H₂O₂ concentration 0.08 %v/v; temperature 30°C) (a) Pt, (b) Pd, and (c) Rh. Error bars are the standard deviations (n=3). *P* value for (a-c) <.05.

Figure 36 shows PGM recovery during two-step bioleaching from non-reduced and reduced SAC at pulp density 4 %w/v in the absence (control) and presence of GSH (0.6 g/L) and PVP (0.4 g/L) (pH 9.4; glycine concentration 10 g/L; H₂O₂ concentration 0.08 %v/v; temperature 30°C). Error bars are the standard deviations (n=3). *P* value <.05.

Figure 37 shows the effect of initial solution pH on bioreduction efficiency (a) Pt recovery and (b) Pd recovery. Error bars are the standard deviations (n=3).

Figure 38 shows the effect of initial concentrations of Pt(II) and Pd(II) ions on bioreduction efficiency (a) Pt recovery and (b) Pd recovery. Error bars are the standard deviations (n=3).

Figure 39 shows the effect of initial concentrations of Pt(II) and Pd(II) ions on bacterial growth at pH 6 during two-step bioreduction. Error bars are the standard deviations (n=3).

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Figure 40 shows the effect of viable vs. non-viable cells on the bioreduction efficiency of Pt(II) and Pd(II) at pH 6 (Pt(II) concentration = 150 ppm, Pd(II) concentration = 100 ppm). Error bars are the standard deviations (n=3).

Figure 41 shows the effect of biosynthesized Pt and Pd NPs on bacterial growth.

- 5 **Figure 42** shows TEM micrograph of an unreacted *C. metallidurans* cells (a) fixed and stained cells and (b) whole mount of unstained cells.

Figure 43 shows TEM micrograph of Pt and Pd nanoparticles produced by *C. metallidurans* cells. Image was taken 24 hours after the start of the bioreduction experiment.

- 10 **Figure 44** shows the size distribution histogram and Gaussian fitting of Pt and Pd nanoparticles produced by *C. metallidurans* cells. Results were taken 24 hours after the start of the bioreduction experiment.

- Figure 45** shows Energy dispersive X-ray spectroscopy (EDX) spectra of Pt and Pd nanoparticles produced by *C. metallidurans* cells. Spectrum was taken 24 hours after
15 the start of the bioreduction experiment.

- Figure 46** shows TEM micrograph of Pt and Pd nanoparticles produced by *C. metallidurans* cells. (a) cubical and rhombic dodecahedral shaped NPs, (b) rhombic dodecahedral shaped NPs, (c) cubical shaped NPs, (d) nanospheres, (e) single nanocube, (f) cubical and spherical shaped NPs, (g, h and i) spherical, cubical, and rod-shaped NPs.
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Figure 47 shows TEM micrograph of Pt and Pd nanoparticles produced by *C. metallidurans* cells at high initial concentrations of Pt-cyanide and Pd-cyanide complexes. Image was taken 24 hours after the start of the bioreduction experiment.

- Figure 48** shows HRTEM, FFT, and IFFT micrograph of Pd NP. HRTEM image of Pd NP
25 showing a fringe spacing of 1.94 Å and its corresponding FFT and IFFT image.

Figure 49 shows selected area electron diffraction pattern of PdO.

Figure 50 shows selected area electron diffraction pattern of Pt NP.

Figure 51 is a schematic diagram of the method to extract PGM.

30 **Detailed description**

The inventors believe that biometallurgical processes are generally more environmentally benign, sustainable, and close to natural biogeochemical cycles, thus reducing the demand for natural resources such as ores, energy, and landfill space.

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The term bioleaching is defined as "mobilization of metal ions from insoluble materials by biological oxidation and complexation processes". In bioleaching, microbes produce secondary metabolites that dissolve the target metals and leave behind an unwanted complex matrix. Some heterotrophic microorganisms (bacteria and fungi) are reported
5 to be capable of recovering precious metals from wastes and secondary sources. These microorganisms produce cyanide in an aqueous medium by oxidative decarboxylation of glycine and form soluble metal-cyanide complexes of the respective metal ions. Bioleaching is commonly considered an environmentally benign metal recovery process. The unique abilities of microbial metabolism and mineral transformations of the
10 microorganisms make bioleaching a simple and efficient processing technique. Moreover, the biological approach is considered as economical for processing small deposits, low-grade ores, secondary wastes, and complex ores. However, its potential exploitation for the recovery of precious metals from secondary sources is not well documented in the literature.

15 Unfortunately, the potential of biometallurgical processes for the recovery of PGM is largely unexplored. Despite its advantages over conventional metal recovery techniques, biorecovery of PGM from SAC is particularly challenging mainly because of the following: (i) complex nature of SAC, (ii) toxicity imposed by the interfering metals
20 on microorganisms, (iii) low yield of the lixiviant, (iv) competition for metal-cyanide complexes between target and non-target metals, (v) low metal recovery at high pulp density, (vi) low metal recovery in the presence of metal oxides, and (vii) slow leaching kinetics. To overcome at least some of these challenges, the present invention provides an effective, sustainable, and environment-friendly process via the bio-extraction of
25 PGM from SAC.

Further, the synthesis of nanoparticles (NPs) has gained immense research interest in recent times. The unique crystalline, catalytic, optical, and surface-related physicochemical properties of these nanoparticles find applications in numerous areas
30 such as biomedical, pharmaceuticals, optics, biosensors, fuel cells, semiconductors, photonics, electronics, petrochemicals, and catalysis. Several methods are used for synthesizing NPs, such as chemical, physical, and biological methods. Physicochemical methods were used during the early phases of synthesizing NPs. In physical methods, high temperatures or laser ablations are used to generate metal atoms from bulk metals
35 followed by coalescing of atoms to form nanoparticles. In chemical methods, wet

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chemistry is used to produce the metal atoms which involve two elements: a reducing agent and a source of metal ions. An additional capping agent may be used to control the shape, size, and dispersity of NPs. The utilization of toxic substances during physicochemical synthesis approaches led to interest in "green synthesis" of eco-friendly materials. Hence, biosynthesis of NPs categorised as microbial synthesis and phytosynthesis have been introduced. The biosynthesis of NPs uses proteins and/or peptides as the green reducing agents and reduces the environmental footprint. Moreover, biosynthesized NPs are biocompatible, chemical-free, sustainable, eco-friendly, and cost-effective. However, the limitations associated with the biological synthesis of NPs include challenges in controlling shape and size, stability, and aggregation. Although chemical methods produce stable NPs with different shapes and sizes, the biomedical applications of these NPs are limited due to biosafety concerns.

Bioreduction is an enzymatically-assisted metal precipitation process where the metal ion is reduced from a high valence to a zero valence state. Bacteria produce NPs extra- or intra-cellularly by the reduction mechanism that converts the metal ions to NPs using intracellular signalling pathways which involves bacterial enzymes. In leached liquor, microbial bioreduction results in the transformation that plays a critical role in the recovery of the metals. It must be recognized that all the studies on the biosynthesis of Pt and Pd NPs used either aqueous solutions comprising only of PGM-chloride complexes or model synthetic solutions i.e., metal salts as a source of metal ions. The model synthetic solutions contain only one or two metals of interest and are way too simplistic compared to real waste leachate. In model synthetic solutions, the adverse effects of metal toxicity on microbial functions are low, thus, these solutions are easy to process. Conversely, in real waste leachate, the composition of metal ions is very complex, containing several metals at high concentrations, pose negative effects for proper microbial functioning, and limits the process efficiency. Spent autocatalyst (SAC) contains a large number of heavy metals that challenges bioleaching efficiency. The presence of these metals poses toxicity to viable cells. Amongst these metals, certain metals utilize cyanide for metal-cyanide complexation. Therefore, the presence of metal ions of these undesired metals makes real waste leachate more difficult to process. The inventors are not aware of any study which has dealt with the biorecovery of Pt and Pd from SAC leachate.

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Accordingly, it is desirable to (1) develop and optimize an efficient pretreatment technique that removes interfering elements (copper, zinc, iron, and titanium) from SAC before bioleaching, (2) optimize the biomobilization of PGM using hydrogen cyanide forming (HCN) bacteria and to optimize the leaching rate to enhance the bioleaching efficiency, (3) study microbial-metal interactions and to minimize adverse effects of metal toxicity and oxidative stress in bacteria during bioleaching, in order to enhance PGM extraction at high pulp densities, and (4) explore the bioreduction mechanism for the bacteria-mediated biorecovery of Pt and Pd and green synthesis of Pt and Pd NPs from SAC leachate and the effect of various process parameters on the bioreduction efficiency, and size and shape of synthesized NPs.

The present invention provides a method of extracting platinum group metals (PGM) from a material, the method comprising:

- a) surface oxidising the material;
- b) ultrasonicated the surface oxidised material of step a) in the presence of an acid; and
- c) bioleaching the ultrasonicated material of step b) in the presence of cyanide in order to form a leachate comprising PGM, the cyanide being formed from a cyanide producing microbe in the presence of a cyanide precursor.

The material can be a catalytic converter or a spent automotive catalyst. Such material in general comprises metals such as Pt, Pd, Rh, Cu, Zn, Fe, Ti, Al, Ba, Ce, Zr, Ni, Ca, and/or Mg, and in other instances can also comprise V, Mn, Nb, Cd, Sn, Sb, and/or Hf. These metals can be present in varying amounts, depending on the manufacturer.

Surface oxidation was found to reduce the dissolution of the PGM from the material in step b). Additionally, the ultrasonication step (step b)) substantially removes metals which may interfere with the extraction of PGM. These metals may include Cu, Zn, Fe and Ti. Accordingly, the recovery of PGM is improved.

The present invention also provides a method of extracting platinum group metals (PGM) from a material, the material further comprising Cu, Zn, Fe, Ti or a combination thereof, the method comprising:

- a) surface oxidising the material;

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b) ultrasonically treating the surface oxidised material of step a) in the presence of an acid; and

c) bioleaching the ultrasonicated material of step b) in the presence of cyanide in order to form a leachate comprising PGM, the cyanide being formed from a cyanide

5 producing microbe in the presence of a cyanide precursor.

In some embodiments, the surface oxidation is performed at about 700 °C to about 900 °C, or preferably at about 850 °C. In some embodiments, the temperature is about 750 °C to about 900 °C, about 800 °C to about 900 °C, or about 800 °C to about 850 °C.

10 The surface oxidation can be performed for about 2 h to about 10 h, or preferably for about 4 h. In some embodiments the duration is about 3 h to about 10 h, about 4 h to about 10 h, or about 4 h to about 8 h.

In some embodiments, the surface oxidised material of step a) is ultrasonicated at a

15 frequency of about 30 kHz to about 80 kHz, or preferably about 37 kHz to about 80 kHz.

In some embodiments, the surface oxidised material of step a) is ultrasonicated at a power of about 30% to about 100%, or preferably of about 70% to about 80%. For example, the sonication power can be varied from about 30% to about 100% when the

20 frequency is at 37 kHz. When the frequency is at 37 kHz, 100% of sonication power is about 120W. For example, the sonication power can be varied from about 30% to about 100% when the frequency is at 80 kHz. When the frequency is at 80 kHz, 100% of sonication power is about 100W. Accordingly, the power can be about 30W to about 120W.

25

In some embodiments, the surface oxidised material of step a) is ultrasonicated for a duration of about 10 min to about 150 min, or preferably for about 70 min to about 80 min. In some embodiments, the duration is about 20 min to about 150 min, about 30 min to about 150 min, about 40 min to about 150 min, about 50 min to about 150 min,

30 about 60 min to about 150 min, or about 70 min to about 150 min.

In some embodiments, the surface oxidised material of step a) is ultrasonicated at a temperature of about 30 °C to about 80 °C, or preferably about 70 °C.

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In some embodiments, the acid is selected from nitric acid, hydrochloric acid, sulphuric acid, or a combination thereof.

5 In some embodiments, the acid has a concentration of about 2 M to about 16 M, or preferably about 8 M to about 9 M. In some embodiments, the concentration is about 4 M to about 16 M, about 6 M to about 16 M, about 6 M to about 14 M, about 6 M to about 12 M, about 6 M to about 10 M, or about 8 M to about 10 M.

10 In some embodiments, the surface oxidised material of step a) is ultrasonicated at a frequency of about 37kHz, a temperature of about 70 °C, a power of about 80% (about 96W to about 100W), a duration of about 80 min, and the acid is nitric acid at about 8 M to about 8.5 M.

15 In some embodiments, the method further comprises a step after step b) of reducing the ultrasonicated material of step b) in the presence of a reducing agent.

20 In some embodiments, the reducing agent selected from formic acid, ascorbic acid, glycolic acid, malonic acid, or a combination thereof. In some embodiments, the reducing agent selected from formic acid, ascorbic acid, or a combination thereof.

In some embodiments, the reducing agent has a concentration of about 1 % v/v to about 15 % v/v, or preferably about 5 % v/v to about 10 % v/v. In some embodiments, the concentration is about 2 % v/v to about 15 % v/v, about 3 % v/v to about 15 % v/v, about 4 % v/v to about 15 % v/v, or about 5 % v/v to about 15 % v/v.

25 In some embodiments, the ultrasonicated material of step b) is reduced for a duration of about 30 min to about 120 minutes, or preferably of about 60 min to about 90 min. In some embodiments, the duration is about 40 min to about 120 min, about 50 min to about 120 min, about 60 min to about 120 min, or about 60 min to about 100 min.

30 In some embodiments, the ultrasonicated material of step b) is reduced at a temperature of about 50 °C to about 90 °C, or preferably of about 50 °C to about 80 °C.

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In some embodiments, the ultrasonicated material of step b) is reduced at a reducing agent concentration of about 5 vol%, duration of about 90 min, and temperature of about 80°C.

- 5 In the presence of a cyanide producing microbe, the cyanide precursor is converted into cyanide. The cyanide is then used to leach PGM from the material. The cyanide precursor can for example be glycine.

- 10 In some embodiments, the cyanide precursor has a concentration of about 0.5 g/L to about 20 g/L, or preferably about 10 g/L. In some embodiments, the concentration is about 1 g/L to about 20 g/L, about 2 g/L to about 20 g/L, about 3 g/L to about 20 g/L, about 4 g/L to about 20 g/L, about 5 g/L to about 20 g/L, about 6 g/L to about 20 g/L, about 7 g/L to about 20 g/L, about 8 g/L to about 20 g/L, about 9 g/L to about 20 g/L, or about 10 g/L to about 20 g/L.

15

- In some embodiments, the ultrasonicated material of step b) is bioleached at a pulp density of about 0.1% w/v to about 12% w/v, or about 0.5% w/v. Pulp density refers to a ratio of a solid component to a liquid component, or a mass of spent automotive catalysts (SAC) or material in unit volume of reagents. In other embodiments, the pulp density is about 0.1% w/v to about 11% w/v, about 0.1% w/v to about 10% w/v, about 0.1% w/v to about 9% w/v, about 0.1% w/v to about 8% w/v, about 0.1% w/v to about 7% w/v, about 0.1% w/v to about 6% w/v, about 0.1% w/v to about 5% w/v, about 0.1% w/v to about 4% w/v, about 0.1% w/v to about 3% w/v, about 0.1% w/v to about 2% w/v, about 0.1% w/v to about 1% w/v, about 0.1% w/v to about 0.8% w/v or about 25 0.1% w/v to about 0.5% w/v.

In some embodiments, the ultrasonicated material of step b) is bioleached at a pH of about 7 to about 11, or preferably about pH 9 to about 10.5.

- 30 In some embodiments, the ultrasonicated material of step b) is bioleached at a temperature of about 22 °C to 38 °C, or preferably about 30 °C.

- In some embodiments, the ultrasonicated material of step b) is bioleached at a cyanide precursor concentration of about 10 g/L, a pulp density of about 0.5 % w/v, a pH of 35 about 9 to about 10.5, and temperature of about 30 °C.

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The bioleaching step can be performed in a single step by mixing the cyanide producing microbe and cyanide precursor with the ultrasonicated material. In this regard, the cyanide is produced *in situ*. In some embodiments, the ultrasonicated material of step
5 b) is bioleached in the presence of the cyanide producing microbe and cyanide precursor under aerobic conditions, the microbe characterised by a HCN synthase operon (*hcnA*, *hcnB*, and *hcnC*) in its genome.

The bioleaching step may be performed as a two-step process, by first separately pre-
10 incubating the cyanide producing microbe and cyanide precursor under aerobic conditions in order to produce the cyanide, and then subsequently mixing the cyanide producing microbe and cyanide precursor with the ultrasonicated material of step b). The cyanide producing microbe can be pre-incubated under aerobic conditions in order to achieve mid-log growth phase of the microbe, following which the cyanide precursor
15 can be mixed in. Additionally, once maximum cyanide production is reached, the pH can be adjusted to achieve optimum cyanide concentration. This has the advantage of improving the bioleaching efficiency as the toxicity of the metals can cause a loss in bacterial activity.

20 Accordingly, in some embodiments, the bioleaching step comprises:
i) pre-incubating the cyanide producing microbe and the cyanide precursor under aerobic conditions in order to produce the cyanide; and
ii) mixing the cyanide producing microbe and the cyanide precursor of step i) with the ultrasonicated material of step b).

25 Alternatively, the bioleaching step can be performed as spent medium leaching. In spent medium leaching, the spent medium (cell free medium) is obtained by first pre-incubating the cyanide producing microbe and cyanide precursor under aerobic conditions in order to produce the cyanide, and then subsequently isolating the cyanide
30 from the cyanide producing microbe. The spent medium is then mixed with the material. This has the advantage of improving the bioleaching efficiency as the toxicity of the leached metals can cause a loss in bacterial activity. The absence of cells ensures a higher concentration of the DO available for PGM-cyanide complexation. There is no consumption of cyanide by the cells, and hence thus results in enhanced PGM recovery.

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The leaching can also be performed under alkaline conditions to minimize the loss of cyanide lixiviant and enhanced PGM recovery.

Accordingly, in some embodiments, the bioleaching step comprises:

- 5 i) pre-incubating the cyanide producing microbe and the cyanide precursor under aerobic conditions in order to produce the cyanide; and
- ii) isolating the cyanide from the cyanide producing microbe in order to form a cell-free medium and mixing the cell-free medium with the ultrasonicated material of step b).

10

This has the advantage of improving the bioleaching efficiency as the toxicity of the metals can cause a loss in bacterial activity.

In some embodiments, step i) comprises pre-incubating the cyanide producing microbe
15 under aerobic conditions in order to achieve mid-log growth phase of the microbe, followed by mixing the pre-incubated cyanide producing microbe with the cyanide precursor. In some embodiments, step i) further comprises a step of adjusting a pH in order to increase cyanide concentration.

20 The cyanide producing microbe can be characterised by a HCN synthase operon (hcnA, hcnB, and hcnC) in its genome.

In some embodiments, the aerobic condition is an O₂ % saturation of about 30%.

25 In some embodiments, the microbe is selected from *Chromobacterium violaceum*, *Pseudomonas fluorescens*, *Bacillus megaterium*, or a combination thereof.

In some embodiments, the pre-incubating step is performed at pH of about 7 to about
11.

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In some embodiments, the step of pre-incubating the cyanide producing microbe comprises incubating the cyanide producing microbe at a pH of about 7.5, followed by incubating the microbe at a pH of about 9. The pH may be maintained by using a base such as sodium hydroxide or an acid such as hydrochloric acid.

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In some embodiments, the cell-free medium has a pH of about 10.5.

