An in vitro Anti-microbial Photodynamic Therapy (aPDT) with Blue LEDs to Activate Chlorophylls of Alfalfa Medicago sativa L on Aggregatibacter actinomycetemcomitans

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Abstract

Aggregatibacter actinomycetemcomitans is one of bacteria which play role in aggressive periodontitis. A. actinomycetemcomitans has been implicated as the reason that aggressive periodontitis does not respond to conventional therapy alone. These pathogens are known to remain in the tissues after therapy to reinfect the pocket. A. actinomycetemcomitans as a dominan periodontopathic bacteria and the discovery that this organism penetrates the tissues offered another perspective to the pathogenesis of aggressive periodontitis. Anti microbial photodynamic therapy (aPDT) is a medical treatment that utilizes light to activate a photosensitizing agent. The exposure of light to photosensitizer results in the formation of oxygen species, causing localized photodamage and cell death. The aim of this study was to investigate the effect of aPDT LED with various of quantum yield density or laser irradiation energy dose to activate chlorophyll of Alfalfa Medicago sativa L on A. actinomycetemcomitans bacteria.

To determine the antimicrobial effect on A. actinomycetemcomitans, samples were distributed to 3 groups as follow: (1) Groups A (treated with MIC of chlorophylls and LED 453 nm with varying quantum yield density (4.09; 7.73; 12.28; 16.38, and 20.48 J/cm2), (2) Group C- (negative control, no exposure to either chorophylls), (3) Group C+ (treated only with chlorophylls). The suspension was planted on sterile media and incubated at a temperature of 37oC for 24 hours. After incubation, the number of colony-forming units per milliliter (CFU/ml) was determined. The results were analyzed by analysis of variance (ANOVA) and the Tukey test. A P value ≤0.05 was considered to indicate a statistically significant difference.

The chlorophyll of Alfalfa Medicago sativa L absorption for LED 435 nm exposure is 77,2%. Irradiation of LED with various of quantum yield (4.09; 7.73; 12.28; 16.38 and 20.48) J/cm2 can activate chlorophyll of Alfalfa Medicago sativa L to produce ROS that cause damage to the bacterial cell. The control (+) and control (-) group did not significantly differ each other ($p>0.05$). The blue LED treatment group resulted in statistically significant decrease of CFU ($p<0.05$) compared to the control group. The Tukey post hoc test result that the highest quantum yield density 20.48 J/cm2 reduce CFU of A. actinomycetemcomitans up to 81%.

Irradiation of LED with various of quantum yield can activate chlorophyll of Alfalfa Medicago sativa L to produce ROS that cause damage to the bacterial cell. An increase in the density of the quantum yield reduce the number of bacterial viability. The effectiveness of quantum yield for producing a particular ROS type depends on photosensitizer, the availability of oxygen, and the reaction environment.

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Introduction

Periodontitis, an inflammation of the gingiva and the adjacent attachment apparatus, is characterized by loss of connective tissue attachment and alveolar bone. The primary etiology is bacterial plaque, which can initiate destruction of the gingival tissues and periodontal attachment apparatus[1-2]. It may be sub-classified based on etiology, clinical presentation, or associated complicating factors. The microbial etiology of periodontitis has been extensively

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studied and it is not associated with a single microorganism, but a group of bacteria participating in the initiation and progression of periodontitis. For periodontopathic bacteria to cause periodontal diseases, it is essential that they are able to colonize subgingival pockets and produce virulence factors that directly damage host tissue [3].

