ABSTRACT: Sustainable resource recovery is the key to manage the overburden of various waste entities of mining practices. The present study demonstrates for the first time a novel approach for iron recovery and biodiesel yield from two acid-adapted microalgae, *Desmodesmus* sp. MAS1 and *Heterochlorella* sp. MAS3, grown in synthetic acid mine drainage (SAMD). Virtually, there was no difference in the growth of the strain MAS3 both in Bold’s basal medium (control) and SAMD. Using the IC50 level (200 mg L\(^{-1}\)) and a lower concentration (50 mg L\(^{-1}\)) of iron in SAMD, the cell granularity, exopolysaccharide (EPS) secretion, iron recovery, and biodiesel were assessed in both the strains. Both cell granularity and accumulation of EPS were significantly altered under metal stress in SAMD, resulting in an increase in total accumulation of iron. Growth of the microalgal strains in SAMD yielded 12–20% biodiesel, with no traces of heavy metals, from the biomass. The entire amount of iron, accumulated intracellularly, was recovered in the residual biomass. Our results on the ability of the acid-adapted microalgal strains in iron recovery and yield of biodiesel when grown in SAMD indicate that they could be the potential candidates for use in bioremediation of extreme habitats like AMD.

1. INTRODUCTION

The heavy global demand for minerals over the years led to the depletion of their resources, and the intensive mining resulted in several hazards from derelict mines.\(^1,2\) The environmental challenges from the mining process are also of great concern.\(^3\) The acidic effluent produced from the rock interaction or oxidation of iron sulfide (FeS\(_2\)), termed as acid mine drainage (AMD), is the most hazardous form of post-mining activity.\(^4\) The environmental impact of AMD is very high, contributing to the bioavailability of heavy metals (HMs) principally in water and soils. Because of the widespread occurrence of AMD, nearly 19,000 km of streams and 72,000 ha of lakes and reservoirs are affected throughout the world.\(^5\) The water from Maurliden mine in Sweden contains high concentrations of iron (400 mg L\(^{-1}\)) and zinc (450 mg L\(^{-1}\)) besides the presence of other metals such as Mn and Cd in trace concentrations.\(^6\) In South Africa, the iron concentration in acidic effluents (pH 2.1–3.1) from coal and gold mines was >800 mg L\(^{-1}\). At the mining-impacted area of Iberian pyrite belt in Spain, the iron concentration in water samples collected at different locations varied from 21.8 to 2000 mg L\(^{-1}\).\(^7\) The above reports imply that iron is the most predominant metal in AMD irrespective of the conditions at geological strata of the mining areas.

Current mining waste management practices are aligned toward linear economic thinking (take-make-waste), necessitating the implementation of sustainable approaches to reuse and efficiently manage the resources.\(^8\) The recovery of metals like iron from AMD is a valuable approach to meet their

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discharge limits while maintaining sustainable economic approach. Limestone drains, open limestone channels, and limestone diversion wells are the standard options in the passive treatment AMD. Hammarstrom et al. reported that the use of pulsed limestone bed systems for treating AMD reduced the formation of coatings besides hindering treatment efficiency. On the other hand, the active treatment processes generate copious sludge volumes enriched with various metals thus requiring further treatment. Also, the recovery of metals from AMD using chemical precipitation produces sludge, and the metal recycling is not a sustainable process. Adsorption, coagulation, chemical precipitation, and integrated filtration and chemical precipitation are some of the other techniques used for iron recovery from AMD. Though biological methods for recovery of metals through passive bioreactors are promising viable alternatives, they require electron donors or substrates to promote and sustain the process. Microalgae that thrive in any extreme habitats from desert to AMD by their ability to tolerate harsh environmental conditions are implicated in a wide array of biotechnological applications. Most studies on metal recovery from synthetic solutions used dead algal biomass as the adsorbent, but the potential of acidophilic microalgae in metal recovery from AMD has not been understood so far. The approach of biofuel production, especially as an alternative to other fossil fuels, cannot compete with the conventional technology due to the costs associated with the yield of biomass and biofuel. However, the “green technology” involving microalgae is significant in circular economy related to bioremediation, resource recovery, and generation of value-added products. Very recently, we identified two strains of acid-tolerant microalgae, Desmodesmus sp. MAS1 and Heterochlorella sp. MAS3, that can grow well at pH 3.5 and remove HMs and sustainably produce biomass for the yield of biodiesel. In the present study, for the first time, we tested whether these acid-tolerant microalgal strains upon prolonged adaptation for over 100 generations to acidic conditions at pH 3.5 have the potential for sustainable iron recovery and biodiesel production for appropriate bioenergy feedstock when grown in a synthetic AMD (SAMD).