In some embodiments, the ultrasonicated material of step b) is bioleached in the presence of the microbe and H_2O_2 , wherein H_2O_2 is at a concentration of about 0.02 % v/v to about 0.16 % v/v, or preferably about 0.08 % v/v.

In some embodiments, the ultrasonicated material of step b) is bioleached in the presence of a ROS scavenger and/or a dispersant. In some embodiments, the ultrasonicated material of step b) is bioleached in the presence of glutathione and polyvinylpyrrolidone (PVP).

In some embodiments, glutathione is added at least 10 h after mixing the reduced material with the microbe. Glutathione acts as an antioxidant or ROS scavenger to minimize oxidative stress in the presence of a high concentration of metals. Other antioxidants may be used, such as nicotinamide adenine dinucleotide phosphate (NADPH), vitamin A, vitamin C, beta-carotene, Ubiquinones, flavonoids, fullerene, alginate.

In some embodiments, the antioxidant (such as glutathione) has a concentration of about 0.2 g/L to about 2 g/L, or preferably about 0.6 g/L.

A dispersant may be added to minimize bacteria-metal interaction. For example, a non-ionic dispersant such as polyvinylpyrrolidone (PVP) may be used. Other dispersants may also be used, such as poly-(ethylene oxide) (PEO), poly(vinyl alcohol-co-vinyl acetate) (PVAL), carboxymethyl cellulose (CMC), polyethylene glycol octylphenyl ether, cetyl trimethyl ammonium bromide (CTAB), ethylene glycol, poly(ethylene glycol) (PEG), sodium dodecyl benzene sulfonate (SDBS), sodium dodecyl sulfate (SDS), sodium polyacrylate, sodium lignin sulfonate (SLS). In some embodiments, the dispersant has a concentration of about 0.2 g/L to about 1 g/L, or preferably about 0.4 g/L.

The material from which PGM (such as Pt, Pd, Rh) is being extracted can be a spent catalyst. Catalysts gradually lose their catalytic activity, usually through structural changes, poisoning, overheating or the deposition of extraneous material such as coke. A catalyst is "spent" when it no longer exhibits the necessary activity or specificity required by the user.

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In some embodiments, the material is a spent catalytic converter or a spent automotive catalyst. A catalytic converter is an exhaust emission control device that converts toxic gases and pollutants in exhaust gas from an internal combustion engine into less-toxic pollutants by catalysing a redox reaction. Catalytic converters are usually used with internal combustion engines fuelled by gasoline or diesel, including lean-burn engines, and sometimes on kerosene heaters and stoves. The catalytic converter is generally a multicomponent material, containing rhodium, platinum and palladium, ceria (CeO_2), γ -alumina (Al_2O_3), and other metal oxides. It typically consists of a ceramic monolith of cordierite ($2\text{Mg}\cdot 2\text{Al}_2\text{O}_3\cdot 5\text{SiO}_2$) with strong porous walls enclosing an array of parallel channels. Cordierite is used because it can withstand the high temperatures in the exhaust, and the high rate of thermal expansion encountered when the engine first starts – typically, the exhaust gas temperature can reach several hundred degrees in less than a minute. Metallic monoliths can also be used. To achieve a large surface area for catalysis, the internal surfaces of the monolith are covered with a thin coating (30–50 μm) of a highly porous material, known as the washcoat. The washcoat generally consists of alumina (70–85%) and oxides which serve as structural promoters (stabilisers to maintain surface area, such as BaO) and chemical promoter (such as CeO_2). This system becomes the support for the PGM (Pt, Pd and Rh). A catalytic converter can be spent when unburn fuel passes through the catalytic converter. As the catalytic converter can become very hot during use, the unburnt fuel can ignite inside the converter resulting in complete or partial or melting of the ceramic catalyst. Other fluids such as antifreeze or oil can also make their way into a vehicle's exhaust system. This creates a thick soot and carbon that coats and clogs the air passages in the ceramic honeycomb catalyst of the converter.

In some embodiments, the method is characterised by a volume of at least 1L.

In some embodiments, the method further comprises a step of bioreducing the leachate of step c) in order to form nanoparticles.

In some embodiments, the leachate of step c) is bioreduced using *Cupriavidus metallidurans*. *Cupriavidus metallidurans* is a non-spore-forming, Gram-negative bacterium which may be adapted to survive heavy metal stress.

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In some embodiments, *C. metallidurans* is pre-incubated.

In some embodiments, the leachate of step c) is bio-reduced at a pH of about 4 to about 8, preferably about 6.

5

In some embodiments, the nanoparticles are characterised by an average size of about 10 nm to about 80 nm.

10 In some embodiments, the nanoparticles are characterised by a hydrodynamic diameter of about 80 nm to about 110 nm.

In some embodiments, the nanoparticles are characterised by polydispersity index of about 0.2 to about 0.3.

15 In some embodiments, the method comprises:

- a) surface oxidising the material;
 - b) ultrasonicated the surface oxidised material of step a) in the presence of an acid; and
 - c) bioleaching the ultrasonicated material of step b) in the presence of cyanide in
- 20 order to form a leachate comprising PGM, the cyanide being formed from a cyanide producing microbe in the presence of a glycine;
- wherein the cyanide producing microbe characterised by a HCN synthase operon (*hcnA*, *hcnB*, and *hcnC*) in its genome.

25 In some embodiments, the method comprises:

- a) surface oxidising the material;
 - b) ultrasonicated the surface oxidised material of step a) in the presence of an acid; and
 - c) bioleaching the ultrasonicated material of step b) in the presence of cyanide in
- 30 order to form a leachate comprising PGM, the cyanide being formed from a cyanide producing microbe in the presence of a glycine;
- wherein the cyanide producing microbe is selected from *Chromobacterium violaceum*, *Pseudomonas fluorescens*, *Bacillus megaterium*, or a combination thereof.

35 The present invention also provides a method of pretreating a material comprising

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platinum group metals (PGM), the method comprising:
ultrasonicing the material in the presence of an acid;
wherein at least a surface of the material is oxidised.

- 5 In some embodiments, the oxidised surface of the material comprises PtO_2 , PdO and/or Rh_2O_3 .

In some embodiments, the acid is nitric acid.

- 10 The present invention provides a method of pretreating a material comprising platinum group metals (PGM), the method comprising:
reducing the material in the presence of a reducing agent;
wherein at least a surface of the material is oxidised.

- 15 In some embodiments, the reducing agent is an organic acid.

In some embodiments, the reducing agent is selected from formic acid and/or ascorbic acid.

- 20 In some embodiments, the method further comprises a step before the reduction step of ultrasonicing the material in the presence of an acid.

The present invention also provides a method of extracting platinum group metals (PGM) from a material, comprising:

- 25 a) bioleaching the material in the presence of cyanide in order to form a leachate comprising PGM, the cyanide being formed from a cyanide producing microbe in the presence of a cyanide precursor.

In some embodiments, the method comprises:

- 30 a) bioleaching the material in the presence of cyanide in order to form a leachate comprising PGM, the cyanide being formed from a cyanide producing microbe in the presence of a cyanide precursor, and additionally in the presence of a ROS scavenger and/or a dispersant.

- 35 In some embodiments, the method comprises:

- 25 -

a) bioleaching the material in the presence of cyanide in order to form a leachate comprising PGM, the cyanide being formed from a cyanide producing microbe in the presence of a cyanide precursor, and additionally in the presence of a ROS scavenger and/or a dispersant;

- 5 wherein the ROS scavenger has a concentration of about 0.2 g/L to about 2 g/L; and wherein the dispersant has a concentration of about 0.2 g/L to about 1 g/L.

In some embodiments, the method comprises:

- a) bioleaching the material in the presence of cyanide in order to form a leachate comprising PGM, the cyanide being formed from a cyanide producing microbe in the presence of a cyanide precursor, and additionally in the presence of a ROS scavenger and/or a dispersant;

wherein the ROS scavenger has a concentration of about 0.2 g/L to about 2 g/L;

wherein the dispersant has a concentration of about 0.2 g/L to about 1 g/L; and

- 15 wherein the ROS scavenger is added at least 10 h after mixing the material with the cyanide producing microbe.

An exemplary description of the workings of the invention is laid out below. In the embodiments that follows, the invention is described in relation to some conditions for consistency to showcase the present invention. However, the skilled person would understand that the invention is not limited to such.

Examples

1. Ultrasound-assisted nitric acid pretreatment for enhanced PGM biorecovery

- 25 The elemental composition of the metal-bearing solids presents challenges to sensitive biological processes. SAC contains a mixture of metal oxides additives and other metals besides PGM. Unfortunately, metals, in particular, copper, zinc, iron, and titanium are present at a high concentration and competitively form stable metal-cyanide complexes which reduce the recovery efficiency of the PGM. Besides these metals, other metals present in SAC are toxic and directly affect bacterial growth and activity. Moreover, the presence of metal oxides creates passivation layers on the surface, thus making it difficult for the leaching lixiviants to extract the desired metals. Therefore, it is necessary to remove these interfering metals during the early stages of the metal recovery process. As the use of ultrasound-assisted nitric acid pretreatment and its effect on the bioextraction of PGM from SAC has not been reported, the inventors

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examine the effect of ultrasound-assisted nitric acid pretreatment of SAC to achieve maximum removal of competing metals copper, zinc, iron, and titanium.

To avoid the loss of Pd during ultrasonication, surface oxidation of Pd presents in SAC was performed prior to ultrasonication. This is because Pt and Rh have minimal solubility in nitric acid while Pd is soluble in nitric acid. The removal of larger quantities of Pd during pretreatment effectively reduces the amount of Pd that can be recovered during bioleaching. Surface oxidation of Pd was performed by heating SAC in a furnace in laboratory air to form a protective oxide layer on the surface to reduce its dissolution in ultrasound-assisted nitric acid pretreatment. The oxidation of PGM at lower temperatures is characterized by the formation of a thin tarnish film, visible or invisible, on their surface. In general, PGM is resistant to oxidation in air. Since Pd is soluble in nitric acid and leached during ultrasonic pretreatment in nitric acid, surface oxidation of Pd was performed prior to ultrasonication to minimize its removal during SAC pretreatment.

Multivariate optimization is a rapid, accurate, and time-saving approach that reduces the total number of experiments that need to be executed for the development of the mathematical models and the assessment of the statistical significance of the factors being studied. Response surface methodology (RSM) is a powerful statistical tool used to evaluate interaction effects and to develop a functional relationship between responses of interest and the process variables. RSM identifies the optimum conditions of the variables which lead to a minimum or a maximum value of a response over a range of interest. To examine the effect of various process variables on ultrasound-assisted pretreatment of SAC, central composite design (CCD) of RSM was applied and a statistical model was built for the optimal percentage removal of Cu, Zn, Fe, and Ti. A multi-objective approach was applied to optimize the various process parameters for SAC pretreatment. The selected parameters and their levels designed by CCD of RSM are shown in Table 1.

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Table 1 Factors and their levels used for ultrasonic-assisted nitric acid pretreatment of SAC

Factors	Units	Low axial	Low factorial	Center point	High factorial	High axial
A-Ultrasound	min	10	50	80	110	150

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duration						
B-Ultrasound power	%	30	40	60	80	100
C-Ultrasound frequency	kHz	-	37	-	80	-
D-Nitric acid conc.	M	2	6	9	12	16
E-Temperature	°C	30	40	55	70	80

The empirical relationship between Cu, Zn, Fe, and Ti extraction in terms of coded factors are given in the following second-order polynomial equation 1, 2, 3, and 4 respectively.

5

$$\text{Cu Extraction (\%)} = 64.32 - 1.47A + 4.59B - 5.64C - 3.32D + 3.52E + 0.28AB - 0.19AC - 0.45AD - 0.42AE - 0.33BC - 0.61BD + 0.01BE - 1.02CD - 1.52CE - 0.95DE - 4.14A^2 - 2.54B^2 - 5.27D^2 - 2.46E^2 \quad (1)$$

$$10 \quad \text{Zn Extraction (\%)} = +71.24 - 1.83A + 4.09B - 6.19C - 3.37D + 3.64E + 0.32AB - 0.80AC - 0.59AD - 0.85AE - 0.50BC - 0.78BD + 0.55BE - 0.56CD - 1.47CE - 0.78DE - 4.01A^2 - 3.35B^2 - 5.80D^2 - 2.95E^2 \quad (2)$$

$$15 \quad \text{Fe Extraction (\%)} = +47.11 - 1.06A + 3.91B - 4.91C - 1.86D + 4.42E + 0.38AB - 0.69AC - 0.24AD - 0.71AE - 0.09BC - 0.24BD + 0.90BE - 0.93CD - 1.16CE - 0.73DE - 3.31A^2 - 2.02B^2 - 3.85D^2 - 1.91E^2 \quad (3)$$

$$20 \quad \text{Ti Extraction (\%)} = +58.42 - 0.91A + 3.96B - 4.86C - 1.37D + 5.01E - 0.41AB - 0.20AC - 0.09AD - 0.12AE - 1.29BC + 0.99BD - 0.31BE - 0.09CD - 0.41CE - 0.66DE - 4.79A^2 - 2.84B^2 - 2.93D^2 - 1.44E^2 \quad (4)$$

A, B, C, D, and E represent ultrasound duration (min), ultrasound power (%), ultrasound frequency (kHz), nitric acid concentration (M), and temperature (°C), respectively. The coefficient of each of the factors (A, B, C, D, and E) in the empirical model (equations 1 to 4) provides a direct indication of their effect on the overall process. An examination of the linear coefficient of these variables in these equations developed by fitting the experimental results shows that:

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- 5 i. For copper removal (%), the order of significance of parameters followed the following order: ultrasound frequency > ultrasound power > temperature > nitric acid > ultrasound duration. Amongst these, ultrasound frequency, nitric acid, and ultrasound duration have a negative linear effect while ultrasound power and temperature have a positive linear effect.
- 10 ii. For zinc removal (%), the order of significance of parameters followed the following order: ultrasound frequency > ultrasound power > temperature > nitric acid > ultrasound duration. Amongst these, ultrasound frequency, nitric acid, and ultrasound duration have a negative linear effect while ultrasound power and temperature have a positive linear effect.
- 15 iii. For iron removal (%), the order of significance of parameters followed the following order: ultrasound frequency > temperature > ultrasound power > nitric acid > ultrasound duration. Amongst these, ultrasound frequency, nitric acid, and ultrasound duration have a negative linear effect while ultrasound power and temperature have a positive linear effect.
- 20 iv. For titanium removal (%), the order of significance of parameters followed the following order: temperature > ultrasound frequency > ultrasound power > nitric acid > ultrasound duration. Amongst these, ultrasound frequency, nitric acid, and ultrasound duration have a negative linear effect while ultrasound power and temperature have a positive linear effect.

An analysis of variance (ANOVA) is required to test the significance and adequacy of the quadratic model and to investigate the effect of process parameters on each response.

25 **Selective extraction of copper, zinc, iron, and titanium**

An analysis of variance (ANOVA) is used to investigate the effect of process parameters on the extraction of Cu, Zn, Fe, and Ti. Statistical analysis was applied to determine the significant terms and process variables and their individual as well as the interactive effect on Cu, Zn, Fe, and Ti removal.

- 30 For Cu, Zn, and Fe extraction, the F-value of the models (21.25 for Cu, 26.61 for Zn, and 23.69 for Fe) and the P-value (<0.0001) indicate that models are highly significant; the likelihood of occurrence of an error, at less than 0.01%, validates the accuracy of the models. In these models, parameters A, B, C, D, E, CE, A², B², C², D², and E² are
- 35 significant terms with higher F-values and p-values less than 0.05. In other words, the

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linear effects of variables A, B, C, D, and E; quadratic terms A^2 , B^2 , C^2 , D^2 , and E^2 ; and interaction between interactive term CE were significant in Cu, Zn, and Fe extraction. The coefficient of variation is 7.68%, 6.57%, and 8.41% for Cu, Zn, and Fe, respectively, suggesting that the models are reproducible and account for the response adequately.

For Ti extraction, the F-value of the model (38.15) and the P-value (<0.0001) indicate that model is highly significant; the likelihood of occurrence of an error, at less than 0.01%, validates the accuracy of the model. In this model, parameters A, B, C, D, E, BC, BD, A^2 , B^2 , C^2 , D^2 , and E^2 are significant terms with higher F-values and p-values less than 0.05. The coefficient of variation is 5.22% for Ti, suggesting that the model is reproducible and explained the response adequately. All models have a relatively high value of R^2 (0.94 for Cu, 0.95 for Zn, 0.94 for Fe, and 0.96 for Ti).

15 **Effect of individual process variables on metal extraction**

The effect of each of the process variables on Cu, Zn, Fe, and Ti extraction are given in Figures 1, 2, 3, and 4, respectively.

Ultrasound duration: The one-factor plot in Figures 1a, 2a, 3a, and 4a shows the relationship between ultrasound duration and percentage extraction of Cu, Zn, Fe, and Ti, respectively with the other parameters kept constant at their central values. An increase in ultrasound duration of up to 70 to 80 minutes led to the maximum extraction of these metals. Sonication results in enhanced metals dissolution due to de-agglomeration and breakage of SAC particles, increase in contact between SAC and liquid medium, and generation of heat caused by ultrasound, but prolonged exposure to sonication results in decreased concentration of dissolved metals. This is due to the adsorption of dissolved metals on oxide surfaces. This phenomenon has been observed in conventional leaching process of metals such as nickel, copper, and cobalt.

Ultrasound power (%): Ultrasound power is the second most important parameter for Cu and Zn, and the third most important parameter for Fe and Ti extraction. The one-factor plot in Figures 1b, 2b, 3b, and 4b shows the relationship between ultrasound power and percentage extraction of Cu, Zn, Fe, and Ti, respectively with the other parameters kept constant at their central values (except for ultrasound frequency which was kept at 37kHz). An increase in ultrasound power of up to 80% resulted in maximum

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metal extraction due to increasing acoustic cavitation at higher power. Further increase in power led to a decrease in metals extraction possibly due to the greater vibrational amplitude at high ultrasound power which results in decoupling between the vibrating plate and the solution, and lower energy transfer efficiency between the sonicator and the liquid medium. This results in lower acoustic cavitation intensity. Such an effect on metals extraction has previously been reported for Cu recovery. Another possible reason is the precipitation of the metals at higher sonication power which results in reduced metals extraction.

- 10 **Ultrasound frequency:** The one-factor plot in Figures 1c, 2c, 3c, and 4c show the relationship between ultrasound frequency and percentage extraction of Cu, Zn, Fe, and Ti, respectively with the other parameters kept constant at their central values. Maximum metal extraction occurred at lower ultrasound frequency. The relationship between ultrasound intensity (I) and frequency (f) is given in equation 5.

$$15 \quad I = \left(\frac{1}{2}\right) \rho c f^2 A^2 \quad (5)$$

where A is the vibrational amplitude, ρ and c is the density and velocity of sound in the liquid medium respectively. At a constant intensity I , the amplitude is higher at low ultrasound frequency, thus generating large cavitation bubbles which result in strong hydromechanical shear forces, and enhanced metals extraction.

- 20 The models described in equations 1-3 show that ultrasound frequency has the most significant effect in the extraction of Cu, Zn, and Fe, while the model described in equation 4 shows that ultrasound frequency is the second most significant parameter in the Ti extraction.