Aggressive periodontitis is an early-onset, destructive disease that shows a high rate of periodontal progression and distinctive clinical features. Population studies show that the disease is more prevalent in certain geographic regions and ethnic groups. Aggressive periodontitis is an infectious disease, and recent data show that in affected subjects the subgingival microbiota is composed of a mixed microbial infection, with a wide heterogeneity in the types and proportions of microorganisms recovered. There is also evidence that the *Aggregatibacter actinomycetemycomitans* (*A actinomycetemcomitans*) may play an important role in the development of the aggressive periodontitis. *A actinomycetemcomitans* primary human ecologic niche is the oral cavity. It is found in dental plaque, periodontal pockets, and buccal mucosa in up to 36% of the normal population. The organism can apparently seed from these sites to cause severe infections throughout the human body, such as brain abscesses and endocarditis [4-5]. There is a large body of evidence which implicates *A. actinomycetemcomitans* as an important microorganism in the etiology of aggressive periodontitis[6]. Neither mechanical plaque removal nor flushing or rinsing with disinfectants allows the complete eradication of bacterial reservoirs within the periodontal pocket. The same holds true for slow-release antimicrobial devices impregnated with antibiotics. In aggressive periodontitis or in subjects refractory to therapy, treatment with antibiotic drugs is advisable. At recent there is increasing awareness of resistance-related phenomena. Resistance development may be the consequence of injudicious use of antibiotics in common bacterial or viral infections. In general, the use of antibiotic drugs has advantages and disadvantages, and this is true also with respect to resistance issues of periodontal bacteria [7-9]. Systemic administration of drugs may cause adverse effects, mostly as gastrointestinal disturbances. Therefore, it is necessary that other alternative methods be more effective and safe in maintaining oral health and preventing periodontitis , as well as be eco-friendly, one of which is the photodynamic method[10-11].

Photodynamic therapy (PDT) is a medical treatment that utilizes light to activate a photosensitizing agent (photosensitizer). The exposure of light to the photosensitizer results in the formation of oxygen species, such as singlet oxygen and free radicals, causing localized photodamage and cell death[12-14]. Antimicrobial PDT also known as photodynamic inactivation (PDI)[12], lethal photosensitization, photoactivated disinfection (PAD) or photodynamic antimicrobial chemotherapy (PACT)[15] represents an alternative treatment for drug resistant pathogens and has made a comeback as a possible approach to treat multidrug resistant infections. Drug resistant bacteria can be effectively eliminated by PDT [16] and as of yet there are no reports of microbes becoming resistant to PDI despite numerous attempts to induce resistance by repeated cycles of semi-lethal PDT and microbial regrowth [17].

Photodynamic therapy represents an alternative antibacterial, antifungal, and antiviral treatment for drug-resistant organisms. Various studies have shown that Gram-positive bacteria are most susceptible to aPDT[17-18]. Special interest is concentrated on aPDT effects in bacteria resistant to antimicrobial drugs. Reliable method of terminating the strains is important in treating hospital infections such as Pseudomonas aeruginosa or MRSA. which has been attained by aPDT [19]. The resistance of bacteria can be overcome by cell wall modification or by the selection of appropriate sensitizing dyes [20-23]. Experiments in rats have shown that lethal photosensitization of Porphyromonas gingivalis is possible in vivo, and that it results in decreased bone loss. Thus, it was suggested that photodynamic therapy might be useful as an alternative approach for the antimicrobial treatment of periodontitis [10-11, 24-26].

PDT requires a source of light that activates the photosensitizer by exposure to low-power visible light at a specific wavelength. More than 400 compounds are known with photo-sensitizing properties including dyes, drugs, cosmetics, chemicals, and many natural substances[27]. Organic photosensitizer is generally extracted from natural materials such as chlorophyll from green plants and

photosynthetic bacteria. There are two types of chlorophyll: chlorophyll-a ($C_{55}H_{72}MgN_4O_5$) and chlorophyll-b ($C_{55}H_{70}MgN_4O_6$), pheophytin *a* & *b* and chlorophyllide *a*[20, 28,29]. The width of the absorption spectrum of chlorophyll is very wide (light absorption peak of chlorophyll-a is 430-662 nm, and chlorophyll-b is a 453-642nm). The main functions of chlorophyll in photosynthesis process are absorbing light, transferring energy, and separating charge on the photosynthetic membrane[30]. Chlorophyll has photophysics properties similar to porphyrins but with wider absorption area and higher intensity than porphyrins, so chlorophyll more applicable to be developed for PDT in tumors and cancer[31] . High energy absorption during the process of photosynthesis is caused by long excitation process (≤ 10^{-8}seconds). The longer excitation process of singlet chlorophyll, the larger the electronic energy conversion from the ground state to the triplet excited state may occur. The excess energy at triplet excited state gives opportunities to chlorophyll to transfer energy to oxygen molecule around them. This reaction produces reactive oxygen singlet (ROS) which is responsible for aPDT[32].