2. RESULTS AND DISCUSSION

2.1. Growth of Acid-Adapted Microalgae in SAMD.

The acid-adapted microalgae, Desmodesmus sp. MAS1 and Heterochlorella sp. MAS3, were grown in SAMD that contained 50% of phosphates originally present in AMD (Table 1). In fact, this reduction in phosphates in SAMD is to avoid complexation in the culture medium and increase iron bioavailability as determined by Visual MINTEQ modeling. Initially, the growth of the microalgal strains in Bold’s basal medium (BBM) and SAMD, in terms of chlorophyll, was compared (Figure 1a). Overall, the growth of the strain MAS1 in SAMD was significantly more than that of the strain MAS3. The calculated doubling time for the strain MAS1 was two-fold higher in BBM compared to SAMD. Interestingly, no such difference in generation time was observed for the strain MAS3, indicating its better tolerance to the elevated concentrations of metals in SAMD. The growth inhibition in the microalgal strains in SAMD could be due to the competition between H+ and metal ions for cell surface binding sites, especially under acid pH. In the presence of a mixture of metals such as Cu and Ni and standard BG11 phosphates, Rugnini et al. reported significant inhibition in the photosynthetic activity of Desmodesmus sp. and Chlorella vulgaris. Under balanced growth conditions at pH 2.6, the energy balance, based on amount of absorbed energy, and the pigment content per cell were reduced in an acidophilic microalga, Chlamydomonas acidophila, due to changes in the environmental conditions that force the microalga to adapt by reorganizing photosynthetic apparatus and metabolic pathways. Concentration-dependent growth inhibition observed at the end of 96 h by using Fe concentrations ranging from 25 to 800 mg L−1 in both the

![Figure 1.](https://dx.doi.org/10.1021/acsomega.0c00255)

**Table 1. Characteristics of AMD and Synthetic AMD (SAMD)**

| characteristic | AMD | SAMD |
|---------------|-----|------|
| pH            | 3.0 ± 0.1 | 3.5 ± 0.2 |
| iron (Fe)     | 208.0 ± 9.95 | 1 ± 0.02 |
| manganese (Mn)| 14.48 ± 0.16 | 20 ± 0.1 |
| copper (Cu)   | 0.052 ± 0.002 | 0.5 ± 0.02 |
| zinc (Zn)     | 0.0151 ± 0.004 | 0.5 ± 0.01 |
| cadmium (Cd)  | 0.005 ± 0 | 0.5 ± 0.1 |
| nitrate (NO3−)| 129.65 ± 0.61 | 90 ± 1.5 |
| total phosphate (PO4−) | 6.77 ± 1.07 | 3.3 ± 0.2 |

*a n = 5. b n = 3. The concentrations are in mg L−1.*
microalgal strains revealed an IC₅₀ value of 200 mg L⁻¹ (R² = 0.94; 0.97 for MAS1 and MAS3, respectively) (Figure S1). Spijkerman suggested that high concentrations of iron in the medium tend to limit the phosphate uptake that is essential for acidophilic algae. Especially higher concentrations of iron decrease the bioavailable fraction of phosphates by complexation or lowering P uptake rates due to adsorption process, a phenomenon that is common in iron-rich acidic lakes. Also, ferric ion concentration exceeding 5 mmol L⁻¹ was too toxic for phytoplankton in an acidic river in Spain, leading the algae to consume more ATP resulting in growth inhibition. Moreover, Visual MINTEQ data (Table S1) revealed that iron accumulation with phosphates is about 0.26 and 0.32% at 50 and 200 mg L⁻¹, respectively. Based on the above observations, only the two concentrations, 50 and 200 mg L⁻¹, were used in further experimentation.