- 25 **Nitric acid concentration:** The one-factor plot in Figures 1d, 2d, 3d, and 4d shows the relationship between nitric acid concentration and percentage extraction of Cu, Zn, Fe, and Ti, respectively with the other parameters kept constant at their central values. Maximum metal extraction was attained at a nitric acid concentration between 8 to 8.5 M. A decrease in metal recovery at higher nitric acid concentration is due to the oxidizing potential of nitric acid which results in oxidation of metals and the formation of a passivation layer on the SAC and hence reduce metal extraction. Similar findings on the role of nitric acid concentration on metals leaching have been reported.
- 30

Temperature: The one-factor plot in Figures 1e, 2e, 3e, and 4e show the relationship between sonication temperature and percentage extraction of Cu, Zn, Fe, and Ti, respectively with the other parameters kept constant at their central values. The increase in temperature had a positive effect on the metal extraction i.e., metal extraction increased with an increase in temperature and maximum metal extraction was attained at a temperature of 70°C. Higher temperature accelerates the reaction between metals and acid, increase the rate of reaction, and enhance the metal extraction.

10 **Interactive effect of process variables on metal extraction**

Response surfaces were plotted between two independent process variables while keeping the other independent variables constant at their central values. The plots for the interactive effect of process variables on the extraction of Cu, Zn, Fe, and Ti are given in Figures 5, 6, 7, and 8, respectively. These plots are based on the experimental conditions set by CCD at their respective responses. The 3D surface graphs show the interactive effect between the two parameters (variables) and their respective response. In 3D surface graphs, the two variable parameters are given on the x-axis while all the other parameters are kept constant at their centre values. The interaction plot shows the interactive effect between the significant interactive term and their respective response.

Ultrasound power (B) and ultrasound frequency (C) are the most important parameters for Cu extraction, and the coefficient in CE show the significant interactive effect on Cu extraction (equation 1). Figure 5 (a-e) show the interactions between the most significant parameter i.e., ultrasound frequency and all the other parameters. Maximum Cu extraction (%) occurred at an ultrasound frequency 37kHz and temperature of 70°C [Figure 5 (a and e)]. Figure 5 (b and d) show that maximum Cu extraction (%) occurred at 8-8.5M nitric acid and at a sonication duration of 80 minutes. Further increase in sonication duration resulted in lower Cu removal due to adsorption of dissolved species on SAC particles, with greater loss at higher acid concentration. At high acid concentration (12M), maximum Cu removal occurred at sonication duration of 75 minutes. The decrease in metal extraction is attributed to the high oxidizing potential of nitric acid at high concentrations which results in oxidation of metals and the formation of a passivation layer on SAC particles. The greater loss in Cu extraction with longer sonication duration and at higher nitric acid concentration is attributed to the

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coupling effect of adsorption of dissolved species and the passivation of the SAC particles. A similar decrease in copper extraction with longer sonication duration has previously been reported.

- 5 Maximum Cu extraction occurred at low ultrasound frequency while a higher concentrated nitric acid is required to extract a reasonable amount of Cu at high ultrasound frequency. High-frequency results in low Cu removal due to the adsorption of dissolved species and small cavitation effect. Therefore, it is concluded that the coupling effect between SAC passivation, small vibrational amplitude, and weak
10 hydromechanical shear forces are responsible for a large decrease in Cu extraction.

Figure 5c shows the interaction between ultrasound frequency and ultrasound power. Maximum Cu extraction (%) occurred at an ultrasound power 80%. Similar findings on the effect of ultrasound frequency and ultrasound power on Cu removal has previously
15 been reported. The interaction between ultrasound power and ultrasound duration at different nitric acid concentrations show that an optimal nitric acid concentration of 8.5M yielded maximum Cu removal. Higher acid concentration resulted in decreased Cu removal possibly due to a significant passivation effect. Overall, several conclusions can be drawn from Figures 5 (a-e): (i) maximum Cu extraction (%) occurred at sonication
20 duration of 80 minutes, (ii) lower metals extraction occurred at higher acid concentration especially with prolonged sonication, (iii) ultrasound power 80% yielded optimal Cu extraction (%), (iv) higher temperature results in higher Cu extraction, and (v) low ultrasound frequency yielded optimal Cu removal and required lower acid concentration.

25 For Zn extraction, ultrasound power (B) and ultrasound frequency (C) are the most important parameters; the coefficient in CE show a significant interactive effect on Zn extraction (equation 2). Figure 6 (a-e) showed the interactions between the most significant parameter i.e., ultrasound frequency and all the other parameters. A similar
30 trend has been observed for the extraction of Cu and Zn. A low concentration of acid (8.5M) favors the dissolution and extraction of Zn for sonication time 80 minutes before it starts decreasing due to adsorption and precipitation on SAC particles. Both Zn and Cu showed an increased removal at low acid concentrations, possibly due to the low passivation effect. An increase in temperature results in higher extraction of Zn and Cu.

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Figure 6c shows the relation between ultrasound power and frequency at constant sonication duration and a constant nitric acid concentration. High ultrasound frequency resulted in lower Zn removal; the decrease was more obvious at low ultrasound power and duration of sonication. This low Zn removal was attributed to small vibrational amplitude and small cavitation effect at high frequency, low ultrasound intensity and insufficient time for metal dissolution. Previous studies have reported that prolonged sonication results in decreased metals solubility but do not affect the rate of dissolution of certain metals including zinc. Our findings on the effect of ultrasound duration on Zn removal show that sonication duration as an individual parameter has the least effect on Zn removal, although interactions with other parameters such as ultrasound power especially at high ultrasound frequency increased its effectiveness. It is concluded that the optimal conditions for Cu extraction favor the Zn removal.

For Fe and Ti extraction, ultrasound frequency (C) and temperature (E) are the most important parameters; the coefficient in CE show a significant interactive effect on Fe extraction while the coefficients in BC and BD show a significant interactive effect on Ti extraction (equations 3 and 4). Figures 7 (a-d) and 8 (a-d) show the interactions between ultrasound frequency and all the other parameters for the extraction of Fe and Ti, respectively. Both Fe and Ti exhibit the similar behavior of individual parameters as was observed for the extraction of Cu and Zn. Similar to Cu and Zn extraction, the coefficient in CE shows the most significant interactive effect on Fe extraction [equation 3, Figure 7 (e)]. However, the coefficients in BC and BD show the most significant interactive effects on Ti extraction [equation 4, Figure 8 (e and f)]. The reaction temperature shows a positive effect on the extraction of Cu, Zn, Fe, and Ti i.e., an increase in temperature results in higher metal removal due to higher reaction rate and increased reaction kinetics, and optimal metal removal was observed at temperature 70°C.

To determine the normality of a data set, a normal probability plot was obtained as shown in Figure 9 (a, c, e, and g). The presence of the points largely on the straight line indicated normal distribution and supported the adequacy of the least square fit for the developed model. The relatively high R^2 values for Cu, Zn, Fe, and Ti show that quadratic models are suitable for representing the experimental data.

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Figure 9 (b, d, f, and h) show the predicted versus actual data for metal extraction. The strong correlation between the predicted and experimental responses showed a good model fit and a high degree of significance for all models. The proximity of most of the points to the 45° line validated the accuracy of the models for response prediction.

5

Optimization and validation

Significant factors and optimal conditions for the extraction of Cu, Zn, Fe, and Ti from SAC in ultrasound-assisted pretreatment were established from fitted regression models. Based on numerical optimization, the maximum extraction of Cu, Zn, Fe, and Ti (80.74%, 86.68%, 59.14, and 70.44, respectively) occurred under optimal condition (i.e., ultrasound power 80%, nitric acid concentration 8.5M, ultrasound duration 80 minutes, ultrasound frequency 37kHz, and temperature 70°C). A validation experiment under this optimal condition showed extraction of Cu (82%), Zn (88%), Fe (60), and Ti (72), which are consistent with the model. The percentage removal of metals during the optimised ultrasound-assisted pretreatment of SAC is given in Table 2.

15

Table 2 Percent metals recovery during ultrasound-assisted nitric acid pretreatment of SAC

Metals removed (%)											
Pt	Pd	Rh	Mg	Fe	Ni	Cu	Zn	Al	Ba	Ca	Ti
5 ± 0.04	17 ± 0.13	7 ± 0.08	39 ± 0.11	60 ± 0.15	29 ± 0.06	82 ± 0.13	88 ± 0.18	30 ± 0.07	94 ± 0.20	84 ± 0.22	72 ± 0.13

It is evident that ultrasound irradiation removed considerable amounts of most of the non-PGM that may otherwise interfere with the recovery of PGM by forming stable complexes with cyanide. Ultrasonic pretreatment resulted in a reduction in the particle size since acoustic cavitation causes breakage of the particles. At lower frequency, this pre-treatment increases particle collision, thereby causing size reduction and increasing surface area which enhances subsequent leaching of metals.

25

2. Enhanced biorecovery of PGM from SAC using *C. violaceum*

To enhance cyanide production and bioleaching efficiency, a BSL-2 organism *C. violaceum* was used. *C. violaceum* is a Gram-negative non-spore forming facultative anaerobe. This bacterium can exploit a wide range of energy sources by using appropriate oxidases and reductases and has been used for the bio-dissolution of the

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- 35 -

metals because of its cyanide-associated metabolic activities. The HCN synthase operon (hcnA, hcnB, and hcnC) in its genome, encoding a formate dehydrogenase and two amino acid oxidases, respectively, are involved in cyanic acid synthesis. Cyanide synthesis occurs in the presence of low levels of oxygen, in which four electrons
5 produced by HCN synthase are transferred to oxygen. Therefore, *C. violaceum* produces HCN under aerobic conditions, with cyanide produced as a secondary metabolite. Cyanogenic bacteria have the intrinsic capability to degrade cyanide; *C. violaceum* and *B. megaterium* synthesize the enzyme β -cyanoalanine synthase which converts cyanide into β -cyanoalanine during the late stationary and early death phases. Nearly all
10 transition metals (except lanthanides and actinides) form stable water-soluble complexes with cyanide which show very high chemical stability.

The low yield of cyanide production in the wild strain of *C. violaceum* and its tight regulation under quorum control has led to the construction of an engineered strain to
15 enhance cyanide production. A metabolically engineered strain of *C. violaceum* was constructed by Tay et al., 2013 at the Department of Biochemistry, Faculty of Medicine, NUS, which is incorporated by reference herein. The engineered strain, named *C. violaceum* pBAD hcnABC carries two sets of cyanide producing operons (while wild strain carries one set of cyanide producing operons) and requires L-(+) – Arabinose as an
20 inducer to induce the expression of the duplicated cyanide producing operons. The metabolically engineered strains of *C. violaceum* were used in bioleaching studies. Pretreated SAC was used for the two-step bioleaching.

The growth (OD_{600nm}) and cyanide production of *C. violaceum* pBAD hcnABC strain at
25 an initial pH 7.5, 8, 9, 10, and 11 were observed. Optimal bacterial growth was observed at pH 7.5, and a long lag phase was observed at pH 10 and 11 due to the alkaline medium. The highest cyanide production was achieved at pH 9 even though the bacterial growth was higher at pH 7.5. The cyanide production peaked towards the early stationary phase. After 30 hours, the cyanide produced by *C. violaceum* pBAD hcnABC
30 at pH 7.5, 8, 9, 10, and 11 was 30.23 mg/L, 33.87 mg/L, 40.44 mg/L, 21.57 mg/L, and 10.18 mg/L, respectively.

Dissolved oxygen (DO) is an important parameter in the biological process as it directly affects bacterial growth, cyanide production, and leaching. Oxygen consumption is
35 regarded as an indicator of metabolic activity, since DO plays a significant role during

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the bacterial growth and leaching as DO levels decreased significantly within 24 hours due to bacterial respiration. To investigate the role of DO on bacterial growth and cyanide production, cell counts (CFU/mL) and cyanide production at an initial pH 7.5, 8, 9, 10, and 11 were measured over six days. The pH values were set before the addition of the inoculum and glycine was added at the mid logarithmic phase. Hydrogen peroxide (H₂O₂, 35% w/w) was used as an additional oxygen source for the bacteria. A predetermined amount of H₂O₂ (0.04 %v/v) was added to the culture 12 hours before sampling from Day 1 to Day 6. The bacterium showed higher growth and produced more cyanide in the presence of H₂O₂. After 6 days, in the absence of H₂O₂, the cyanide produced by *C. violaceum* pBAD *hcnABC* at pH 7.5, 8, 9, 10, and 11 was 33.29 mg/L, 38.27 mg/L, 48.30 mg/L, 35.26 mg/L, and 20.17 mg/L, respectively. After 6 days, in the presence of H₂O₂, the cyanide produced by *C. violaceum* pBAD *hcnABC* at pH 7.5, 8, 9, 10, and 11 was 35.33 mg/L, 41.36 mg/L, 52.20 mg/L, 39.30 mg/L, and 24.37 mg/L, respectively.

Two-step bioleaching was used to recover the PGM from SAC using the metabolically engineered *C. violaceum* pBAD *hcnABC*. Multivariate optimization and RSM technique were used to evaluate individual and interaction effects and to develop a functional relationship between responses of interest and the process variables.

20

Two-step bioleaching using central composite design (CCD)

Two-step bioleaching was carried out in 250 mL Erlenmeyer flasks with 100 mL of culture medium. In two-step bioleaching, bacteria were first grown at pH 7.5 (in the absence of SAC) to achieve optimal bacterial growth followed by a change in pH (before SAC addition) to achieve optimum cyanide concentration. Pre-determined amounts of glycine and pretreated SAC were then added to the culture medium at the mid-log phase of the bacterial growth and 24 hours of inoculation (Day 0), respectively. Samples were taken daily from Day 0 to Day 5 to monitor the cell density, pH, free cyanide concentration, and PGM recovery. Hydrogen peroxide (H₂O₂, 35% w/w) was used as an additional oxygen source for the bacteria. A predetermined amount of hydrogen peroxide (H₂O₂) was added to the culture three hours before the sampling from Day 1 to Day 5. Pretreated SAC was added to the culture medium when cell density and cyanide yield were optimum. The effects of the following five parameters on bioleaching efficiency for PGM recovery were studied: glycine concentration, pulp density, pH, H₂O₂ concentration, and temperature. Central composite design (CCD) of RSM was applied

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and a statistical model was built for the optimal percentage recovery of Pt, Pd, and Rh from SAC. The selected parameters and their levels designed by CCD of RSM are shown in Table 3.

5 **Table 3** Factors and their levels used for two-step bioleaching of SAC batch II

Factors	Units	Low axial	Low factorial	Center point	High factorial	High axial
A-Glycine concentration	g/L	0.5	4	10	16	20
B-Pulp density	% w/v	0.1	0.5	1	2	4
C-pH		7.5	8	9	10	11
D-H ₂ O ₂ concentration	% v/v	0.02	0.04	0.08	0.12	0.16
E-Temperature	°C	22	26	30	34	38

Quadratic models for the recovery of Pt, Pd, and Rh were developed and the empirical relationship between the metal recovery and the coded factors are given in the second-order polynomial equations 6, 7, and 8, respectively.

10

$$\text{Pt Recovery (\%)} = +55.63 + 1.49A - 10.92B + 2.60C + 1.04D + 0.23E + 0.09AB - 0.25AC - 0.25AD + 0.13AE - 0.26BC - 0.18BD - 0.16BE - 0.53CD - 6.55A^2 + 0.73B^2 - 2.59C^2 - 1.29D^2 - 2.74E^2 \quad (6)$$

15

$$\text{Pd Recovery (\%)} = +62.32 + 1.45A - 9.35B + 2.51C + 1.27D + 0.29E - 0.48AB - 0.25AC - 0.13AD + 0.25AE - 0.03BC - 0.34BD - 0.43BE - 0.49CD - 6.58A^2 + 0.21B^2 - 2.29C^2 - 1.13D^2 - 2.30E^2 \quad (7)$$

20

$$\text{Rh Recovery (\%)} = +90.42 + 0.76A - 7.56B + 1.95C + 0.74D + 0.64E + 0.04AB - 0.56AC - 0.19AD + 0.62AE + 1.18BC + 0.71BD + 0.16BE - 0.04CD - 4.09A^2 - 0.28B^2 - 3.21C^2 - 0.36D^2 - 2.08E^2 \quad (8)$$

25

A, B, C, D, and E represent glycine concentration (g/L), pulp density (% w/v), pH, H₂O₂ concentration (% v/v), and temperature (°C), respectively. The coefficient of each of the factors (A, B, C, D, and E) in the empirical model (equations 6 to 8) provides a direct indication of their effect on the overall process. An examination of the linear coefficient of the variables in the equations developed by fitting the experimental results shows the following order of significance of parameters: pulp density > pH > glycine

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concentration > H₂O₂ concentration > temperature. Amongst these, pulp density has a negative linear effect while pH, glycine concentration, H₂O₂ concentration, and temperature have a positive linear effect. For Pt and Pd, the parameters glycine concentration, pulp density, pH, and H₂O₂ concentration showed a significant (p < 0.001) linear effect while for Rh, all the parameters showed a significant (p < 0.001) linear effect.

An analysis of variance (ANOVA) is required to test the significance and adequacy of the quadratic model and to investigate the effect of process parameters on each response.

10

Selective extraction of platinum, palladium, and rhodium

An analysis of variance (ANOVA) of the second order polynomial was used to investigate the effect of process parameters on the recovery of Pt, Pd, and Rh. Statistical analysis technique was applied to determine the significant terms and process variables and their individual as well as the interactive effect on PGM recovery.

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The F-value of the models (60.40 for Pt, 76.85 for Pd, and 80.15 for Rh) and the P-value (<0.0001) indicate that models are highly significant; the likelihood of occurrence of an error, at less than 0.01%, validates the accuracy of the models. In the quadratic models for Pt and Pd recovery, parameters A, B, C, D, E, A², B², C², D², and E² are significant terms with higher F-values and p-values less than 0.05. The coefficient of variation (CV) is a measure of the residual variation of the data relative to the size of the mean. A relatively lower value of the CV indicates a higher precision and reliability of the experimental data. The respective CV at 4.08% and 2.92% for Pt and Pd models suggests that the models are reproducible and accounts for the response adequately. In the quadratic model for Rh recovery, parameters A, B, C, D, E, BC, BD, A², C², and E² are significant terms with higher F-values and p-values less than 0.05. The CV of the Rh recovery model at 1.56% suggests that the model is reproducible and accounts for the response adequately. All models have a relatively high value of R² (0.99 for Pt, 0.98 for Pd, and 0.99 for Rh).

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Individual effect of process variables on PGM recovery

The individual effect of process variables on Pt, Pd, and Rh recovery are given in Figures 10, 11, and 12, respectively. These figures present the one-factor plot in which the relationship between the individual process variable and percentage recovery of PGM is

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shown. In the one-factor plot, one variable was varied over the range of the experimental conditions while the other parameters were kept constant at their central values.

5 **Glycine concentration:** The one-factor plot Figures 10a, 11a, and 12a show the relationship between glycine concentration and recovery of Pt, Pd, and Rh, respectively. An increase in glycine concentration led to the enhanced recovery of Pt, Pd, and Rh. Maximum recovery of PGM was achieved at 10 g/L glycine concentration. Further increase in glycine concentration diminished the PGM recovery. As mentioned earlier,
10 glycine is a precursor in cyanide production. While high concentrations of glycine favor cyanide production and directly effects PGM recovery, too high a concentration of glycine inhibited bacterial growth, thus reduced PGM recovery. Previous studies have reported that glycine beyond an optimal concentration inhibits the synthesis of peptidoglycan in bacterial cell wall.