In the past, photosensitizer activation was achieved *via* a variety of light sources, LED and laser[10-11,33]. Light sources, such as dye lasers, these laser systems are complex and expensive. For treatment of larger areas, non-coherent light sources, such as tungsten filament, quartz halogen, xenon arc, metal halide, and phosphor-coated sodium lamps, are in use. Recently, non-laser light sources, such as light emitting diodes (LED), have also been applied in PDT[34-36]. These light sources are much less expensive and are small, lightweight, and highly flexible.

The aim of this study was to investigate the effect of aPDT LED with various of quantum yield density or laser irradiation energy dose to activate chlorophyll of Alfalfa Medicago sativa L on A. actinomycetemcomitans bacteria.

Materials and methods

Bacterial Strain and Culture Conditions

The sample strains used on this research was pure culture bacteria from *A. actinomycetemcomitans* bacteria collected from tooth of patients diagnosed aggressive periodontitis in Dental Hospital Airlangga University Surabaya Indonesia.

Materials

Chlorophyll of *Alfalfa Medicago sativa L* (K-Link liquid chlorophyll) with concentrations of 8 mg/ml. To know the absorption spectrum of photosensitizer, characterization using UV-Vis spectrophotometer 1800 Shimadzu was conducted.

Light Source

Light source was carried out with light emitting Diode (LED) local product with specification power 1 W, electric current 750 mA, electrical voltage 3,3 V and emission peaks 453 nm. The performance of source lights was characterized by measure power stability against temperature and distance

Minimum Inhibitory Concentration

A broth microdilution method was used to determine the minimum inhibitory concentration (MIC) of chlorophylls against *A. actinomycetemcomitans*. Briefly, testing was done using 96- well flat-bottomed microplate (Nunc, Denmark), with an assay volume of 200 µl/well. First, Tryptocase Soy Broth (TSB) (Merck, Darmstadt, Germany) was added (90 µl) to each well. The chlorophylls of *Alfalfa Medicago sativa L* (0; 0.025; 0.05; 0.075; 0.1) % w/v was added (90 µl/well) to wells and serially diluted two-fold across the plate. The plates were then inoculated with a 20 µl/well of fresh TSB bacterial cultures, with a concentration of 10^7 CFU/ml for *A. actinomycetemcomitans*. The final bacterial cell concentration in the wells was 10^5 CFU/ml. Then the microplates were incubated for 24 hours at 37°C, under microaerophilic conditions. The MIC was defined as that concentration of the substance that will inhibit the visible growth of microorganism after 24 hours of incubation. All tests were repeated at 5 times. The results were analyzed by analysis of variance (ANOVA) and the Tukey test. A P value ≤0.05 was considered to indicate a statistically significant difference.

Antimicrobial Effect of Treatments Against *A. actinomycetemcomitans* with Total Plate Count (TPC)

To determine the antimicrobial effect of treatments on *A. actinomycetemcomitans*, samples were distributed to 3 groups as follow: (1) Groups A (treated with MIC of chlorophylls and LED 453 nm), (2) Group C^- (negative control, no exposure to either chorophylls), (3) Group C^+ (treated only with chlorophylls), for each group the experiment was repeated at least 3 times. All

treated with various quantum yield density (4.09; 7.73; 12.28; 16.38, and 20.48) J/cm^2. The suspension was planted on Tryptocase Soy Agar (TSA) (Merck, Darmstadt, Germany) sterile media and incubated at a temperature of 37^{o}C for 24 hours. After incubation, the number of colony-forming units per milliliter (CFU/ml) was determined. The results were analyzed by analysis of variance (ANOVA) and the Tukey test. A P value ≤0.05 was considered to indicate a statistically significant difference. The percentage decrease in the number of bacterial colonies growth defined as: | (Σ sample colony - Σ control colony)/ Σ control colony | x 100%.

Results

Light Source Characterization

Characterization of the light source includes a wavelength and temperature during light exposure. The characterization of light source results of light source in various electrical power and temperature at a distance of 2 cm are shown in Figure 1(a) and 1(b) respectively. Figure 1(a) shows wavelength of LED versus electrical power, and Figure 1(b) temperature of LED versus time.