2.2. Microalgal Response to Iron Levels in SAMD. Cell granularity, measured through side scatter (SSC) following flow cytometry, and exopolysaccharide (EPS) accumulation were determined to assess the microalgal response to iron at 1 mg L⁻¹ as contained in SAMD (Table 1) and higher supplemental levels of 50 and 200 mg L⁻¹ (Figure 1b). The cell granularity, attributed mostly to intracellular changes under stress, was very less when the microalgal strains were grown in SAMD that contained 1 mg L⁻¹ of iron. Increased iron concentrations in SAMD (50 and 200 mg L⁻¹) decreased the SSC signal in both the strains, indicating significant intracellular changes. However, the change in granularity was significant in strain MAS3 as compared to strain MAS1. Such a decrease in cell granularity in response to metals such as Cu and Ni was also observed in a microalga, Chlorella. Metals in high concentrations alter the cell membrane by increasing permeability leading to enlargement of cells due to the accumulation of photosynthetic products. Also, increased cellular granularity may be due to changes in the ultrastructure as observed in Chlamydomonas reinhardtii when exposed to Cd. At high concentrations of Zn (4.4 mM) in the presence of Mn and Ni at pH 3.5, Ulothrix sp., an acidophilic alga isolated from AMD, exhibited completely disoriented thylakoids in chloroplasts. The secretion of EPS, as isolated from AMD, exhibited completely disoriented thylakoids in chloroplasts. 37 The secretion of EPS, as isolated from AMD, exhibited completely disoriented thylakoids in chloroplasts. 37

Intradieal accumulation was higher which accounted for 80% of the total accumulated iron, at 50 mg Fe L⁻¹ in strain MAS1. However, the extracellular accumulation was very significant (90% of the total) when this strain was grown in the presence of 200 mg L⁻¹ of Fe. The increase in total accumulation of iron in strain MAS1 when iron concentration in SAMD increased from 50 to 200 mg L⁻¹ was 3-fold, whereas the corresponding increase in MAS3 was only 1.2-fold. Interestingly, the extracellular accumulation of iron was significantly higher in strain MAS3 grown in the presence of 50 or 200 mg L⁻¹ of iron in SAMD. The mode of adsorption and accumulation of iron is not understood although iron uptake in microalgae is likely to include one of the two pathways (i) active surface adsorption and passive intracellular accumulation or (ii) passive surface adsorption and active intracellular accumulation. 41 In the presence of Fe and Zn that are essentially involved in photosynthesis, Euglena gracilis (an acidophilic alga) accumulated 60% of Cd in chloroplasts, but Ni accumulation was limited in the presence of other essential divalent metals such as Zn, Mn, and Cu. 42 Nearly 80% of total accumulated iron accounted for extracellular accumulation in MAS1 grown in SAMD with 200 mg L⁻¹ of iron. In fact, higher EPS secretion associated with elevated concentrations of iron (Figure 1b) resulted in increased external accumulation of iron. Garcia-Meza et al. 42 also observed significant extracellular metal accumulation in photosynthetic biofilms from mine tailings facilitated by more EPS secretion. Similarly, in AMD environments that contained mixed metals, the increased accumulation of EPS was attributed to the alleviation of metal toxicity in microalgae present in biofilms. Biodiesel was extracted after in situ transesterification of the biomass collected from the microalgal cultures grown in BBM (control) and SAMD containing 1, 50, or 200 mg Fe L⁻¹ (Figure 3a). In all, the biodiesel yield increased significantly when the strains were grown in SAMD. Among three concentrations of iron included in SAMD, 50 mg L⁻¹ was significantly effective in yielding biodiesel. Again, it is quite interesting to note that particularly 50 mg L⁻¹ of iron in SAMD significantly enhanced biodiesel yield in strain MAS1. Thus, the per cent biodiesel yield in strain MAS1 grown in 1,
Laurens et al. also observed that the biodiesel yield grown in BBM supplemented with 20 mg L\(^{-1}\) was only 9% of the biomass (based on dry wt) from residual biomass obtained after extracting biodiesel. The mean values \((n = 3)\), related to a microalgal strain, carrying the same letter on the bars are not significantly \((P \leq 0.05)\) different from each other according to DMR test.