15

Pulp density: Pulp density shows a significant negative linear effect and is the most important parameter for PGM recovery. The one-factor plot in Figures 10b, 11b, and 12b shows the relationship between pulp density and recovery of Pt, Pd, and Rh, respectively. Maximum recovery of PGM was achieved at 0.5% w/v pulp density; an
20 increase in pulp density significantly reduced PGM recovery. The decrease in PGM recovery at high pulp densities may be attributed to several reasons:

(1) In the presence of higher concentrations of (heavy) metals, toxicity of the metals increases, and viable cell counts decreases. The increased toxicity inhibits bacterial
25 growth and thus cyanide production. Although two-step bioleaching significantly reduced the negative effect of metal toxicity on bacterial growth, a decrease in viable cell counts and cyanide production throughout bioleaching is inevitable. Previous studies on bioleaching of gold from electronic waste have reported similar findings i.e., high pulp density resulted in a negative effect on gold recovery.

30

(2) The presence of higher concentrations of Cu, Zn, Fe, and Ti compete for cyanide lixiviants, thus lower concentration of cyanide is available to form PGM-cyanide complex which results in lower recovery. Figure 13 (a-c) show the effect of pulp density on PGM recovery. It is evident that maximum recovery of Pt, Pd, and Rh was achieved at pulp
35 density of 0.5 %w/v, and higher pulp density led to a decrease in metal recovery.

- 40 -

pH: pH shows a significant positive linear effect and is the second most important parameter for PGM recovery. The one-factor plot in Figures 10c, 11c, and 12c show the relationship between pH and recovery of Pt, Pd, and Rh, respectively. An increase in pH from 7 to 9 significantly enhanced PGM recovery. Recovery of PGM was maximum at pH 9-9.5 and decreased with increase in pH (beyond pH 10). Several reasons are postulated for the pH effect:

(1) Although optimum bacterial growth was observed at pH 7.5 and 8, maximum free cyanide was observed at pH 9-9.5. The cyanide lixiviant occurs in solution as free cyanide which includes the non-dissociated hydrocyanic acid (HCN) and cyanide anion (CN⁻). At pH 7.5 and 8, cyanide in the system is largely present as HCN (which is volatile). Therefore, less cyanide is available for PGM-cyanide complex formation. Despite the optimum bacterial growth at pH 7.5 and 8, the volatility of the cyanide resulted in low metal recovery. However, at pH 9-9.5, free cyanide is largely present as cyanide anion which form PGM-cyanide complexes. Therefore, optimum PGM recovery was achieved at pH 9-9.5.

(2) A low bacterial growth with a long lag phase was observed in an alkaline medium (pH 10 and 11). The alkaline medium inhibits bacterial growth which leads to limited production of cyanide for PGM-cyanide complex formation.

Figure 14 (a-c) show the effect of pH on PGM recovery. It is evident that maximum recovery of Pt, Pd, and Rh was achieved at pH 9.5.

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H₂O₂ concentration: While DO decreases significantly within 24 hours due to respiration of the bacteria, oxygen is also needed for PGM-cyanide complex formation. Figure 10d, 11d, and 12d show that DO plays an important role during bioleaching, and addition of H₂O₂ significantly enhanced bioleaching efficiency. Maximum recovery of PGM was achieved at an optimum H₂O₂ concentration of 0.08 %v/v. Similar findings have been reported in a previous study where the addition of H₂O₂ enhanced gold recovery. The effect of H₂O₂ on PGM recovery is shown in Figure 15 (a-c), where it is evident that addition of H₂O₂ significantly enhanced the recovery of PGM, with the optimal pH at 9.5.

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Temperature: Equations 6, 7, and 8 reveal that of the five parameters examined, temperature is the least significant parameter during bacterial growth and bioleaching and shows a positive linear effect on bacterial growth and PGM recovery. Figure 10e, 11e, and 12e show that within the range of temperature examined (i.e., 22°C to 38°C), maximum PGM recovery was achieved at 30°C, which is also the optimal temperature for the growth of *C. violaceum*.

Interactive effect of process variables on PGM recovery

Response surfaces were plotted between two independent process variables while all other independent variables were kept constant at their central values. The plots for the interactive effect of the process variables on the recovery of Pt, Pd, and Rh are given in Figures 16, 17, and 18, respectively. The 3D surface graphs show the interactive effect between two parameters (variables) and their respective response. In 3D surface graphs, the two parameters are given on the x-axis while all the other parameters are kept constant at their centre values. The interaction plots show the interactive effect between the significant interactive terms and their respective response.

Figures 16 (a-d), 17 (a-d), and 18 (a-d) show the interactions between the most significant parameter (pulp density) and all the other parameters and their relevant response for Pt, Pd, and Rh, respectively. An increase in glycine concentration of up to 10 g/L increased PGM recovery, with this effect more evident at alkaline pH. Any further increase in glycine concentration decreased PGM recovery, possibly due to its negative effect on bacterial growth and cyanide production. Moreover, a greater reduction in PGM recovery was observed at lower concentrations of H₂O₂, with this effect more evident at pH 8. Similarly, maximum PGM recovery was obtained at an optimum glycine concentration of 10 g/L and pulp density of 0.5% w/v. An increase in pulp density significantly reduced PGM recovery, with this effect more evident at higher glycine concentration. This is because higher pulp density and glycine concentrations negatively affect bacterial growth and thus, bioleaching efficiency. The interactive effects between glycine and H₂O₂ concentrations, and glycine and temperature were more obvious at lower glycine concentrations. Lower glycine concentrations in the presence of lower amounts of H₂O₂ resulted in decreased PGM recovery. Similarly, a greater reduction in PGM recovery was observed at lower glycine concentration and extreme conditions. The perturbation plots in Figures 16 (e), 17 (e), and 18 (e) show the sensitivity of the process variables and display the comparative effect of factors at a particular point

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(center point); a steep slope or curvature in a factor shows that the response is sensitive to that factor. A relatively flat line shows insensitivity to change in that factor. The perturbation plots and second-order polynomial equations 6, 7, and 8 show that pulp density, pH, and glycine concentration affect PGM recovery most significantly.

5 Temperature and H₂O₂ showed the least impact, over the range of values examined.

To determine the normality of a data set, a normal probability plot was obtained and shown in Figure 19 (a, c, and e). The presence of the points largely on the straight line indicated normal distribution and supported the adequacy of the least square fit for the developed model. The high R^2 values for Pt (0.99), Pd (0.99), and Rh (0.99) show that
10 quadratic models are suitable for representing the experimental data. Figure 19 (b, d, and f) show the predicted versus actual data for metal extraction. The strong correlation between the predicted and experimental responses shows a good model fit and a high degree of significance for all models. The proximity of most of the points to the 45° line
15 validate the accuracy of the models for response prediction.

Optimization and validation

Significant factors and optimal conditions for the recovery of Pt, Pd, and Rh from SAC were established from the fitted regression models. Based on numerical optimization,
20 the maximum extraction of Pt, Pd, and Rh (at 68.47%, 73.10%, and 97.51%, respectively) was predicted under optimal condition (i.e., glycine concentration 10 g/L, pulp density 0.5%w/v, pH 9.4, H₂O₂ concentration 0.08 %v/v, and temperature 30°C). Validation experiments with three replicates were performed under the optimal conditions and the results were compared with that predicted by the models to validate
25 the surface response models developed. The validation experiments under this optimal condition resulted in the extraction of Pt (69%), Pd (74%), and Rh (99%), a result consistent with the model.

Spent medium leaching at alkaline pH

30 Spent medium leaching under alkaline conditions resulted in higher metal recovery compared to two-step bioleaching. This is due to the following reasons:

(1) In two-step bioleaching, bacteria consume oxygen for respiration, leaving less oxygen available for the metal complex formation. In spent media leaching, decoupling
35 of the bacterial growth and metal complexation lead to the availability of more DO for

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metal complexation. This is a result of cells being separated from the culture after it reached maximum cell density and cyanide production and only cell-free metabolites were used for (spent medium) leaching. For example, the bacteria were grown at pH 9 until the cyanide concentration peaked, after which cell-free medium (spent medium) was collected (via filtration) and the pH was adjusted to pH 10.5, followed by the addition of pretreated SAC. The absence of cells in the leaching medium ensured a higher concentration of the DO available for PGM-cyanide complexation.

(2) Bacteria produce cyanide during the late exponential and early stationary phases. During the stationary phase, the bacteria produce and consume cyanide at the same time. During the late stationary phase, the bacteria convert cyanide into the non-toxic β -cyanoalanine. In cell-free spent medium leaching, there is no consumption of cyanide by the cells, and hence thus resulted in enhanced PGM recovery.

(3) pH influences the dissociation of hydrogen cyanide (HCN, pKa 9.3) as free cyanide ion (CN^-) and H^+ , with low pH favoring a loss of cyanide (as HCN) via volatilization, and high pH favoring dissociation and a higher concentration of free cyanide ion (CN^-). Spent media leaching under alkaline conditions minimized the loss of cyanide lixiviant and enhanced PGM recovery.

Overall, in spent medium leaching, enhanced recovery of Pt, Pd, and Rh result from the absence of (i) conversion of cyanide to β -cyanoalanine, (ii) sorption of metal ions onto biomass, as well as (iii) cyanide loss by outgassing. Spent media leaching experiments were performed under optimum conditions of glycine concentration (10 g/L) and temperature (30°C), in the absence of H_2O_2 . Maximum PGM recovery [Pt (76%), Pd (81%), and Rh (100%)] under alkaline pH was achieved at 0.5 % w/v pulp density.

Table 4 compares the recovery of PGM under two-step bioleaching and spent medium leaching. Spent media leaching at alkaline pH results in the highest PGM recovery.

Table 4 Pt, Pd, and Rh extraction (%) of pretreated SAC (at various pulp densities) by engineered strain of *C. violaceum* in two-step and spent medium leaching.

PGM	*Metal recovery (%)			
	4 %w/v	2 %w/v	1% (w/v)	0.5% (w/v)
Pt	30 (32)	42 (47)	62 (68)	69 (76)

Pd	34 (36)	49 (57)	69 (74)	74 (81)
Rh	62 (67)	74 (93)	96 (98)	99 (100)

* Data (without parentheses) show two-step bioleaching at pH 9.4.
Data (in parentheses) show spent media leaching at pH 10.5

Scale-up of PGM recovery in a bioreactor

5 The present invention also provides a scale-up (from 100 mL to 1 L working volume) of
the PGM recovery through a collective optimization of significant factors. As shown
above, the transition from a small-scale experiment to a large-scale setup that is
feasible for industrial application is not straightforward. The recovery process involves
a multitude of factors which act and/or counteract with and/or against each other, and
10 thus can be antagonistic. Through this study, the inventors have found a condition range
which would provide a synergistic (or at least additive) effect. As examples, scale-up
studies were carried out in a bioreactor using the engineered *C. violaceum* strain at 1
%w/v pulp density under optimized conditions (at glycine concentration 10 g/L, pH 9.4,
and temperature 30°C) in two-step bioleaching. In shake flask experiments, H₂O₂ was
15 added as an additional source of oxygen for the bacteria at specific intervals. In scale-
up studies, dissolved oxygen was supplied and maintained continuously using purified
oxygen (with DO measure as % saturation) during bioleaching. Scale-up studies were
performed under batch and fed-batch modes. In the batch mode, only base and acid
(for pH control), oxygen, and antifoam were added in the sterile culture medium after
20 inoculation in the 1 L culture. In the fed-batch mode, fresh medium was added during
bioleaching (together with the addition of acid, base, DO, and antifoam); with an initial
working volume of 0.5 L, an additional 0.5 L fresh medium was added two days after
SAC addition. Fed-batch mode was applied to enhance bacterial growth, cyanide
production, and metal-cyanide complexation by providing fresh nutrients. The scale-up
25 studies were performed for DO at 10, 20, 30, 40, and 50 % saturation.

Samples were taken daily from Day 0 to Day 5 to monitor the cell density, pH change,
free cyanide concentration, and PGM recovery. The results of the bacterial growth and
cyanide concentration during two-step bioleaching are reported in Figure 20 which also
30 show the impact of DO on bacterial growth and cyanide production. The optimum cell
counts and cyanide concentration during two-step bioleaching occurred in the presence
of DO at 30% saturation. Higher concentrations of DO result in low bacterial growth and
thus, cyanide production due to the following reasons:

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(1) High concentrations of DO were attained at a high airflow rate and stirrer speeds. The shear forces from the impeller result in shear stress which damage the cell wall and hence cell viability.

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(2) At high concentrations of DO, reactive oxygen species (ROS) such as superoxide (O_2^-) accumulate as a by-product of aerobic metabolism which creates oxidative stress. These ROS are toxic to the cells since they are more reactive than molecular oxygen.

10 A comparison of PGM recovery in two-step bioleaching for shake flask experiments and scale-up studies in bioreactor showed that the latter resulted in marginally higher PGM recovery. Using shake flasks, maximum PGM recovery of Pt (62%), Pd (69%), and Rh (96%) was achieved at 1 % w/v pulp density. In the scale-up studies under batch mode, maximum PGM recovery of Pt (64%), Pd (70%), and Rh (98%) was achieved under the
15 same operating conditions, and with DO at 30% saturation. Under fed-batch mode, maximum PGM recovery of Pt (65%), Pd (72%), and Rh (100%) were achieved. Compared to batch mode, the fed-batch mode resulted in only marginally higher PGM recovery. Spent media leaching under batch mode at the optimum conditions resulted in maximum PGM recovery of Pt (70%), Pd (75%), and Rh (100%) at 1 % w/v pulp
20 density. The 1-Liter study in bioreactor not only validated the results of shake-flask experiments but also showed that a marginally higher PGM recovery can be achieved with a better control of DO in the process.

3. Reduction pretreatment for fast and enhanced recovery of PGM

25 As mentioned earlier, the major drawbacks of bioleaching include toxicity of the substrate when operating at high pulp density, slow leaching rate, and low efficiency. The slow leaching rate and low leaching efficiency were caused by the presence of other competing metals that interfere with the leaching process and the presence of a passivating oxide film on the solids. To overcome the former, ultrasonication
30 pretreatment removed competing metals such as Cu, Zn, Ti, and Fe. However, the latter impedes PGM recovery and renders the process slow and less efficient. Upon heating up to 350°C, Pt is converted to PtO_2 by forming a thin solid oxide film which restricts Pt biomobilization. The formation of PdO and Rh_2O_3 takes place at 800-840 and 600 °C, respectively. Both PdO and Rh_2O_3 form a protective layer on the surface which impedes
35 biomobilization. Such temperatures commonly occur in catalytic converters which result

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in the transformation of a portion of PGM to their oxides. These PGM-oxides, though not present in significant quantity, form a protective layer on PGM which leads to low metal recovery. To overcome this problem, SAC reduction pretreatment was performed before leaching to enhance recovery and to increase the rate by reducing PGM-oxides to the elemental forms. For reduction pretreatment, the following three parameters were studied: reducing agent concentration, reduction time, and reduction temperature. Two reducing agents i.e., formic acid and ascorbic acid were used for reduction pretreatment. Reduction pretreatment was performed under the following experimental conditions: reducing agent concentration (1, 5, 10, 15 vol%), reduction time (30, 60, 90, 120 minutes), and reduction temperature (50, 60, 70, 80, 90 °C). Reduction experiments were carried out in a shaking water bath at an agitation rate of 250 rpm. A schematic diagram of the experimental design used in this study is given in Figure 21. A sequential pretreatment technique was applied in which SAC samples were first oxidized with heat (pre-oxidation) followed by ultrasonic-assisted nitric acid treatment (optimized). The SAC samples were then reduced using formic acid or ascorbic acid following by spent media leaching of the reduced SAC under optimized conditions (pH 10.5, glycine concentration 10 g/L, and temperature 30°C).

Effect of process parameters on SAC reduction and PGM recovery

Ultrasonic-assisted nitric acid pretreatment not only removed the competing metals from SAC but also reduced the particles size and thus enhanced its surface area, thus leading to higher leaching efficiency. Figure 22 (a-c) show the effect of SAC reduction on leaching efficiency at pulp density 0.5 %w/v for the recovery of Pt and Pd, and at pulp density 4 %w/v for Rh recovery. Since non-reduced SAC resulted in 100%, 98%, and 93% Rh recovery at pulp densities 0.5, 1, and 2 %w/v, respectively, under spent media leaching, a high pulp density was used to determine the effect of SAC reduction on Rh recovery. Spent medium leaching of the reduced SAC was conducted under pH 10.5, glycine concentration of 10 g/L, and at 30°C.

Reducing agent concentration: Both formic acid and ascorbic acid allow the reduction of PGM-oxides to the metallic forms. These acids were used as both are biodegradable. Figure 22 (a) shows that the optimum concentration for the reducing agent was 5 and 10 %vol for formic acid and ascorbic acid, respectively, and that higher concentration showed no enhancement in leaching efficiency. The leaching efficiency for Pt, Pd, and Rh increased (from 76%, 81%, and 65%) to 81%, 85%, and 69%, for formic acid, and

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84%, 87%, and 69%, for ascorbic acid. Formic acid is a strong reducing agent compared to ascorbic acid; therefore, a lower concentration of formic acid results in high activity and reduction of the passive oxide layers of PGM-oxides. On the other hand, ascorbic acid is a mild reducing agent.

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Reduction time: Figure 22 (b) shows the effect of reduction time on SAC reduction and leaching efficiency. Compared to formic acid which required 90 min to achieve optimum leaching, ascorbic acid required 60 min possibly due to its high concentration. For formic acid SAC reduction, the leaching efficiency for Pt, Pd, and Rh increased marginally from 81%, 85%, and 69%, to 85%, 88%, and 71%, respectively, with an increase in reduction time from 60 min to 90 min. Prolonged reduction durations (>90 min for formic acid and >60 min for ascorbic acid) showed no further enhancement in leaching efficiency, possibly due to the complete reduction at these durations.

15 **Reduction temperature:** Figure 22 (c) shows that an increase in reduction temperature from 50°C to 80°C for formic acid and 50°C to 60°C for ascorbic acid led to an increase in reduction of PGM-oxide and enhanced subsequent leaching. The leaching efficiency for Pt, Pd, and Rh increased from 83%, 87%, and 71%, respectively, to 91%, 95%, and 74%, respectively, for formic acid and from 86%, 88%, and 69%, respectively, to 88%, 90%, and 72%, respectively, for ascorbic acid. Further increase in reduction temperature led to a decrease in PGM recovery due to an increase in the decomposition rate of the reductant.

25 The optimum conditions determined for SAC reduction were reducing agent concentration of 5 and 10 vol% for formic acid and ascorbic acid, respectively; reduction time of 90 and 60 minutes for formic acid and ascorbic acid, respectively; and reduction temperature of 80 and 60 °C for formic acid and ascorbic acid, respectively. Overall, SAC reduction with formic acid resulted in higher PGM recovery. Figure 23 compares PGM recovery from SAC with and without reduction under spent media leaching at different pulp densities (0.5, 1, 2, and 4 %w/v) under optimized conditions. Reduced SAC resulted in higher recovery compared to non-reduced SAC. The maximum recovery of PGM i.e., 91, 95, and 100 % of Pt, Pd, and Rh, respectively, at pulp density 0.5 %w/v; 87, 91, and 100 % of Pt, Pd, and Rh, respectively, at pulp density 1 %w/v; 61, 68, and 100 % of Pt, Pd, and Rh, respectively, at pulp density 2 %w/v; and 39, 45, and

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74 % of Pt, Pd, and Rh, respectively, at pulp density 4 %w/v were achieved under spent media leaching (Day 2) with formic acid reduction.