Figure 1a shows that LED has a range of wavelengths between 444 and 462 nm, with the emission peaks 453 nm. LED temperature measurement result (Figure 1.b) shows that temperature produced by LED increases over time. Unfortunately, the increase of temperature is in range optimization growth of bacteria *A. actinomycetemcomitans*. Therefore, it is possible that overtime exposure of laser can make bacteria grows better.

The absorption spectrum of chlorophyll of *Alfalfa Medicago sativa L* as photosensitizer in wavelength of 300 – 900 nm from UV-Vis spectrophotometer is shown in Figure 2. Based on the test results, the absorption percentage of chlorophyll of *Alfalfa Medicago sativa L* at wavelength of 453 nm (LED) can be calculated as follows[37]:

Abs= log 1/T (Abs:Absorbance, T: transmittance)
For 453 nm LED: 0.6416= log 1/T ; T = 0.228,
So the chlorophyll of *Alfalfa Medicago sativa L* absorption for LED 435 nm exposure in % is = (1-0,228) x 100% = 77,2%. From this result, the quantum yield density of photodynamic treatment can be calculated for various exposure times. Table 1 shows quantum yield density of LED exposure with chlorophylls of *Alfalfa Medicago sativa L* as photosensitizer.

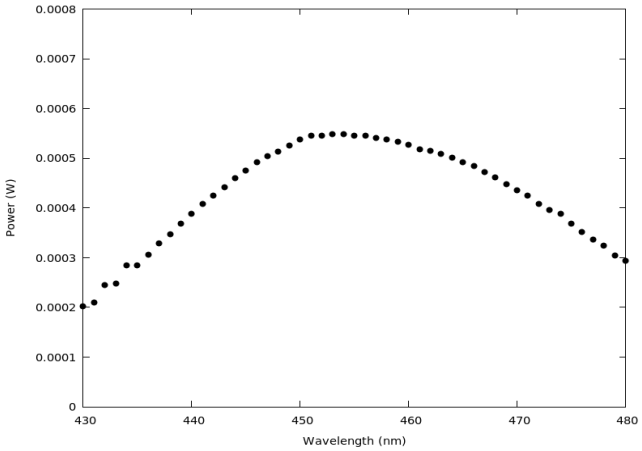

Figure 1a. Wavelength of LED versus electrical power.

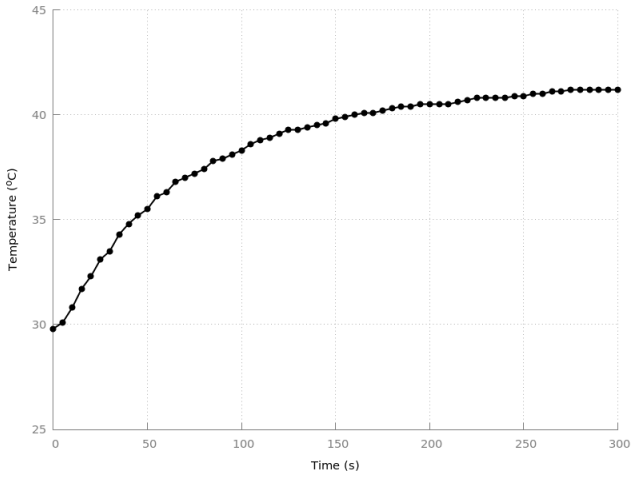

Figure 1.b. Temperature of LED versus exposure time.

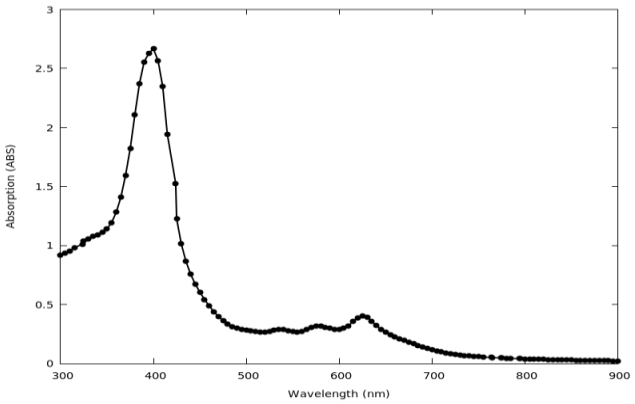

Figure 2. Absorption spectrum of chlorophyll in wavelength of 300 – 900 nm.