50, and 200 mg Fe L\(^{-1}\) was 13, 20, and 15, whereas the corresponding values for MAS3 were 12, 16, and 14. Evidently, there was an 18–25% decrease in biodiesel production in cultures grown in SAMD supplemented with 200 mg L\(^{-1}\) of iron when compared with the concentration of 50 mg L\(^{-1}\). Laurens et al. also observed that the biodiesel yield determined following in situ transesterification in *Chlorella* and *Nannochloropsis* was only 9–10% of the biomass (based on dry wt). Lipids, primarily triacylglycerols (TAGs), are synthesized under unfavorable conditions as energy storage molecules in microalgae. The significant enhancement in intracellular accumulation of iron and increased biodiesel production in MAS1 when grown in the presence of 50 mg Fe L\(^{-1}\) clearly support the likely triggered accumulation of TAGs which are the precursors for biodiesel enhancement. We observed earlier that the same strains MAS1 and MAS3 when grown in BBM supplemented with 20 mg L\(^{-1}\) of Fe at pH 3.5, the yield of fatty acid methyl esters was ~25% on dry wt basis. Obviously, the observed lower yield of biodiesel in this study could be due to the presence of toxic HMs in SAMD. Concas et al. suggested that increased iron concentration could trigger oxidative stress in microalgal species, resulting in enhanced accumulation of lipids. Also, Ren et al. reported that higher iron concentrations in the culture medium induced lipid synthesis through metabolic alteration in *Chlorella vulgaris*.

Although iron was supplemented even at 200 mg L\(^{-1}\) in SAMD, no traces of iron or any other HM were detected in biodiesel obtained from both the microalgae strains, indicating that the biodiesel is of good quality and can be safely used as an energy source. Raikova et al. determined bio-oil production by hydrothermal liquefaction from metal-grown *Spirulina* biomass and observed that metals were accumulated in biomass residue than in oil. Similarly, biodiesel obtained from microalgae grown in metal-contaminated flue gas showed no traces of metals. Interestingly, a major portion of iron that accumulated inside the microalgal cells remained in the residual biomass recovered after extraction of biodiesel (Figure 3b). In fact, the iron content in residual biomass collected from both the cultures grown in the presence of 50 mg L\(^{-1}\) was significantly more as compared to the higher concentration used in SAMD. For instance, the recovery of iron from the residual biomass was 75% of the amount accumulated intracellularly in strain MAS1 grown in SAMD with 50 mg Fe L\(^{-1}\), and the corresponding value for strain MAS3 was 98%. Our data demonstrate that most of the iron accumulated in microalgal strains could be recovered from the residual biomass left after extraction of biodiesel. Also, the present study indicates the great potential of the two acid-tolerant microalgae in resource recovery and biodiesel production while remediating an AMD.

### 3. CONCLUSIONS

Identifying suitable microorganisms for metal recovery and biodiesel yield is of utmost importance in bioremediation of extreme habitats like AMD. Two acid-tolerant strains of microalgae, *Desmodesmus* sp. MAS1 and *Heterochlorella* sp. MAS3, grew well in SAMD samples that contained iron at 200 mg L\(^{-1}\); a concentration close to that present in real AMD. While growing in SAMD with higher concentrations of iron (200 mg L\(^{-1}\)), both the strains could remove significant amounts of the metal and this recovery was directly related to the altered cellular granularity and increased secretion of EPS under the conditions of stress imposed by metals. Growth of both the strains at 200 mg Fe L\(^{-1}\) resulted in an enhanced extracellular accumulation of iron facilitated by increased production of EPS. In situ transesterification of metal-enriched biomass yielded 12–20% biodiesel at high concentrations of iron in SAMD. The entire amount of iron in the biomass was recovered from the residual biomass left after solvent extraction for biodiesel. This is the first study that demonstrated the great potential of acid-tolerant microalgae in sustainable recovery of iron and biodiesel yield when grown in extreme conditions as exist in AMD, thus paving for the green circular economy.

### 4. EXPERIMENTAL SECTION

#### 4.1. Microalgae and Analysis of Growth Response.

The acid-tolerant microalgal strains, *Desmodesmus* sp. MAS1 and *Heterochlorella* sp. MAS3, initially maintained in BBM, were adapted to acidic conditions for more than 100 generations by growing in BBM at pH 3.5 under the culture conditions described earlier. The microalgal cells growing...
at log phase were centrifuged at 8000g for 10 min and washed with ultrapure water twice, and the pellet was resuspended either in BBM or SAMD (Table 1). The composition of metals used in SAMD is based on our earlier studies related to toxicity and uptake of HMs\textsuperscript{26,27} and wherever necessary the concentrations of HMs in SAMD were changed. The microalgal suspension was added to 20 mL of sterilized BBM or SAMD samples contained in 50 mL Erlenmeyer flasks to obtain a final density of 5 × 10^2 cells mL\textsuperscript{−1} for initially determining the relative growth. Varying concentrations of Fe were prepared using a stock solution of FeSO_4\textsubscript{2}•7H_2O (Sigma-Aldrich, USA) in ultrapure water and passed through a sterile 0.22 μm disposable syringe filter. Initially, the microalgae were tested for their survival in SAMD composed of higher concentrations of HMs than those in AMD because they exhibited tolerance to Mn, Cu, Zn, and Cd, but not Fe.\textsuperscript{27,28}