4. Control measures for microbial-metal interaction and oxidative stress for improved bioleaching at high pulp density

The major drawbacks of biorecovery include slow leaching rate and low efficiency, especially due to toxicity of the substrate which arises when operating at high pulp density. To overcome low leaching efficiency, ultrasonic-assisted nitric acid pretreatment of SAC was applied, and an engineered strain of *C. violaceum* was used to enhance biorecovery. SAC reduction was also performed prior to bioleaching to optimize the process. Ultrasonic pretreatment removed significant concentration of the interfering metals that pose toxicity to bacteria and adversely impact bioleaching efficiency. The engineered strain of *C. violaceum* produced higher concentrations of cyanide that enhanced metal mobilization while SAC reduction increased PGM extraction by removing the passivating oxide film present on the substrate. SAC reduction significantly enhanced PGM recovery at pulp densities of 0.5, 1, 2, and 4 %w/v.

Known strategies employed to enhance leaching efficiency from urban waste include the use of mixed culture of cyanogenic bacteria and application of ultrasound. It has been reported that mixed cultures of *P. aeruginosa* and *C. violaceum* exhibited higher leaching capability than other combinations of mixed cultures examined, as well as single cultures, possibly due to higher tolerance to metal toxicity. Another study on the use of pure and mixed cultures of cyanogenic bacteria reported that pure culture of *C. violaceum* produced more cyanide (20 mg/L) compared to mixed cultures of *C. violaceum* and *P. aeruginosa* (15 mg/L) although the latter showed the highest gold recovery per unit cyanide produced, possibly due to higher tolerance to metal toxicity. However, no information was provided on how a mixed-culture microbial consortium has developed higher tolerance to metal toxicity. It has been reported that sonication can enhance bacterial growth and bioleaching by reducing resistance in the boundary layer between bacterial cell wall and nutrients, improving oxygen and nutrient transport, and increasing the transport of cellular waste products away from the cells. Again, no explanation was given on how sonication enhances bioleaching efficiency without any detrimental and toxic effect on the bacteria, since sonication results in localized high temperature and pressure, and creates stress on bacteria which is detrimental to growth. Moreover, these studies failed to discuss how mixed cultures of cyanogenic

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bacteria and sonication during bioleaching can improve bioleaching efficiency at high pulp density.

Although SAC reduction has been shown to be efficient for enhanced and higher rate of PGM recovery, further study was carried out to develop a more efficient bioleaching technique that can overcome pulp density limitation, reduce microbial-metal interaction, and recover PGM with high efficiency at higher pulp densities. This study aims to investigate microbial-metal interaction and pulp density limitation for the recovery of Pt, Pd, and Rh from SAC at high pulp densities. The specific objectives of this study are to examine oxidative stress on bacteria at high pulp density, to develop a strategy to reduce cytotoxicity of metal ions and oxidative stress that are detrimental for bacterial growth, to minimize particles sorption on bacterial cells, and to enhance bacterial growth, cyanide production and PGM recovery.

15 **Oxidative stress and reactive oxygen species (ROS)**

Oxygen is essential for aerobic bacteria. However, while its concentration below a certain level causes hypoxia which leads to cell death, oxygen concentration over a certain level causes oxidative stress. Besides this, other factors such as ionizing radiation, UV light, ozone, nitrogen oxides, and metals cause bacteria to experience oxidative stress. High concentration of metals leads to oxidative stress, osmotic stress, and metal ion stress in bacteria, due to cytotoxicity of the metal ions. Much of the published work deals with oxidative stress caused by elevated levels of oxygen. It has been reported that microorganisms have developed protective responses to tolerate environmental oxygen concentrations. Reactive oxygen species (ROS) accumulate as a by-product of aerobic metabolism when oxygen concentration surpasses the air saturation level. ROS can be generated by endogenous as well as exogenous sources. These ROS which include superoxide anion radicals, hydroxyl radicals, and hydrogen peroxide are more reactive than molecular oxygen and are toxic to bacteria.

Higher concentrations of H₂O₂ in shake flask experiments and molecular oxygen in bioreactor scale-up exert negative effect on the overall bioleaching efficiency. This occurs due to oxidative stress caused by elevated concentrations of these molecules. Previous studies have focused on the mechanisms by which these compounds are produced intracellularly and damage cellular components. The physiological response of bacteria exposed to high concentrations of metals, metal toxicity, and osmotic stress

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which produce oxidative stress as a secondary reaction to survive in the presence of high concentrations of heavy metals has not been reported. High concentration of metals produces oxidative stress which creates an imbalance between the generation and elimination of ROS (through endogenous and exogenous antioxidants). The resultant increase in concentration of ROS exerts a negative effect on bacterial growth, metabolism, enzyme activity, gene expression and causes damage to DNA, protein, and lipids which finally lead to cell damage and cell death.

Bacteria possess enzymatic and non-enzymatic defense mechanisms and repair systems which protect them against damage caused by oxidative stress and controls the damage by inactivating ROS. The native antioxidant defense mechanisms are generally inadequate to protect them against oxidative damage, and antioxidant additives are generally used. Exposure to high concentrations of ROS compromises the endogenous antioxidant system, and exogenous antioxidants are required to compensate for the deficiency of antioxidants. Even though studies have reported the detrimental effects of ROS on bacterial growth and cell functions, its role during bioleaching at high pulp density appears to be largely overlooked. One such study reported that accumulation of copper ions (Cu^{+2}) can lead to increased intracellular ROS and death of *Acidithiobacillus ferrooxidans*. High concentrations of iron reportedly lead to oxidative stress which damages biomolecules (carbohydrates, lipids, proteins, and nucleic acids) and causes cell death. It has been shown that SAC comprises many heavy metals at high concentrations which, at a pulp density of 4 %w/v, led to cell death. What is not yet clear however is the toxic effect of metals on bacterial growth, cyanide production, and bioleaching efficiency at high pulp density in terms of ROS and oxidative stress. Therefore, it is important to investigate the influence of ROS and oxidative stress on bioleaching efficiency.

Microbial-metal interaction

Microbial-metal interaction also influences metal recovery, especially in a high pulp density system. In bioleaching, metallic particles not only agglomerate due to gravitational forces but specific sorption of these particles on the cells also occurs. Since bioleaching is a diffusion-controlled mechanism, the sorption of cells on metal particles and particle agglomeration restricts bioleaching and limits metal recovery. Extracellular polymeric substances (EPS) in which biofilm microorganisms are embedded are responsible for the attachment of biofilms to the particle surface. Although the complete

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profile of EPS is not fully established, it is held that EPS consist of polysaccharides, proteins, glycoproteins, and glycolipids. Due to the presence of reactive groups in EPS, the polymers are affected by chelators, ions, and surface-active compounds. Sorptive EPS consists of charged groups and binds various metals, nutrients, ROS, and
5 contaminants. Although EPS has an important role in the removal of heavy metal from the environment due to its ability to bind metal ions from solutions (which prevents their entrance into bacterial cells, and protects bacteria against environmental stress), the sorption of cells and EPS on the particle surface effectively reduces bioleaching efficiency. Therefore, it is speculated that bioleaching efficiency can be enhanced by
10 minimizing the attachment of cells and EPS to metal particles.

Accordingly, we introduced a strategy that further enhances bioleaching efficiency at higher pulp densities by simultaneously controlling the oxidative stress and the negative impact of microbial-metal interaction. A two-step bioleaching technique was developed
15 that minimizes oxidative stress in the presence of a high concentration of metals by introducing an antioxidant glutathione (GSH), and minimizes bacteria-metal interaction by the addition of a dispersant polyvinylpyrrolidone (PVP). Two-step bioleaching was carried out in 250 mL Erlenmeyer flasks with 100 mL of culture medium. Bioleaching experiments were carried out without (control) and with GSH and PVP. PVP was added
20 in the culture at the time of the addition of SAC while GSH was added 12 hours after the addition of SAC. Overproduction of ROS in cells was determined by using three different oxidative-stress-sensitive probes which are commonly used to measure intracellular ROS. Three probes namely dihydrorhodamine 123 (DHR 123), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), and dihydroethidium (DHE) were used.
25 The effect of PVP on bioleaching was determined by measuring zeta potential.

Two-step bioleaching at pulp densities of 0.5, 1, 2, and 4 %w/v

Two-step bioleaching by *C. violaceum* was carried out to investigate the effect of pulp density on bacterial growth, cyanide production, pH variations, and bioleaching
30 efficiency at pulp densities at 0.5, 1, 2, and 4 %w/v. Figure 24 (a-c) shows the effect of pulp density on PGM recovery. The figure shows that maximum recovery of Pt, Pd, and Rh was achieved at a pulp density of 0.5 %w/v and that an increase in pulp density results in decreased PGM recovery. At low pulp densities of 0.5 and 1 %w/v, a slight increase in recovery was observed from day 1 to day 4 indicating bacterial activity and
35 cyanide production. At pulp densities of 2 and 4 %w/v, negligible increase in PGM

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recovery was generally observed after day 1, indicating loss of bacterial activity (see Figure 25). The bioleaching efficiencies of Pt, Pd, and Rh at pulp densities 0.5, 1, 2, and 4 %w/v were 69 %, 74 %, and 99 %; 62 %, 67 %, and 95 %; 42 %, 48 %, and 84 %; and 30 %, 33 %, and 81%, respectively. This is because high concentrations of metals
5 adversely affect viable cells thus leading to low cyanide production and resulting in low metal recovery.

The results presented in Figure 24 are consistent with the data in Figure 25 which shows the free cyanide concentration and cell counts over five days during two-step
10 bioleaching. Figure 25 (a-b) shows that a gradual decrease in cell counts and free cyanide concentration was observed from day 1 to day 4 at pulp densities of 0.5 and 1 %w/v, indicating the presence of viable cells, bacterial activity, and production of cyanide. At pulp density of 2%w/v, viable cells observed after day 3 were low while at pulp density of 4%w/v, no viable cells were observed from day 3. The free cyanide
15 concentration at pulp densities of 0.5, 1, 2, and 4 %w/v were 6.44, 4.35, 2.14, and 0.80 mg/L on day 4. The decrease in viable cells and cyanide production due to an increase in pulp density during bioleaching demonstrated the decrease in the bacterial activity which suppressed the bioleaching efficiency.

20 The toxicity of metals was the direct cause of the loss of bacterial activity and bioleaching efficiency. The unfavourable conditions (such as changes in pH and depleted nutrient supply and dissolved oxygen) might be another cause of decreased bioleaching efficiency. Bioreactor studies (at 1 litre) under fed-batch mode where pH and elevated levels of oxygen were maintained throughout the bioleaching showed similar findings.
25 Therefore, it is concluded that toxicity of the metals at high pulp densities limits the bioleaching efficiency. To determine the effect of metal toxicity (especially at high pulp density) on bioleaching, all subsequent two-step bioleaching experiments were carried out at pulp densities of 4 %w/v, 8 %w/v, and 12 %w/v. It is important to note that SAC is acidic, and that the addition of SAC in the culture resulted in a decrease in pH,
30 especially at high pulp densities. The pH of the culture after the addition of SAC at pulp density 8 and 12 %w/v were between pH 3 to pH 4. The pH of the culture during two-step bioleaching was maintained at pH 9.4 (i.e., the optimal pH for maximum cyanide production and PGM recovery) after the addition of SAC to the culture.

35 **Generation of intracellular ROS during bioleaching**

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SAC has a very complex composition and contains a variety of metals (as shown in Table 2). At high concentrations, these metals are toxic to the bacteria which leads to a loss in bacterial growth and cyanide production and hence bioleaching activity. The toxicity imposed by the presence of multi-metals creates metal stress in bacteria which leads to oxidative stress and induces excessive intracellular ROS, which leads to cell death. ROS are generated as by-products of aerobic metabolism and exposure to various natural and synthetic toxicants. It is reported that exposure of microbes to clinical antibiotics, solvents, metals, and heat results in oxidative stress. It is assumed that any stress that physically impairs redox enzymes could cause oxidative stress. Elevated levels of ROS are potentially damaging for proteins, lipids, and nucleic acids. At low pulp density, bacterial growth and cyanide production are high, and any adverse effects of metal toxicity, metal and oxidative stress are low. However, at high pulp densities, the synergistic effect of metal toxicity, metal and oxidative stress adversely affects bioleaching efficiency.

The production of intracellular ROS during bioleaching was monitored using oxidative stress-sensitive probes dihydrorhodamine-123 (DHR-123), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), and dihydroethidium (DHE). DHR-123 is a specific ROS mitochondrial stain while DCFH-DA is a cytosolic stain. The fluorescent rhodamine 123, the product of nonfluorescent DHR-123 oxidation, is positively charged and binds selectively to the inner mitochondrial membrane of living cells. The oxidation of DHR-123 is catalysed by the enzyme peroxidase and measured using excitation and an emission wavelength of 505 and 535 nm, respectively. Upon uptake by living cells, the acetyl groups in DCFH-DA are removed by membrane esterases to form 2',7'-dichlorodihydrofluorescein (DCFH). The nonfluorescent DCFH is highly sensitive to ROS (such as RO, RO₂, HOCl, OH, and ONOO⁻) and is oxidised to the highly fluorescent 2',7'-dichlorofluorescein and measured using excitation and an emission wavelength of 504 and 524 nm, respectively. DHE is specific for O₂⁻ (one-electron reduction product of O₂) with minimum oxidation induced by HOCl, ONOO⁻, and H₂O₂. However, O₂⁻ can lead to the formation of H₂O₂, [•]OH, and reactive nitrogen species (RNS). The oxidation of DHE by O₂⁻ produces 2-hydroethidium (EOH) and intermediate products. The possibility of the oxidation of intermediate products to fluorescent ethidium (E⁺) by H₂O₂ and [•]OH is less. E⁺ fluorescence is measured at an excitation of 500–530nm and emission of 590–620nm, respectively. The EOH fluorescence is measured at excitation and an emission

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wavelength of 480 and 567 nm, respectively. The fluorescence values are positively correlated with the ROS content.

The changes in intracellular ROS content with time during two-step bioleaching at pulp densities of 4, 8, and 12 %w/v are given in Figure 26 (a-c). These figures show that the intracellular ROS content increased with an increase in metal concentrations and over time. At high pulp density, the accumulation of intracellular ROS content was greater compared to low pulp density. The increase in intracellular ROS content adversely affects bacterial growth, cyanide production, and bioleaching efficiency. The corresponding value of cell counts and free cyanide concentration during two-step bioleaching at different pulp densities are given in Figure 27 (a). The free cyanide concentration was 52.04 mg/L on day 0. In control experiments, the free cyanide concentration was 56.14 mg/L on day 2 whereas the free cyanide concentration at pulp densities of 4, 8, and 12 %w/v were 4.45 mg/L, 2.31 mg/L, and 1.13 mg/L on day 2. In control experiments, a gradual decrease in cell counts and a slight increase in cyanide concentration was observed indicating bacterial activity. In contrast, a decrease in cell count and cyanide concentration was observed in the presence of SAC, indicating loss of bacterial activity and cyanide production, and cyanide consumption within 48 hours of the bioleaching process. Therefore, it is concluded from Figures 26 (a-c) and 27 (a) that the toxicity of metals creates oxidative stress in cells which results in the accumulation of intracellular ROS and causes cells death. The resulting PGM recovery is given in Figure 27 (b). The maximum recovery of PGM i.e., 29, 32, and 61 % of Pt, Pd, and Rh, respectively, at pulp density 4 %w/v; 24, 26, and 39 % of Pt, Pd, and Rh, respectively, at pulp density 8 %w/v; and 18, 23, and 36 % of Pt, Pd, and Rh, respectively, at pulp density 12 %w/v were achieved on day 2.

Effect of exogenous antioxidant on intracellular ROS, bacterial activity, and metal recovery

The dramatic increase in endogenous ROS by oxidative stress constitutes threat to cells viability. However, microorganisms have mechanisms to scavenge intracellular ROS and to maintain a balance between generation and elimination of ROS within the cells. There exist non-enzymatic antioxidants and intracellular enzymatic antioxidant systems in microorganisms that scavenge excess ROS and minimize oxidative stress within the cells. However, endogenous ROS scavenging system may not be sufficient to regulate intracellular ROS and prevent oxidative stress, since the endogenous antioxidant system

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is compromised at a high concentration of ROS. It is important to carefully evaluate the activity and capacity of exogenous antioxidant. The time and concentration of the antioxidant required to inhibit a defined concentration of the ROS is an important factor to be considered.

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Glutathione (GSH) is made up of three amino acids viz. glutamate, cysteine and glycine and has antioxidant, immune-boosting, and cellular detoxifying properties. It is used to eliminate intracellular ROS to protect cells against ionic, osmotic, and oxidative damage, and has been used to improve the growth activity of bacteria. The use of GSH as ROS

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Exogenous GSH was added 12 hours after the addition of the SAC (i.e., after the start of the bioleaching) at the following concentrations: 0, 0.2, 0.4, 0.6, 0.8, 1, 1.5, and 2 (g/L). GSH-free (control) bioleaching experiments were conducted to distinguish the effect of GSH on intracellular ROS, bacterial growth, cyanide production, and bioleaching activity. The initial bioleaching experiments were conducted at pulp density 4 %w/v. Figure 28 (a-c) shows the intracellular ROS content at a pulp density of 4 %w/v and different GSH concentrations during two-step bioleaching. The intracellular ROS content in the GSH-free group increased continuously (from 12 hours to 96 hours) arising from induced oxidative stress in the bacteria due to the presence of metals. A closer examination at the GSH-containing group showed that the addition of 0.6 g/L of GSH resulted in the maximum inhibition of ROS; any increase or decrease in GSH concentration resulted in decreased inhibition of ROS. A continuous increase in ROS content was observed (as in the GSH-free control) after the addition of GSH at a low concentration of 0.2 g/L and high concentrations of 1.5 and 2 g/L, although the magnitude of increase was lower compared to the GSH-free group, possibly due to low activity of GSH at low concentration and negative impact on cells at high concentrations. A recent study on the bioleaching of Li and Co from LiCoO_2 reported that the addition of 0.3 g/L GSH increased the bioleaching efficiency of Li from 87.6% to 98.1% and Co from 87.0% to 96.3% at 5.0% pulp density of LiCoO_2 .

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The corresponding cell counts at different GSH concentrations during two-step bioleaching at pulp density of 4 %w/v are given in Figure 29. A decrease in viable cell counts was observed in the GSH-free group with no viable cells were observed after day 3 whereas cells viability (and thus activity) was observed in GSH-containing groups over

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four days. The optimum number of viable cells were found in the culture in the presence of 0.6 g/L of GSH, which corroborated the results presented in Figure 28 i.e., the addition of GSH (optimum value of GSH: 0.6 g/L) inhibited ROS accumulation, eliminated oxidative stress, and increased bacterial activity (growth and cyanide production). Since bacteria possess enzymatic and non-enzymatic defense mechanisms against oxidative stress, a possible reason for ROS inhibition is that the addition of exogenous GSH possibly enhanced intracellular ROS scavenging and antioxidant activities of bacteria which results in enhanced bacterial activity. Another possible reason is that the addition of exogenous GSH can stimulate the production of non-enzymatic small molecule antioxidants.

Figure 30 shows PGM recovery during two-step bioleaching at a pulp density of 4 %w/v and at different GSH concentrations. At high pulp density, metal toxicity induced oxidative stress in bacteria which killed all the viable cells within two days after the addition of SAC. The loss of bacterial activity reduced cyanide production and correspondingly lowered bioleaching efficiency. The addition of exogenous GSH significantly enhanced intracellular ROS scavenging activity, reduced the intracellular ROS content, and greatly enhanced the bacterial activity, cyanide production, and thus bioleaching efficiency. As shown in Figure 30, significantly higher bioleaching efficiency was achieved after the addition of GSH. The maximum recovery of Pt, Pd, and Rh achieved was 30%, 33%, and 62%, respectively, in the absence of GSH (control) and 56%, 64%, and 80%, respectively, in the presence of GSH, representing an increase of 87%, 94%, and 29%, respectively.