LED Wavelength (nm)	Absorption	energy of LED (J/cm^2)	Quantum Yield density (J/cm^2)	Decrease percentage bacterial growth (%)
444 - 462	77%	5.31	4.09	10,31
		10.03	7.73	20,24
		15.94	12,28	30,27
		21.27	16,38	37,33
		26.60	20,48	81,04

Table 1. Percent decrease in bacterial viability at various quantum yield density of LED exposure with chlorophylls as photosensitizer.

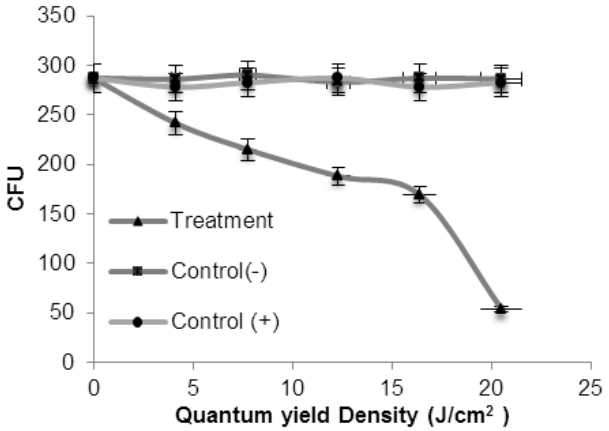

Figure 3a. Viability bacterial collonies in various quantum yield density of LED.

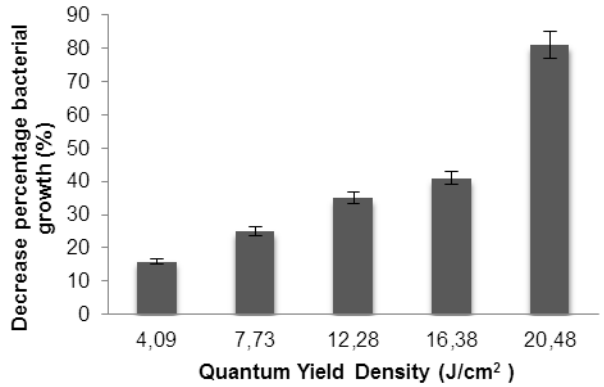

Figure 3.b. Decrease percentage bacterial growth in various quantum yield density of LED exposure.

The minimum inhibitory concentration (MIC) test of chlorophylls of *Alfalfa Medicago sativa L* demonstrated that this dye did not present any toxic effects against *A. actinomycetemcomitans*. The chlorophylls concentration (0; 0.025; 0.05) % in statistically did not significantly differ each other (p>0.05). So in the next treatment, chlorophylls 0.025% w/v added to the bacterial suspension.

The control (+) and control (-) group did not significantly differ each other (p>0.05) (Fig.3a). The blue LED treatment group resulted in statistically significant decrease of CFU (p<0.05) compared to the control group. The Tukey post hoc test result that exposure of LED with quantum yield density of 20.48 J/cm^2 highest decreases the number of bacteria *A. actinomycetemcomitans.* Figure 3 show viability bacterial collonies and decrease percentage bacterial growth (%) in various quantum yield density of LED exposure with chlorophylls of *Alfalfa Medicago sativa L*. The results in Table 1 and Figure 3 show that exposure of LED at distance of 2 cm with quantum yield density of 20.48 J/cm^2 decreases the number of bacteria *A. actinomycetemcomitans* by 81%. These results are mainly due to use of Chlorophyll of *Alfalfa Medicago sativa L* as exogenous photosensitizer, which can utilize 77% of the light with wavelength of 453 nm.

Discussion

Aggressive periodontitis is an infectious disease, and recent data show that in affected subjects the subgingival microbiota is composed of a mixed microbial infection. There are significant differences in the microbiota of the disease among different geographic regions and ethnicities. There is also evidence that the *A. actinomycetemycomitans* may play an important role in the development of aggressive periodontitis[7-9]. Scaling and root planing has been considered the gold standard treatment for periodontitis. However, scaling and root planing does not lead to major clinical improvements in all subjects, especially in cases of advanced disease and deep periodontal pockets, because scaling and root planing alone does not cause a sufficiently deep change in the subgingival microbial composition to achieve and maintain a profile compatible with periodontal health. Scaling and root planing does not target specific bacterial species. After scaling and root planing, the first colonizers as well as the Actinomyces species, re-emerge in greater proportions and the pathogens of the red and orange complexes recolonize more slowly[38]. Therefore, numerous studies have assessed the effects of locally delivered antiseptics and antibiotics as adjuncts

to periodontal treatment[39]. Local antimicrobial therapy has more commonly been used during the maintenance phase, for treating remaining and isolated active pockets. Systemic antibiotics adverse drug reactions, uncertain patient compliance and lower concentration of the drug at subgingival sites[3].