Subsequently, iron concentrations ranging from 25 to 800 mg L\textsuperscript{−1} were included in SAMD to determine IC\textsubscript{50} (the concentration of iron required for 50% growth inhibition) values for iron in both the strains. Finally, to determine microalgal growth and their potential in iron recovery and yield of biodiesel, concentrations of only 50 and 200 mg L\textsuperscript{−1} of iron that correspond to a lower and IC\textsubscript{50} value, respectively, were used. Each treatment was replicated thrice, and all of the flasks were incubated under constant illumination (60 μmol m\textsuperscript{−2} s\textsuperscript{−1}) with 100 rpm shaking at 25 °C.\textsuperscript{26}

The microalgal growth was determined, in terms of relative fluorescence units (RFUs) of chlorophyll, in a microplate reader using 100 μL aliquots of the cultures withdrawn at desired intervals as described earlier.\textsuperscript{26,27} After 96 h of growth at pH 3.5, IC\textsubscript{50} values for iron were determined by referring to the dose–response curve (inhibition) following nonlinear regression and using GraphPad prism software (version 8, USA). The content of EPSs in the strains were analyzed by the ATR–FTIR technique at a wave region of 900–1200 cm\textsuperscript{−1} following FTIR spectroscopy (Agilent Technologies, USA), and the peak area was calculated using Resolutions pro software (Agilent Technologies). Cell granularity was measured by flow cytometry using side scatter signal collected through autoelectronics laser (695/40 nm band pass filter) in a BD FACS Canto flow cytometer (Becton Dickinson Instruments) as described previously.\textsuperscript{26}

### 4.2. Iron and Biodiesel Analysis

Ten milliliters of the microalgal cultures, in triplicates, from each treatment were withdrawn for iron analysis only after 96 h for iron analysis. The iron content in the samples was analyzed in inductively coupled plasma–mass spectrometer (ICP-MS, Agilent Technologies, USA) as described earlier.\textsuperscript{27,28} The total, intracellular, and extracellular concentrations of iron accumulated in the biomass were determined to account for the extent of iron recovery from SAMD, and the values were expressed as mg g\textsuperscript{−1} of biomass (dry wt).

For analysis of biodiesel after 96 h, 10 mL of culture suspensions were centrifuged and washed twice with 0.025 M ethylenediaminetetraacetic acid (EDTA) to remove EPS together with metals adsorbed onto the cell surface. Following solvent extraction using HCl–MeOH mixture and in situ transesterification, biodiesel was then extracted with hexane. The hexane layer was removed for quantification of biodiesel gravimetrically, and the yield values were expressed as percentages on the basis of dry wt of biomass.\textsuperscript{27} The leftover aqueous phase together with the residual biomass was then concentrated in a vacufuge concentrator (Eppendorf) at 60 °C for 2 h. The biomass pellet was digested and the digest was diluted 10 times with 5% nitric acid and used for iron analysis in ICP-MS (Agilent technologies, USA). The biodiesel obtained was also analyzed for iron after diluting with 5% nitric acid. The values for iron partitioned in biodiesel and the residual biomass were expressed as percentages.

### 4.3. Statistical Analysis

The standard deviations for the experimental data means (n = 3) were calculated using GraphPad Prism V.8 software, and the statistical significance (P ≤ 0.05) of the means was determined following one-way ANOVA analysis and Duncan’s multiple range test using IBM SPSS statistical software (version 24, USA).

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c00255.

Species distribution of iron supplemented in SAMD as determined by Visual MINTEQ modeling; determination of IC\textsubscript{50} values following growth inhibition, in terms of RFUs of chlorophyll, and in strains of MAS1, MAS3 at various iron concentrations in SAMD (PDF)

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#### Notes

The authors declare no competing financial interest.

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