Effect of exogenous dispersant on microbial-metal interaction and metal recovery

The toxic effects of heavy metals on soil microorganisms have been extensively studied. For example, it is reported that heavy metals contamination has a harmful effect on bacterial respiration and exposure to metals toxicity results in loss of viable cells. However, the negative impact of metals on bacterial activity during bioleaching at high pulp density has not been well addressed. It has earlier been shown that complete growth inhibition occurred at high metal concentrations (Figure 25). Although GSH scavenges intracellular ROS and increases bacterial activity, the formation of biofilm and adsorption of bacteria on metal particles may inhibit bioleaching. It has been reported that cells adsorbed on silver particles resulted in low bioleaching. The functional

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groups in cell wall are responsible for sorption of substrate on cell surface which inhibits metal-cyanide complexation. Metals can also cause ion imbalance by adhering to the cell surface and alter enzymatic functions. Low bacterial growth occurs in the presence of metals, with bacteria developing metal tolerance mechanisms by producing
5 extracellular polymeric substance (EPS) and by forming cell surface complexes. This inhibition in growth arising from the EPS can occur either by decreasing the removal of metabolic products from the bacterial cells or by decreasing oxygen transfer. Therefore, EPS binding and bacterial attachment to metal particles may limit cyanide and oxygen diffusion in diffusion-controlled bioleaching.

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It is known that many chelating agents can reduce metal toxicity. Polyvinyl pyrrolidone (PVP) is a chemically inert, biodegradable, and biocompatible polymer that is extensively used in green chemistry applications. It is pH-stable and temperature resistant and is used in nanoparticle synthesis as a capping agent due to its non-ionic
15 dispersant property. PVP is a water-soluble and non-toxic amorphous polymer with film-forming ability, good binding properties, and adhesive power, and serves as a surface stabilizer and growth modifier, and hampers particle agglomeration. Therefore, it is reasoned that bioleaching efficiency can be enhanced by suppressing nonspecific binding and sorption of cells onto the particles. PVP may be used to disperse bacteria and SAC,
20 and to enhance contact between metal particles and cyanide lixiviants.

Exogenous PVP was introduced with the addition of the SAC (at the start of bioleaching) at the following concentrations: 0, 0.2, 0.4, 0.6, 0.8, 1 (g/L). PVP-free (control) bioleaching experiments were also conducted to distinguish the effect of PVP on bacterial
25 growth, cyanide production, and bioleaching activity. The initial bioleaching experiments were conducted at a pulp density of 4 %w/v. The effect of PVP on bacterial activity was investigated by measuring the zeta potential of the culture. In the absence of PVP, the negatively charged bacterial surface and EPS in which biofilm microorganisms are embedded tend to adsorb the particles onto the biofilm, thus enhancing particle
30 agglomeration and reducing the active surface of the particles.

Table 5 shows the effect of PVP on the zeta potential of bacterial culture at different periods during two-step bioleaching at a pulp density of 4 %w/v. For zeta potential measurements, each experiment was conducted in triplicates and the average data are
35 presented. Since the bacterial surface is negatively charged, it interacts with PVP in an

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- aqueous solution and a large decrease in zeta potential was observed. EPS which contain both hydrophilic and hydrophobic sites interact with particles due to its binding characteristics but the addition of PVP suppress the nonspecific binding between particles and EPS as well as undesired adsorption of cells on particles which result in the
- 5 dispersion of biofilm. PVP has no negative impact on bacterial growth and cyanide production. The decrease in zeta potential was greater at higher concentrations of PVP i.e., 0.4, 0.6, 0.8, and 1 g/L. The binding constant of thiocyanate (SCN^-) anion to PVP is 5.3 showing that PVP has a strong binding affinity to anions. Upon SAC addition to bacterial culture, PGM form complexes with cyanide in the presence of oxygen. The
- 10 PGM-cyanide complexes were formed during bioleaching and an increase in zeta potential was observed which might be due to the greater affinity of PVP towards PGM-cyanide complexes (Table 5). The increase in zeta potential was directly related to the freeing of negative sites on the bacterial surface which was previously occupied by PVP in aqueous solution. PVP not only suppressed the adsorption of cells on SAC particles
- 15 and EPS binding to SAC but also hampered direct contact between SAC and cells which resulted in reduced metal toxicity towards the cells. Therefore, in the presence of PVP, the SAC is less likely to be attached to the biofilm, thus enabling PGM-cyanide complexation to occur.
- 20 PVP reportedly hampers particle agglomeration. The dispersed particles enhanced the reaction between metals and cyanide which resulted in an increased rate of reaction. Moreover, due to the reduced toxicity of metals towards cells, cells growth and cyanide production increased. Since bioleaching is a diffusion-controlled mechanism and metal-cyanide complexation occurs at the solid-liquid interface, it is concluded that the
- 25 addition of PVP results in enhanced metal-cyanide complexation, reduced reaction time, and enhanced bioleaching efficiency. Upon SAC addition, the maximum increase in zeta potential of bacterial culture was achieved at a PVP concentration of 0.4 g/L. A study on the bioleaching of Ag from waste printed circuit boards reported that the addition of 0.24 g/L PVP increased the Ag recovery to 1.8 times.
- 30 The distribution of the SAC particles in the bacteria solutions was observed by TEM and are given in Figure 31 (a-f). Figure 31 (a-c) shows the adsorption of the SAC particles on bacteria in the absence of PVP. Figure 31 c shows that SAC particles are adsorbed on bacteria and encapsulated in a matrix of EPS. Figure 31 d shows that SAC particles
- 35 agglomerate in the absence of PVP due to high surface energy, interfacial energy, and

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- attraction forces. Figure 31 (e-f) shows the distribution of the SAC particles in the bacterial solution after the addition of PVP (0.4 g/L). The addition of PVP hindered the agglomeration of SAC particles as well as the adsorption of SAC particles on bacteria. Figure 32 shows the cell counts and free cyanide concentration during two-step
- 5 bioleaching at a pulp density of 4 %w/v, GSH concentration 0.6 g/L, and PVP concentration 0.4 g/L. The addition of PVP had a positive effect on bacterial growth and cyanide production during two-step bioleaching by minimizing the negative impact of metal toxicity.
- 10 Figure 33 shows the effect of GSH and PVP on PGM recovery during two-step bioleaching at pulp density 4 %w/v. GSH and PVP was added at optimal concentrations (at 0.6 g/L, and 0.4 g/L respectively) at pre-determined time periods. Evidently, the addition of GSH and PVP enhanced PGM recovery. The maximum recovery achieved for Pt, Pd, and Rh was 30%, 33%, and 62%, respectively, in control experiments and 56%, 64%, and
- 15 80%, respectively, in the presence of GSH. The corresponding values for Pt, Pd, and Rh were 68%, 74%, and 86%, respectively, in the presence of GSH and PVP. The overall effect of GSH and PVP resulted in prolonged durations for bacterial growth and cyanide production and thus, bioleaching activity. The scavenging of intracellular ROS and reduced oxidative stress, reduced cells adsorption and EPS binding with particles, and
- 20 increased contact between cyanide lixivants and metals proved to be a useful strategy for enhanced bioleaching efficiency.

Table 5 The effect of PVP on zeta potential of bacterial culture during two-step bioleaching at pulp density 4 %w/v.

Time (Hours)	PVP concentration (g/L)					
	0	0.2	0.4	0.6	0.8	1
Zeta potential (mV) w/o SAC						
24	-32.91	-26.63	-21.44	-21.17	-20.25	-20.23
48	-34.13	-25.34	-20.85	-19.89	-19.04	-19.65
72	-34.09	-25.97	-20.12	-19.46	-17.86	-18.62
Zeta potential (mV) w/ SAC at 4% (w/v)						
24	-24.64	-28.41	-29.33	-26.61	-26.08	-23.34
48	-24.18	-28.72	-32.17	-28.54	-26.47	-24.05

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72	-24.39	-29.26	-32.64	-28.39	-27.12	-24.34
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Two-step bioleaching at high pulp densities

Two-step bioleaching was performed at pulp densities 4, 8, and 12 %w/v in the absence and presence of GSH and PVP. Figure 34 shows the viable cell counts and free cyanide concentration at pulp densities 4, 8 and 12 %w/v. The results were consistent at all pulp densities which shows the effectiveness of the strategy. At all pulp densities, the addition of antioxidant and dispersant resulted in increased growth and higher cyanide production. The corresponding data on the PGM recovery at pulp densities 4, 8 and 12 %w/v are given in Figure 35. The addition of GSH and PVP enhanced the PGM recovery at all pulp densities. In control experiments (when no GSH and PVP was added), bioleaching efficiencies of Pt, Pd, and Rh at pulp densities 4, 8, and 12 %w/v were 30 %, 33 %, and 62 %; 25 %, 27 %, and 40 %; and 18 %, 23 %, and 36 %, respectively. However, when GSH and PVP were added, bioleaching efficiencies of Pt, Pd, and Rh at pulp densities 4, 8, and 12 %w/v were increased to 68 %, 74 %, and 86 %; 53 %, 57 %, and 73 %; and 37 %, 45 %, and 61 %, respectively. It has been shown that reduced SAC resulted in greater PGM recovery compared to non-reduced SAC. This is because the thin oxide layer on the surface of the particles hampers the metal mobilization and hence restricts the bioleaching. Figure 36 compares PGM recovery during two-step bioleaching using non-reduced and reduced SAC at a pulp density of 4 %w/v, in the absence and presence of GSH (0.6 g/L) and PVP (0.4 g/L). Compared to non-reduced SAC, reduced SAC resulted in an overall higher PGM recovery i.e., 73% Pt, 82% Pd, and 90% Rh at pulp density 4 %w/v.

5. Bioreduction of SAC leachate by *C. metallidurans*

SAC leachate was used in green synthesis of Pt and Pd NPs. The initial objective of the reduction study was to identify the optimum conditions for the growth of *Cupriavidus metallidurans*. For this reason, a comprehensive study on bacterial growth at an initial pH of 4, 6, 7, 8, and 10 and at 30 °C and 150 rpm was conducted. The higher bacterial growth was observed at an initial pH of 6, 7, and 8 and the optimal bacterial growth was observed at pH 6. The pH of the bacterial culture tends to increase and stabilize near a pH of 7.5. The growth inhibition at pH 10 might be due to the cell lysis or denaturation of cellular macromolecules.

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The bioreduction of Pt(II) and Pd(II) ions and biosynthesis of Pt and Pd NPs were studied. The spent medium leachate was used for the bioreduction of Pt(II) and Pd(II) ions. Two-step bioreduction was performed where the bacterium was grown separately in peptone meat extract (PME) medium at pH 6. Before bioreduction experiments, the culture was transferred (10% vol./vol. of inoculum at early stationary phase) to fresh medium and grown to early stationary growth phase (in the absence of metal ions) to maximize the amount of metabolically active biomass. Upon reaching the early stationary phase, cells were collected by centrifugation at 5000 rpm for 15 minutes and washed three times with sterile-filtered 0.9 wt.% saline solution. The cell pellets were suspended in 9 g/L saline solution and used as stock for inoculation. The starting bacterial biomass for bioreduction was taken when cell culture was grown until the stationary phase. The pH was adjusted to the predetermined values, followed by the addition of a predetermined amount of SAC spent medium leachate and incubated for 48 hours. Samples were taken at pre-determined sampling intervals, centrifuged at 10,000 rpm for 10 minutes and the supernatant was analyzed for Pt and Pd uptake. The effect of different solution pH and metal ions concentrations on the bioreduction efficiency of viable cells of *C. metallidurans* were studied. The bioreduction efficiency was determined at solution pH of 4, 5, 6, 7, and 8; and initial metal ions concentrations (ppm) of 425, 275, 200, 175, and 150 ppm for Pt(II) and 300, 200, 150, 125, and 100 ppm for Pd(II).

Effect of solution pH

Figure 37 shows the effect of solution pH i.e., 4, 5, 6, 7, and 8 on the bioreduction efficiency in the presence of 150 ppm of Pt(II) and 100 ppm of Pd(II) over 48 hours. The maximum bioreduction for both Pt and Pd was achieved at solution pH 6 followed by pH 7 and 8. The optimum pH value for maximum bacterial growth and bioreduction efficiency was pH 6. The maximum bioreduction efficiency achieved for Pt(II) and Pd(II) at solution pH 6 was 65% and 52%, respectively. The reduction in the concentrations of Pt(II) and Pd(II) during bioreduction experiments associated with the bioprecipitation and formation of Pt and Pd NPs. Uptake of Pt and Pd by viable cells of *C. metallidurans* was high within 24 hours after the addition of SAC spent medium leachate. For an initial pH of 6, the pH of the solution after 24 hours of bioreduction for both Pt(II) and Pd(II) was approximately pH 7 indicated that pH increased slightly during the experiment. The lowest bioreduction efficiency was observed at an initial pH of 4 perhaps due to the low bacterial activity.

Effect of initial metal ion concentration

Figure 38 shows the effect of initial concentrations of Pt(II) and Pd(II) on the bioreduction efficiency at optimum pH i.e., pH 6. The two-step bioreduction was performed to minimize the toxic effect of soluble metals ions (metal ion stress and oxidative stress) on bacteria. The highest bioreduction efficiency was achieved at the lowest concentrations of metal ions i.e., 150 ppm for Pt(II) and 100 ppm for Pd(II). This was perhaps due to the fact that the metal ions-complexes (including PGM-cyanide complexes and other metals-cyanide complexes) hinder the bacterial activity by creating metal ion stress and oxidative stress in the cells that kill the bacterial functions. The reduction began immediately upon exposure to Pt(II) and Pd(II) ions and as the exposure time increased, the immobilized concentrations of Pt and Pd increased. However, the rate of reduction after 24 hours of exposure to SAC leachate was low compared to the rate of reduction within the first 24 hours. The effect of Pt(II)- and Pd(II)-cyanide on bacterial viability was determined using the plate count method. The reacted cells at pre-determined sampling intervals were washed and re-suspended in a filter-sterilized DDI water. The suspension was further undergone for serial dilutions with filter-sterilized DDI water, spread onto PME-agar plates and incubated for 2 days at 30 °C. The CFU was counted to determine the dose-response and toxicity of metals on cell viability. Figure 39 shows the effect of initial concentrations of Pt(II) and Pd(II) ions on cell viability at pH 6 during two-step bioreduction. Prior to the addition of SAC leachate, the number of viable cell counts in the system was 2.8×10^9 CFU/mL. The number of viable cells decreased significantly after 48 hours of bioreduction indicating the osmotic pressure, toxic effect of metal-complexes, and related oxidative stress on viable cells whereas the cell viability of the cultures without Pt(II) and Pd(II) ions showed a slight decrease and remained nearly constant.

Effect of cell metabolic state

The active and passive uptake of Pt and Pd was determined by using viable and non-viable cells. The pH of the growth medium and SAC spent media leachate was adjusted to pH 6. The pH-adjusted SAC spent media leachate supplemented in the growth medium was added to the viable and non-viable cells at the pre-determined conditions i.e., 30 °C and 150 rpm, and incubated for 48 hours. Figure 40 shows the effect of viable vs. non-viable cells on the bioreduction efficiency of Pt(II) and Pd(II) at pH 6. The viable cells resulted in Pt and Pd bioprecipitation and the formation of Pt and Pd NPs. The non-

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viable (dead) cells also resulted in passive sorption of Pt(II)- and Pd(II)-cyanide complexes but no metallic Pt and Pd NPs were observed. In the case of viable cells, Pt and Pd immobilization and NPs formation occurred intracellularly, extracellularly, and on the cell surface. The dead cells can not actively bind Pt and Pd, but passive sorption occurred on the cell surface. The denatured cell membranes and lysed cytoplasmic contents in dead cells provide the active sites for the immobilization of Pt and Pd. The functional groups in the cell envelope such as carboxyl (R-COOH), amine (R-NH₃⁺), phosphoryl (R-OPO₃H₂ and (RO)₂-P(OH)₂), and hydroxyl (R-OH) control the cell surface activity. It has been reported that immobilization of Pt on the cell surface occurred as Pt-organo complexes implying that Pt-organo complexes may be favoured to inorganic complexes.

Antibacterial activity of biosynthesized NPs

The antimicrobial activity of biosynthesized Pt and Pd NPs on Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* was evaluated using the minimal inhibition concentration (MIC) technique. Both optical density and CFU techniques were used to evaluate the MIC concentration of biosynthesized Pt and Pd NPs for bacterial cells. The LB growth medium was inoculated to a recommended final inoculum size of 5×10^5 CFU/mL. Aliquots of bacterial cultures were exposed to equivalent concentrations of Pt and Pd NPs. The maximum and minimum concentrations of both Pt and Pd NPs used for MIC were 30 ppm and 0.5 ppm, respectively. The minimum concentration of NPs was obtained by serial dilutions of the colloids. The aliquots of bacterial culture supplemented with NPs were incubated for 24 hours at 37 °C for *E. coli* and at 30 °C for *P. aeruginosa* and absorbance was measured at 600nm. To visualize the effect of NPs on bacterial growth, cells were spread onto LB-agar plates and exposed to serially diluted NPs dispersions. The agar plates were incubated for 24 hours at 37 °C for *E. coli* and at 30 °C for *P. aeruginosa*. Figure 41 shows the percentage viability of the bacterial cells after 24 hours of incubation in the presence of different concentrations of NPs. The biosynthesized Pt and Pd NPs showed antibacterial activity against tested bacterial strains. Antibacterial activity against *P. aeruginosa* was higher than *E. coli*; the MIC of the NPs (which inhibits visible growth of the bacterial) for *P. aeruginosa* and *E. coli* was found to be 8 ppm and 12 ppm, respectively. The high antibacterial activity of biosynthesized NPs was possibly due to their small size and spherical morphology. The antibacterial activity of NPs depends on many factors such as size, shape, surface area-to-volume ratio, composition, catalytic activity, and concentration. It has been reported

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that smaller NPs which give larger surface area show higher antibacterial activity at low concentrations whereas bigger NPs show antibacterial activity only at high concentrations. The in vivo and in vitro cytotoxicity of the Pt and Pd NPs has not been investigated in detail. Only a limited number of studies investigated the cytotoxicity of Pt NPs and there is no indication of ROS-induced cytotoxicity.

Characterization of biosynthesized Pt and Pd NPs

The electronic, optical, physical, and chemical properties of NPs play an important role in determining material behavior and its use in various applications. The biosynthesized NPs were characterized for composition, morphology, size, hydrodynamic diameter, dispersity index, and zeta potential using the following analytical techniques: field emission transmission electron microscopy (FETEM), energy-dispersive X-ray spectroscopy (EDS), selected area electron diffraction (SAED), dynamic light scattering (DLS), and electrophoretic light scattering (ELS). The biogenic synthesis of Pt and Pd NPs from multi-metal ions leachate was carried out using *C. metallidurans* via bioreduction and bioaccumulation mechanisms. To determine the morphology and size of synthesized NPs, FETEM characterization was performed. Since Pt and Pd NPs were synthesized from multi-metal ions leachate and the resultant solution contains a mixture of Pt and Pd NPs, EDS, HRTEM, and SAED characterization were performed to identify Pt and Pd NPs. HRTEM and SAED are powerful techniques capable of determining nanostructured interface architecture, morphology, crystallography, and chemical composition of NPs.