aPDT combines a nontoxic photoactive dye, photosensitizer, with harmless visible light to generate singlet oxygen and free radicals that kill microbial cells[24-26]. Previous studies on the effectiveness of PDT have focused on different microorganisms and photosensitizers. Methylene blue showed less efficiency on *A. actinomycetemcomitans*, which only presented a 64% bacterial reduction[40]. A more recent study using photosensitizers based on porphyrin skeleton and a red laser showed only a 62% reduction of *A. actinomycetemcomitans*[41]. Bacterial reductions differed from 78% to 95% depending on the quantum yield density used[42].

In this study, Chlorophyll of *Alfalfa Medicago sativa L* as exogenous photosensitizer, which can absorb 77% of LEDs 453 nm. This experiment demonstrated that this dye did not present any toxic effects against *A. actinomycetemcomitans* as photosensitizer 2 mg/ml added to the bacterial suspension without irradiation did not reduce the CFU number.

By irradiation with LED in the visible range of the spectrum with various energy (4.09; 7.73; 12.28; 16.38, and 20.48) J/cm^2, the dye (chlorophyll a) will be excited 2A, 3A and 4A with excited energy (1.87; 2.14; 2.88) eV and intersystem crossing to its triplet state. Activated photosensitizer in the excited triplet state can induce chemical changes in a neighboring molecule that water suspension is composed of oxygen[12-13]. Energy excess is transferred to molecular oxygen to produce the very reactive singlet oxygen ($^1O_2$). Transfer of electrons (or protons) to oxygen or other adjacent molecules to form a radical anion or cation, respectively. These radicals are likely to react with molecular oxygen to produce reactive singlet oxygen (ROS). The quantum yield for a particular ROS type depends on the nature of the sensitizer, the availability of oxygen, and the reaction environment. The photosensitizer was degraded after the irradiation and new photo-products, ROS were formed in suspension. These radicals capable of reacting with biological systems and destroying them [13,43-44]. The photochemical reactions occur in parallel, and the ratio depends on several parameters, with the photosensitizer used and the oxygen concentration being the most important [45-46].

Each chlorophyll molecule can typically produce 10^3-10^5 molecules of singlet oxygen before being degraded through photobleaching or by some other process. The next step is formation of hydrogen peroxide (H_2O_2) due to occurrence of singlet oxygen. High concentrations of hydrogen peroxide and singlet oxygen can form hydroxyl radical (reaction Haber Weiss) which is also produced from metal ions such as iron and copper through Fenton reaction. Hydroxyl radicals easily diffuse through the membrane and cause bacteria cell damage[13]. The results of this study showed that increased energy of quantum yield decreases the amount of bacteria viability. The higest quantum yield density 20.48 J/cm^2 reduced *A. actinomycetemcomitans* by up to 81% of the colony forming units.

Periodontal diseases will be one of the main applications for PDT within the oral cavity. Reasons for such a use are the multispecies infection and multifactorial genesis, the localization of the bacteria and, probably the fact that the method is easy to perform. By photosensitization, even the multi-resistant Gram-negative hospital strains are killed[47]. Besides killing, important virulence factors of Gram-negative bacteria are diminished by PDT, e.g., endotoxins and proteases [25-26,33,48].

Conclusions

Exposure of LED with various of quantum yield can activate chlorophyll of *Alfalfa Medicago sativa L* to produce ROS that cause damage to the bacterial cell. An increase in the density of the quantum yield reduce the number of bacterial viability. The highest quantum yield density 20.48 J/cm^2 reduce CFU of *A. actinomycetemcomitans* up to 81%. The effectiveness of quantum yield for producing a particular ROS type depends on photosensitizer, the availability of oxygen, and the reaction environment.

Declaration of Interest

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