Morphology and size of NPs

TEM analysis was performed to determine the size, morphology, and crystal structure of NPs. The TEM micrographs of whole mounts of both fixed and stained, and unstained cells were obtained for unexposed *C. metallidurans* cells (control) and the results are given in Figure 42. The internal detail of cell is lacking in unstained cells while cell envelope and cytoplasmic contents are visible in stained cells. Figure 42 (b) also shows that bacteria are encapsulated in a matrix of EPS.

The TEM micrographs of cells exposed to SAC leachate solution are given in Figure 43. Figure 43 (a-d) shows that biogenic NPs were synthesized intracellularly and on the cell surface. The average size of the NPs shown in Figures 43 a, b, c, and d is 38 nm, 26 nm, 27 nm, and 37 nm, respectively. The average size of all the NPs shown in Figure

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43 (a-d) is 31.5 ± 0.32 nm with 10% of the NPs showing a diameter smaller than 20 nm, 72% of the NPs showing a diameter between 20 to 40 nm, and 18% of the NPs showing a diameter greater than 40 nm. The frequency distribution (%) of NPs is given in Figure 44. The NPs that are synthesized intracellularly are generally smaller in size
5 compared to the NPs that are synthesized on the surface or extracellularly. The morphology of the biosynthesized NPs was dominated by irregular shape, spherical, and cube. The EDX spectra in Figure 45 confirms the synthesis of the Pt and Pd NPs. Figure 46 shows the biogenic NPs of different size and shapes synthesized extracellularly. The average size of the hexagonal-shaped NPs is 45 nm whereas the average size of the
10 spherical NPs is 51 nm. It is important to note that the initial concentrations of Pt(II) and Pd(II) also play a role in the size-controlled formation of NPs. At high initial concentrations of Pt-cyanide and Pd-cyanide complexes, bigger sized NPs were observed (Figure 47). Figure 47 a and b shows the biogenic NPs that were synthesized intracellularly and extracellularly, respectively, at high initial concentrations of Pt-
15 cyanide and Pd-cyanide complexes. The average size of the NPs shown in Figures 47 a and b is 45 nm and 75 nm, respectively.

The biosynthesized extracellular Pt and Pd NPs occurred through the bioreduction of Pt(II) and Pd(II) ions which implies that biomolecules produced by *C. metallidurans*
20 were oxidised during bioreduction. A study on the biogenic synthesis of gold NPs has reported that the molecules located in the periplasm or cellular membrane of *C. metallidurans* CH34 cells are oxidised during bioreduction. Their findings suggested that a *cop* gene cluster (Methionine residues of CopA and CopB) located within the megaplasmid pMOL30 are involved in the bioreduction of Au(III) to extracellular Au NPs.
25 Another study on the immobilization of Pt has reported that Pt NPs were formed on the cell membrane and within the cytoplasm. Additionally, Pt NPs bound in cytoplasm caused metal toxicity which resulted in cell lysis. The internalized metals inhibit the enzymatic functions and detoxification response of the bacteria i.e., complexation or reduction of metal to a less toxic state, or efflux to the outside of the cell cause oxidative
30 stress which lead to cell death.

The HRTEM image and its corresponding Fourier transform (FFT) and IFFT pattern and Selected area electron diffraction (SAED) pattern were used to identify the NPs. The HRTEM image and its corresponding FFT and IFFT pattern of Pd NP is given in Figure 48.
35 The HRTM image of Pd NPs showed that the fringe spacing of Pd NP was 2.24 Å and

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1.94 Å which corresponds well to the spacing between (111) and (200) planes of rhombic dodecahedral Pd. Also, the presence of PdO phases along with the Pd NPs was observed. The SAED pattern of PdO is given in Figure 49. The patterns of SAED were indexed according to (111), (200), (220), (222), (400), (420), and (422) reflections of PdO based on their *d*-spacings of 3.24 Å, 2.83 Å, 1.99 Å, 1.62 Å, 1.41 Å, 1.25 Å, and 1.14 Å. The SAED pattern of Pt NP is given in Figure 50. The patterns of SAED were indexed according to (200), (220), (400), (331), (420), and (422) reflections of cubic Pt based on their *d*-spacings of 1.95 Å, 1.38 Å, 0.97 Å, 0.91 Å, 0.87 Å, and 0.78 Å. The purpose of this study was to recover Pt and Pd in metallic form from leachate using bioreduction and bioaccumulation mechanisms. The study shows the potential of biosynthesis of Pt and Pd NPs of different shapes and sizes from multi-metal ions leachate. Therefore, it is inferred that this technique can be employed for the selective recovery of Pt and Pd in the form of Pt and Pd NPs from aqueous metal ions. Since bioreduction and bioaccumulation resulted in the simultaneous synthesis of Pt and Pd NPs from multi-metal ions leachate, its potential for the controlled synthesis of bimetallic (core-shell) NPs can also be exploited. Compared to monometallic NPs, bimetallic core-shell NPs exhibits enhanced stability, activity, and selectivity because of lattice strain and unique core-shell interface.

20 **Dispersity index and zeta potential of NPs**

The zeta potential, hydrodynamic diameter (Z-Ave) and polydispersity index (PDI) of colloidal dispersions of the biosynthesized NPs were also determined. The physicochemical characteristics of the NPs play an important role in the use of these NPs as safe, efficient, and stable nanocarriers in drug delivery. Homogenous (monodisperse) NPs are preferred for in vitro and in vivo applications. To obtain the particle size distribution, hydrodynamic diameter and polydispersity index of the NPs was measured. Dynamic light scattering (DLS) technique was used for the in situ measurements of the size and PDI of NPs, and electrophoretic light scattering (ELS) technique was used for the zeta potential measurement of NPs.

30

The hydrodynamic diameter of NPs measured by DLS was 96 nm and 108 nm when NPs were produced by viable cells and by dead cells, respectively. The size distribution of NPs measured by DLS is greater than the size measurements taken by TEM. The difference in size measurements determined by TEM and DLS can be explained by the characteristics of these techniques. The particle diameter measured by TEM is

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proportional to the length of the particles in a solid static state. The size distribution measured by TEM images does not include the width of the capping ligands adsorbed onto the surface of the particles. In contrast, the hydrodynamic diameter obtained by DLS is proportional to the volume of the NPs and represent the accumulation of multiple
5 measurements obtained from NPs dispersions in a dynamic state. Moreover, hydrodynamic diameter measured by DLS is related to the movement of the particles in suspension and it is a reflection of the transport properties of the particle and takes into account the hydration sphere and any protecting or stabilizing layer that may surround the NPs. The size distribution measurements of NPs with heterogeneous size
10 distributions by DLS has certain limitation such as aggregation. The size distribution from TEM will always be smaller than the intensity distribution from DLS for a polydisperse sample. Therefore, it is concluded that aggregation of NPs occurs in the aqueous suspension. The PDI of the NPs produced by alive cells were 0.21 whereas the PDI of the NPs produced by dead cells were 0.26. This difference in PDI can be attributed
15 to the difference in particles size as smaller particles are nicely dispersed while larger particles are agglomerated.

The zeta potential measurement of the NPs by ELS is related to the electrophoretic mobility of particles in the dispersion. The magnitude of the zeta potential is directly
20 related to particle stability; a higher magnitude leads to greater particle stability, resulting in the formation of smaller particles. The zeta potential was measured to determine the stability of the NPs suspension. The samples were centrifuged and filtered using a 0.22-micron filter to remove the bacterial cells before measuring the zeta potential. The zeta potential measurements of the NPs suspension formed by the alive
25 and dead cells were -25.92 and -21.03, respectively. It might possible that some biomolecules capped the NPs, causing a net negative charge over them, and lead to NPs stabilization. It is important to note that the zeta potential measurements described here were taken when bacterial cells were grown and two-step bioreduction was carried out at pH 6 and 30 °C. The higher magnitude zeta potential was observed when two-
30 step bioreduction was carried out at pH 8. Moreover, a low PDI was observed when NPs were produced in the presence of stabilizing agent PVP.

6. Conclusion

Ultrasound-assisted nitric acid pretreatment of SAC can successfully remove non-target
35 metals that would otherwise compete with PGM for cyanide in the metal-cyanide

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complexation. Response surface-based multivariate optimization approach used to determine the optimal conditions for the removal of these interfering metals found that ultrasound frequency, ultrasound power, and temperature were the most important parameters for copper, zinc, iron, and titanium removal in the pretreatment of SAC. The
5 optimized ultrasound-assisted pretreatment of SAC results in the removal of copper (82%), zinc (88%), iron (60%), and titanium (72%) from SAC. Ultrasound-assisted pretreatment increased the surface area and reduced the size of SAC particles.

Central composite design and response surface methodology were employed to optimize
10 the bioleaching conditions for PGM recovery. The response surface empirical models were developed to determine the individual and interactive effect of process variables on PGM recovery, and process optimization was performed. Using central composite design and two-step bioleaching, optimum conditions for maximum PGM recovery i.e., Pt (69%), Pd (74%), and Rh (99%), were determined to be glycine concentration 10
15 g/L, pulp density 0.5%w/v, pH 9.4, H₂O₂ concentration 0.08 %v/v, and temperature 30°C. SAC reduction was found to speed-up the leaching process and enhance PGM recovery. SAC reduction using formic acid before bioleaching resulted in higher and faster PGM recovery, and the maximum recovery of PGM (at 91, 95, and 100 % of Pt, Pd, and Rh, respectively) at pulp density of 0.5 %w/v were achieved under spent media
20 leaching at the following optimized reduction conditions: reducing agent concentration 5 vol%, reduction time 90 minutes, and reduction temperature 80°C.

Bioleaching efficiency greatly improved at high pulp density by controlling microbial-metal interaction between cells and SAC particles, and by eliminating oxidative stress
25 within the bacterial cells. By adopting a strategy of controlling the microbial-metal interaction by using a non-ionic dispersant PVP, and scavenging the intracellular ROS by using antioxidant GSH, higher bacterial growth and higher net availability of cyanide ions for metal mobilization were achieved which resulted in higher PGM recovery. The addition of PVP and GSH reduced the sorption of metals onto the bacterial cell surface,
30 reduced the EPS binding with SAC particles, and reduced the metal toxicity and oxidative stress on bacteria which would otherwise damage cell functions. The dispersion ability of PVP improved the contact between metal and cyanide lixiviant, reduced the non-specific binding between particles, enhanced the conversion rate, and ensured efficient mixing of the reaction mixture to generate more favorable conditions for bioleaching.
35 Overall, this novel strategy allowed higher pulp density to be used during bioleaching

and yielded correspondingly higher PGM recovery. The maximum recovery achieved for Pt, Pd, and Rh was 30%, 33%, and 62%, respectively, at 4 %w/v. The corresponding values for Pt, Pd, and Rh in the presence of GSH and PVP were 68%, 74%, and 86%, respectively, at 4 %w/v. Further, reduced SAC was used to achieve the maximum PGM recovery i.e., 73% Pt, 82% Pd, and 90% Rh at pulp density of 4 %w/v in the presence of GSH and PVP.

In-situ synthesis of Pt and Pd nanoparticles (NPs) using *C. metallidurans* was carried out using SAC leachate containing soluble Pt-cyanide and Pd-cyanide complexes. The effect of various process parameters, including solution pH, reaction time, initial metal ion concentration, and cell metabolic state on the bioreduction efficiency was investigated. The optimal pH for *C. metallidurans* growth and bioreduction was found to be pH 6. Compared to dead cells, viable cells resulted in higher uptake of Pt and Pd within 24 hours from the start of the bioreduction. A high initial concentration of Pt(II) and Pd(II) resulted in lower metal uptake due to toxic effect of metal-complexes, and related oxidative stress on viable cells. The maximum metal uptake i.e., 65% and 52% of Pt(II) and Pd(II), respectively was achieved after 48 hours of bioreduction. NPs of different sizes (average 31.5 ± 0.32 nm) and shapes (cubical, rhombic dodecahedral, hexagonal, spherical, and rod-shaped) synthesized by viable cells of *C. metallidurans* was observed intracellularly, on the cell surface, and extracellularly. The biosynthesized NPs showed excellent antimicrobial activity, low polydispersity index, and high zeta potential.

Table Summary of the operating conditions and PGM extraction (%) under different strategies

S/ N	Bacteria	SAC pretreatment			Bioleaching conditions					PGM recovery (%)			
		Ultrasound	Heat oxidation	Formic acid reduction	pH	Glycine (g/L)	Pulp density (%w/v)	Antioxidant GSH	Stabilizing agent PVP	Leaching technique	Pt	Pd	Rh

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1	<i>C. violaceu</i> <i>m pBAD</i> <i>hcnABC</i>	Y*	Y	N	9.4	10	0.5	N	N	Two-step bioleachin g	69	74	99
2	<i>C. violaceu</i> <i>m pBAD</i> <i>hcnABC</i>	Y*	Y	N	10. 5	10	0.5	N	N	Spent- media leaching	76	81	10 0
3	<i>C. violaceu</i> <i>m pBAD</i> <i>hcnABC</i>	Y*	Y	Y	10. 5	10	0.5	N	N	Spent- media leaching	91	95	10 0
4	<i>C. violaceu</i> <i>m pBAD</i> <i>hcnABC</i>	Y*	Y	N	9.4	10	4	N	N	Two-step bioleachin g	30	33	62
5	<i>C. violaceu</i> <i>m pBAD</i> <i>hcnABC</i>	Y*	Y	N	9.4	10	4	Y	Y	Two-step bioleachin g	68	74	86
6	<i>C. violaceu</i> <i>m pBAD</i> <i>hcnABC</i>	Y*	Y	Y	9.4	10	4	Y	Y	Two-step bioleachin g	73	82	90

Y = Yes, N = No.

*Optimised ultrasound pretreatment was performed under the following conditions: ultrasound at 37kHz, 96W, for 80 minutes at 70 °C, and with nitric acid concentration 8.5M.

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Methods

Spent automotive catalyst (SAC)

SAC was obtained from Environmental Solutions (Asia) Pte Ltd. The catalytic converters were ground to a fine grey colored powder and were further sieved mechanically using standard ASTM sieves to particle size of less than 45 µm. The samples were stored in plastic containers, sealed with parafilm and kept in a dry cabinet at a temperature of 20-22°C.

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Quantification of metal content of SAC

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The samples (0.1 g) were digested in a Titan MPS microwave (PerkinElmer Inc.) equipped with standard 75 mL vessels (maximum temperature 230°C, maximum pressure 40 bar). Aqua regia was added (2.5 mL of 69% HNO₃ + 10 mL of 37% HCl) followed by ramping the temperature up to 210°C over 15 minutes and holding (at 5 210°C) for 30 minutes. The vessels were cooled to room temperature before 2 mL H₂O₂ (35% w/w) was added, and the temperature ramped up to 210°C over 10 minutes and held for 20 minutes. The digestate was cooled to room temperature, diluted with deionized water, and centrifuged (KUBOTA 5100 centrifuge) at 6000 rpm for 15 minutes. The supernatant was filtered using a 0.45 µm syringe filter to remove 10 suspended particulates before storage at 4°C prior to analysis. The SAC residue was dried at 60°C and weighed, and the amount of metal solubilized was measured. Metal concentration was measured using an inductively coupled plasma atomic emission spectrometer (ICP-AES, Thermo Scientific iCAP 6200).

15 **Pretreatment of SAC in ultrasonic bath**

To remove non-PGM, pretreatment was carried out using an ultrasonicator. The ultrasonic bath (Elmasonic P30H) used in this study has the following specifications: (1) two frequency settings: 37 kHz and 80 kHz, (2) maximum ultrasound intensity 2.478 W/cm², (3) temperature varying from 30°C to 80°C, and (4) sonication power which 20 varies from 30% to 100% (where 100% corresponds to 120W for 37kHz, and 100W for 80kHz). To remove non-PGM, ultrasonic-assisted nitric acid pretreatment of SAC was carried out in the ultrasonic bath under "Sweep" mode which allows an equal ultrasound field duration within the water bath. The pretreatment was carried out in 250 mL Erlenmeyer flask, where the SAC was added to nitric acid at a ratio of 1:10 (1g of SAC 25 to 10 mL of HNO₃). The flask containing SAC and nitric acid was sonicated at pre-determined conditions, after which the mixture was then transferred to 50 mL centrifuge tubes and centrifuged at 6000 rpm for 15 minutes. 5 mL of the supernatant was filtered using a 0.45 µm syringe filter to remove suspended particulates before storage at 4°C prior to metal analysis. The residues were washed with deionized water, and re- 30 centrifuged. The process was repeated till the supernatant was clear. The residual SAC was dried in an oven at 60°C overnight before it was weighed to determine the percentage of SAC digested. The dried SAC was then used in the subsequent leaching experiments.

35 **Reduction of pretreated SAC**

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SAC reduction using formic acid (HCOOH, 98%) and ascorbic acid were carried out in a shaking water bath at an agitation rate of 250 rpm for pre-determined time periods. After mixing, the mixture was then centrifuged at 6000 rpm for 15 minutes. The residues were washed with deionized water, and re-centrifuged. The residual SAC was dried in an oven at 60°C overnight. The dried SAC was then used in subsequent leaching experiments for reduced SAC.

Growth of bacterial culture for bioleaching

Metabolically engineered strain of *C. violaceum* was obtained from the Department of Biochemistry, Faculty of Medicine, National University of Singapore. *C. violaceum pBAD hcnABC* carries an additional copy of cyanide hcnABC operon with pBAD promoter and requires L (+) arabinose inducer to induce the expression of additional gene. Bacterial pre-culture was prepared in a 250 mL Erlenmeyer flask by transferring 1 mL of frozen bacterial stock into 100 mL of autoclaved culture media. The flask was kept in an incubator at 30°C and at 150 rpm for 24 hours. To prepare bacterial sub-culture for inoculum, 1 mL of pre-culture was inoculated into 100 mL of autoclaved culture media in 250 mL Erlenmeyer flask. Gentamycin sulphate antibiotics was added to prevent contamination from the wild strain. Gentamycin sulphate was added to the culture to achieve a final working concentration of 15µg/mL. For this, 150 µL of Gentamycin sulphate was added from stock solution of concentration 10 mg/mL. Bacterial culture was grown in an incubator at 30°C and at 150 rpm till the mid-log phase is reached. L (+) arabinose inducer was added to achieve a final working concentration of 0.002% w/v at the mid-log phase to induce the expression of the additional *hcnABC* operon. For this, 1 mL of 0.2% w/v L (+) arabinose was added to bacterial culture. Molecular biology grade Glycine of 99.6% purity was added in all bacterial cultures as a precursor to cyanide production. 5 %w/v of D (+) anhydrous glucose of 99% purity (purchased from Alfa Aesar) was added to the LB-Miller broth for all bacterial cultures as an additional energy source for the bacteria.

Growth of bacterial culture for bioreduction

C. metallidurans strain CH34 purchased from Wako Pure Chemical Ind., Ltd., Osaka, Japan was used in bioreduction experiments. *C. metallidurans* was activated in peptone meat extract medium (PME 8 g/L) at pH 7. To measure the antimicrobial activity of NPs, freeze-dried cells of *E. scherichia coli* (ATCC 53868) and *P.seudomonas aeruginosa* were purchased from American Type Culture Collection (ATCC) and activated in LB-Miller

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broth at pH 7. Bacterial pre-culture was prepared in a 250 mL Erlenmeyer flask by transferring 1 mL of frozen bacterial stock into 100 mL of autoclaved culture media (pH 7). The flasks were kept in an incubator at 30°C for *C. violaceum*, *C. metallidurans*, and *P. aeruginosa* and 37°C for *E. coli*, and at 150 rpm for 24 hours. To prepare bacterial sub-culture for inoculum, 1 mL of pre-culture was inoculated into 100 mL of autoclaved culture media in a 250 mL Erlenmeyer flask.

Leaching studies

Leaching experiments were carried out in 250 mL Erlenmeyer shake flasks housed in a shaking incubator at different operating conditions. The inoculum size of 5% was used in all leaching experiments. Two leaching techniques were used i.e., two-step bioleaching and spent media leaching.

In two-step bioleaching, monocultures of bacterium were grown separately in LB media at an optimal pH (7.00-7.25) in the absence of SAC to reduce toxic effects of SAC on the bacteria. Upon reaching the mid-logarithmic phase in the batch culture, glycine was added at pre-determined concentrations. Upon reaching the maximum cyanide production, the pH was adjusted to pre-determined values, followed by addition of a predetermined amount of either untreated or pretreated SAC (particle size $\leq 45\mu\text{m}$, autoclaved). The two-step bioleaching experiments were run over six days after SAC addition. Control experiments (i.e., bioleaching medium and the SAC) were also conducted under the same conditions in the absence of the bacteria.

In spent medium leaching, bacterium was grown under the same conditions as in the two-step bioleaching until it attained the maximum cell density and cyanide yield. The spent media with cell-free cyanide was then obtained by removing the cells by centrifuging the culture at 10,000 rpm for 15 min and filtration with 0.22 μm filter. The spent medium was then used in leaching experiments. In spent medium leaching experiments, the experiments were run over three days after SAC addition. Control experiments were also conducted using the non-inoculated medium.

All leaching experiments were performed in triplicates. Samples were taken daily, centrifuged (10,000 rpm, for 15 min) and filtered (0.45 μm) before cyanide and metal analyses. All samples were kept at 4°C prior to analysis.

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Scale-up studies were carried out in a bioreactor using the engineered *C. violaceum* strain under optimized conditions for two-step bioleaching. A 2-liter glass (single-well glass vessel) autoclavable bioreactor from Sartorius Stedim Biotech (Model: BIOSTAT® Aplus) was used as the bioreactor. All bioreactor experiments were performed with a total volume of 1 liter. Before each run, the reactor containing the culture media was autoclaved at 121°C for 40 minutes together with reagents, filters, and silicon tubings. Calibration of pH electrode and peristaltic pumps were performed before autoclaving while polarization and calibration of pO₂ electrode was performed after autoclaving. After autoclaving, the vessel was cooled to room temperature before starting the process. Samples were taken daily, centrifuged (10,000 rpm, for 15 min), filtered (0.22 µm), and cyanide and metal analyses were performed. Samples were kept at 4°C prior to analysis.

Bioreduction studies

Bioreduction experiments were carried out using *C. metallidurans* in 250 mL Erlenmeyer shake flasks housed in a shaking incubator using SAC spent media leachate. The inoculum size of 5% was used in all bioreduction experiments. *C. metallidurans* was grown (in the absence of SAC leachate to reduce toxic effects on the bacteria) to mid-logarithmic growth phase at optimal pH (pH 6), 30°C, and at 150 rpm to maximize the amount of metabolically active biomass. The aliquots of the bacterial suspension were harvested by centrifugation at 12,000×g for 5 min. After centrifugation, cells were washed with filter-sterilized deionized water to remove any remaining culture medium and stored in relevant growth medium as the stock for the inoculum. The following three different experimental approaches were used for the bioreduction experiments: (1) The bacterial suspension was centrifuged again under the same centrifugation conditions and bacterial pellets were re-suspended in the SAC spent media leachate (i.e., metal ions solutions where PGM are present in the form of PGM-CN⁻ complex) under various conditions to examine the role of inactive bacteria in the bioreduction of Pt and Pd. (2) The bacteria were grown at optimal pH to early stationary growth phase, pH was adjusted to various values, and pH-adjusted SAC spent medium leachate was added to the bacterial culture to study the effect of initial pH and initial metal ion concentrations on bioreduction efficiency. (3) The bacteria were grown at optimal pH to early stationary growth phase followed by heat sterilization of the cells to obtain non-viable cells. pH was adjusted to optimum value and pH-adjusted SAC spent medium leachate was added to the non-viable cells to study the effect of cell metabolic state on bioreduction

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efficiency. The bacterial cultures were kept for two days after SAC leachate addition. Control experiments were also conducted using the non-inoculated (abiotic) medium. All bioreduction experiments were done in triplicate. Samples were taken at pre-determined sampling intervals, centrifuged (10,000 rpm, for 15 min), filtered (0.22 μm), and metal analyses were performed.

Free cyanide analysis

Thermo Scientific Orion cyanide electrode (Model 9606BNWP) connected to an ion selective electrode (ISE) meter.

Metal analysis

Metal analyses were performed using inductively coupled plasma atomic emission spectrometer (ICP-AES, Thermo Scientific iCAP 6200).

UV-Vis spectroscopy

For measuring the OD and cyanide concentration, Shimadzu Biospec-mini UV-VIS spectrometer was used. For measuring cyanide concentration, samples were first centrifuged at 6000 rpm for 15 minutes to remove bacterial cells.

Measurement of reactive oxygen species (ROS)

Reactive oxygen species (ROS) in cells were determined by the methods reported. The methods were modified according to the experimental conditions. Three different oxidative-stress-sensitive probes namely dihydrorhodamine 123 (DHR 123), 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), and dihydroethidium (DHE) were purchased from Sigma Aldrich.

Following bioleaching experiments at high pulp densities, 5 $\mu\text{g/mL}$ of DHR 123 was added to a 500 μl aliquots samples followed by incubation for 2 hours at 30°C in the dark. The oxidation of nonfluorescent DHR 123 is catalysed by the enzyme peroxidase to the fluorescent rhodamine 123. The mixture was transferred to a transparent 96-well plate, and a micro plate reader was used to measure the oxidation of DHR 123 at excitation and emission wavelengths of 505nm and 535 nm, respectively.

To measure the oxidation of DCFH-DA, 5 mL of pulp was taken and bacteria was dislodged using 0.1% isomeric Tween-20 liquid (purchased from Sigma Aldrich). The

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bacterial suspension was centrifuged at 10000 rpm for 10 minutes and cell pellet was re-suspended in 2 mL of deionized water. DCFH-DA was added at a final concentration of 10 μ M from 1mM stock solution in ethanol followed by incubation for 1 hour at 30°C in the dark. Upon taken up by living cells, the acetyl groups in DCFH-DA are removed
5 by membrane esterases to form 2',7'-dichlorodihydrofluorescein (DCFH). When taken up by viable cells, DCFH-DA forms non-fluorescent 2,7-dichlorodihydrofluorescein (DCFH). The nonfluorescent DCFH is highly sensitive to OH, HOCl, RO, RO₂, and ONOO⁻ and is oxidized to the highly fluorescent compound 2,7-dichlorofluorescein. The mixture was transferred to a transparent 96-well plate, and a micro plate reader was used to
10 measure the oxidation of DCFH-DA at excitation and emission wavelengths of 504 nm and 524 nm, respectively.

DHE is specific for O₂⁻ (one-electron reduction product of O₂) with minimum oxidation induced by HOCl, ONOO⁻, and H₂O₂. However, O₂⁻ can lead to the formation of H₂O₂,
15 [•]OH, and reactive nitrogen species (RNS). The oxidation of DHE by O₂⁻ produced 2-hydroethidium (EOH) and intermediate products. The possibility of the oxidation of intermediate products to fluorescent ethidium (E⁺) by H₂O₂ and [•]OH is less. The E⁺ fluorescence is measured at an excitation of 500–530 nm and emission of 590–620 nm, respectively. The EOH fluorescence is measured at excitation and an emission
20 wavelength of 480 and 567 nm, respectively.

The fluorescence values (relative fluorescence intensity) measured by micro plate reader is positively correlated to ROS. The intracellular ROS content was measured in relative fluorescence units (RFU). The RFU of the solutions were measured in the
25 presence and absence (control) of SAC. Optical density at 600nm (OD₆₀₀) and cell counts were used to measure bacterial growth.

Distribution of SAC particles during bioleaching

The distribution of the SAC particles in the bacteria solutions were examined by Field
30 Emission Transmission Electron Microscope (FETEM; JOEL JEM-2100F).

Nanoparticles characterization

For bioreduction experiments, bacterial cells and the resultant intracellular and extracellular NPs were examined by Field Emission Transmission Electron Microscope
35 (FETEM; JOEL JEM-2100F). Surface elemental composition of the bio-reduced samples

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was examined using an Energy-dispersive X-ray spectrometer (EDX; OXFORD Instruments 6647). EDX analysis was performed using INCA software. Formation of NPs was studied using Selected Area Electron Diffraction (SAED) which is crystallographic technique used to identify crystal structures and analyse lattice matching. For SAED
5 analysis, ImageJ and Gatan Microscopy Suite® (GMS) software were used. For intracellular NPs formation, B-PER Bacterial Extraction Reagent kit (Thermo Fisher Scientific) was used to extract the Pt and Pd NPs.

Measurement of zeta potential and hydrodynamic diameter

10 The zeta potential of bacterial culture during bioleaching and nanoparticles during bioreduction, and hydrodynamic diameter (Z-Ave) of colloidal dispersions of the biosynthesized NPs were measured with Zetasizer Pro (Malvern panalytical) equipped with "ZS Xplorer software suite", using dynamic light scattering (DLS) and electrophoretic light scattering (ELS) for hydrodynamic diameter and zeta potential,
15 respectively.

Statistical analysis and optimization

Statistical analysis of the data was performed by analysis of variance (ANOVA). A 5% significance level was used for all statistical analyses in this study. Statistical
20 optimization of SAC pretreatment and leaching experiments was performed using central composite design of response surface methodology, with Design Expert (version 11.0.3), Minitab (version 18.1), Origin (version 2019b), and JMP (version 14.0.0). Statistical optimization was performed to identify the significance of the individual factors and interactions amongst various factors. Each run in the design matrix was
25 performed thrice.

Experimental errors

To ensure the accuracy of the results, all experiments were conducted in triplicates and experimental errors were quantified using standard deviation of the mean. Error bars in
30 graphs represent standard deviations.

It will be appreciated that many further modifications and permutations of various aspects of the described embodiments are possible. Accordingly, the described aspects are intended to embrace all such alterations, modifications, and variations that fall
35 within the spirit and scope of the appended claims.

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Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers
5 or steps but not the exclusion of any other integer or step or group of integers or steps.

Throughout this specification and the claims which follow, unless the context requires otherwise, the phrase "consisting essentially of", and variations such as "consists essentially of" will be understood to indicate that the recited element(s) is/are essential
10 i.e., necessary elements of the invention. The phrase allows for the presence of other non-recited elements which do not materially affect the characteristics of the invention but excludes additional unspecified elements which would affect the basic and novel characteristics of the method defined.

15 The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

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Claims

1. A method of extracting platinum group metals (PGM) from a material, the method comprising:
 - 5 a) surface oxidising the material;
 - b) ultrasonicated the surface oxidised material of step a) in the presence of an acid; and
 - c) bioleaching the ultrasonicated material of step b) in the presence of cyanide in order to form a leachate comprising PGM, the cyanide being formed from a cyanide
- 10 producing microbe in the presence of a cyanide precursor.
2. The method according to claim 1, wherein the surface oxidation is performed at about 700 °C to about 900 °C, or preferably at about 850 °C.
- 15 3. The method according to claim 1 or 2, wherein the surface oxidised material of step a) is ultrasonicated at a frequency of about 30 kHz to about 80 kHz, or preferably about 37 kHz to about 80 kHz.
4. The method according to any one of claims 1 to 3, wherein the surface oxidised
- 20 material of step a) is ultrasonicated at a power about 30W to about 120W.
5. The method according to any one of claims 1 to 4, wherein the surface oxidised material of step a) is ultrasonicated for a duration of about 10 min to about 150 min, or preferably for about 70 min to about 80 min.
- 25 6. The method according to any one of claims 1 to 5, wherein the surface oxidised material of step a) is ultrasonicated at a temperature of about 30 °C to about 80 °C, or preferably about 70 °C.
- 30 7. The method according to any one of claims 1 to 6, wherein the acid is selected from nitric acid, hydrochloric acid, sulphuric acid, or a combination thereof.
8. The method according to any one of claims 1 to 7, wherein the acid has a concentration of about 2 M to about 16 M, or preferably about 8 M to about 9 M.

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9. The method according to any one of claims 1 to 8, wherein the surface oxidised material of step a) is ultrasonicated at a frequency of about 37kHz, a temperature of about 70 °C, a power of about 100W, a duration of about 80 min, and the acid is nitric acid at about 8 M to about 8.5 M.

5

10. The method according to any one of claims 1 to 9, wherein the method further comprises a step after step b) of reducing the ultrasonicated material of step b) in the presence of a reducing agent.

10 11. The method according to claim 10, wherein the reducing agent selected from formic acid, ascorbic acid, glycolic acid, malonic acid, or a combination thereof.

12. The method according to claim 10 or 11, wherein the reducing agent has a concentration of about 1 % v/v to about 15 % v/v, or preferably about 5 % v/v to about 15 10 % v/v.

13. The method according to any one of claim 10 to 12, wherein the ultrasonicated material of step b) is reduced for a duration of about 30 min to about 120 minutes, or preferably of about 60 min to about 90 min.

20

14. The method according to any one of claims 10 to 13, wherein the ultrasonicated material of step b) is reduced at a temperature of about 50 °C to about 90 °C, or preferably of about 50 °C to about 80 °C.

25 15. The method according to any one of claims 10 to 14, wherein the ultrasonicated material of step b) is reduced at a reducing agent concentration of about 5 vol%, duration of about 90 min, and temperature of about 80°C.

16. The method according to any one of claims 1 to 15, wherein the cyanide precursor has a concentration of about 0.5 g/L to about 20 g/L, or preferably about 10 30 g/L.

17. The method according to any one of claims 1 to 16, wherein the ultrasonicated material of step b) is bioleached at a pulp density of about 0.1% w/v to about 12% w/v, 35 or about 0.5% w/v.

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18. The method according to any one of claims 1 to 17, wherein the ultrasonicated material of step b) is bioleached at a pH of about 7 to about 11, or preferably about pH 9 to about 10.5.
- 5 19. The method according to any one of claims 1 to 18, wherein the ultrasonicated material of step b) is bioleached at a temperature of about 22 °C to 38 °C, or preferably about 30 °C.
- 10 20. The method according to any one of claims 1 to 19, wherein the ultrasonicated material of step b) is bioleached at a cyanide precursor concentration of about 10 g/L, a pulp density of about 0.5 % w/v, a pH of about 9 to about 10.5, and temperature of about 30 °C.
- 15 21. The method according to any one of claims 1 to 20, wherein the ultrasonicated material of step b) is bioleached in the presence of the cyanide producing microbe under aerobic conditions, the cyanide producing microbe characterised by a HCN synthase operon (hcnA, hcnB, and hcnC) in its genome.
- 20 22. The method according to any one of claims 1 to 21, wherein the bioleaching step comprises:
- i) pre-incubating the cyanide producing microbe and the cyanide precursor under aerobic conditions in order to produce the cyanide; and
 - ii) mixing the cyanide producing microbe and the cyanide precursor of step i) with
- 25 the ultrasonicated material of step b);
- wherein the cyanide producing microbe characterised by a HCN synthase operon (hcnA, hcnB, and hcnC) in its genome.
23. The method according to any one of claims 1 to 20, wherein the bioleaching step
- 30 comprises:
- i) pre-incubating the cyanide producing microbe and the cyanide precursor under aerobic conditions in order to produce the cyanide; and
 - ii) isolating the cyanide from the cyanide producing microbe in order to form a cell-free medium and mixing the cell-free medium with the ultrasonicated material of step
- 35 b);

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wherein the cyanide producing microbe characterised by a HCN synthase operon (hcnA, hcnB, and hcnC) in its genome.

24. The method according to any one of claims 1 to 23, wherein the cyanide
5 producing microbe is selected from *C. violaceum*, *P. fluorescens*, *B. megaterium*, or a combination thereof.

25. The method according to any one of claims 22 to 24, wherein the step of pre-
incubating the cyanide producing microbe comprises incubating the cyanide producing
10 microbe at a pH of about 7.5, followed by incubating the cyanide producing microbe at a pH of about 9.

26. The method according to claim 22 or 23, wherein the aerobic condition is an O₂
% saturation of about 30%.

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27. The method according to claim 21 or 22, wherein the ultrasonicated material of
step b) is bioleached in the presence of the microbe and H₂O₂, wherein H₂O₂ is at a
concentration of about 0.02 % v/v to about 0.16 % v/v, or preferably about 0.08 %
v/v.

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28. The method according to any one of claims 21 to 27, wherein the ultrasonicated
material of step b) is bioleached in the presence of a ROS scavenger and/or a dispersant.

29. The method according to claim 28, wherein the ROS scavenger is added at least
25 10 h after mixing the reduced material with the microbe.

30. The method according to claim 28 or 29, wherein the ROS scavenger has a
concentration of about 0.2 g/L to about 2 g/L, or preferably about 0.6 g/L.

31. The method according to any one of claims 28 to 30, wherein the dispersant has
30 a concentration of about 0.2 g/L to about 1 g/L, or preferably about 0.4 g/L.

32. The method according to claim 23, wherein the cell-free medium has a pH of
about 10.5.

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33. The method according to any one of claims 1 to 32, wherein the material is a spent catalytic converter or a spent automotive catalyst.
34. The method according to any one of claims 1 to 33, wherein the material further
5 comprises Cu, Zn, Fe, Ti or a combination thereof.
35. The method according to any one of claims 1 to 34, wherein the method is characterised by a volume of at least 1L.
- 10 36. The method according to any one of claims 1 to 35, wherein the method further comprises a step of bioreducing the leachate of step c) in order to form nanoparticles.
37. The method according to claims 36, wherein the leachate of step c) is bioreduced using *C. metallidurans*.
15
38. The method according to claim 37, wherein *C. metallidurans* is pre-incubated.
39. The method according to any one of claims 36 to 38, wherein the leachate of step c) is bioreduced at a pH of about 4 to about 8, preferably about 6.
20
40. The method according to any one of claims 36 to 39, wherein the nanoparticles are characterised by an average size of about 10 nm to about 80 nm.
41. The method according to any one of claims 36 to 40, wherein the nanoparticles
25 are characterised by a hydrodynamic diameter of about 80 nm to about 110 nm.
42. The method according to any one of claims 36 to 41, wherein the nanoparticles are characterised by polydispersity index of about 0.2 to about 0.3.
- 30 43. A method of extracting platinum group metals (PGM) from a material, comprising:
bioleaching the material in the presence of cyanide in order to form a leachate comprising PGM, the cyanide being formed from a cyanide producing microbe in the presence of a cyanide precursor, and additionally in the presence of a ROS scavenger
35 and/or a dispersant.

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44. A method of pretreating a material comprising platinum group metals (PGM), the method comprising:
ultrasonically the material in the presence of an acid;
5 wherein at least a surface of the material is oxidised.
45. A method of pretreating a material comprising platinum group metals (PGM), the method comprising:
reducing the material in the presence of a reducing agent;
10 wherein at least a surface of the material is oxidised.
46. The method according to claim 45, further comprising a step before the reduction step of ultrasonically the material in the presence of an acid.