Journal of
Bioremediation & Biodegradation

Mezzari et al., J Bioremediat Biodegrad 2017, 8:1
DOI: 10.4172/2155-6199.1000379

Research Article OMICS International

Elimination of Antibiotic Multi-Resistant *Salmonella* Typhimurium from Swine Wastewater by Microalgae-Induced Antibacterial Mechanisms

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Abstract

The effect of microalgae-based swine wastewater treatment on the removal of antibiotic multi-resistant *Salmonella enterica* serovar Typhimurium was investigated. Photobioreactors (PBRs) containing diluted swine digestate with and without microalgae *Scenedesmus* spp. were inoculated with *S.* Typhimurium (10^8 Colony Forming Units per milliliters - CFU mL^{-1}). Viable cells of *S.* Typhimurium were quantified over time by plate counts and qPCR amplification of the *Salmonella* invasion gene activator, *hilA*. In the absence of microalgae, *S.* Typhimurium concentrations increased 1.5 log cells mL^{-1} in 96 h. In the presence of microalgae, *S.* Typhimurium was completely eradicated within 48 h. In the PBRs with controlled pH (6.8 ± 0.8), concentration of *S.* Typhimurium remained constant (2.8 ± 0.2 log CFU mL^{-1}) throughout 96 h. Thus, natural increase in pH>10 due to photosynthesis was detrimental to the antibiotic multi-resistant bacteria survival. Phycoremediation holds promises as an alternative for wastewater treatment process for the elimination of the serious public health threatening antibiotic multi-resistant bacteria, thus effectively avoiding Salmonellosis outbreaks arising from animal farming activities.

Keywords: Antibiotic-resistant bacteria; *hilA* gene; *Salmonella enterica* serovar Typhimurium; *Scenedesmus* spp.; Swine wastewater

Introduction

Major concerns exist over the several invasive and antibiotic resistant organisms thriving in swine wastewaters and that are known to threaten human and animal health. Among them, *Salmonella enterica* serovar Typhimurium deserves special attention since it is the most prevalent antimicrobial resistant serovar in swines, and also frequently related to human infections and outbreaks [1].

Several physicochemical approaches are described to control the proliferation of pathogens, such as exposure to UV irradiation [2,3] use of strong oxidant radicals [2], pH increase [4], and selective membranes [5]. However, most if not all of these approaches are not economically feasible. Therefore, biological anaerobic digestion followed by stabilization ponds are the most common treatment option adopted in swine farming worldwide [3,6,7]. Pathogen elimination can occur under thermophilic conditions, but not under mesophilic conditions that prevail in most digesters [8].

Phycoremediation has been considered as an efficient tertiary treatment method to reduce organic compounds and nutrients from wastewaters [9-12]. Some microalgae produce a wide variety of antibacterial substances that can inhibit or kill pathogens [13,14]. These waterborne pathogens may also be sensitive to high oxygen concentrations and increased pH produced by photosynthesis. Nevertheless, to the best of author's knowledge, the mechanism in which phycoremediation reduce or even eliminate invase antibiotic multi-resistant bacteria from swine wastewaters has not been fully explored. This study demonstrates whether phycoremediation of swine wastewaters could effectively control the proliferation of antibiotic-resistant *Salmonella enterica* serovar Typhimurium. Ancillary objective include the evaluation of a pre-enrichment method for rapid quantification of S. Typhimurium from complex environmental samples.

Materials and Methods

Photobioreactor conditions

Laboratory scale photobioreactors (PBRs) were used to simulate phycoremediation of swine wastewater. Each PBR (3.5 L beakers) was filled with 3 L of diluted swine digestate (6%, v:v). Effluent physicochemical characteristics were (g L^{-1}): pH 7.9, total solids (3-8), total organic carbon (1.5-6.5), total inorganic carbon (0.8-1), total nitrogen (1.5-2), ammonia-N (0.9-1.5), phosphate-P (0.045-0.06). PBRs were subjected to mixotrophic conditions (12 h, light: dark) using red light emission diode light (PGL-RBC 2500, Parus) at 630 nm and 121.5 µmol m^{-2} s^{-1}, room temperature (22 ± 2°C), and continuous mixing. PBRs were inoculated with *Salmonella enterica* serovar Typhimurium (10^5 CFU mL^{-1}) and *Scenedesmus* spp. (30% v/v, 70 mg L^{-1} dry weight biomass), except the negative control without microalgae. To discern microalgae antibacterial effects from pH-derived photosynthesis, pH was adjusted daily in a set of PBRs by adding either 0.1 M HCl or 0.1 M NaOH. All experiments were conducted in duplicate.

Inocula source and identification

Microalgae was collected from a field scale facultative open pond used as tertiary treatment process downstream from a biodigestor at the Brazilian Agricultural Research Corporation (EMBRAPA) swine wastewater facility (Concórdia, SC, Brazil). *Scenedesmus* spp. was the dominant microalgae in the PBRs as described elsewhere [9,10]. The strain was isolated and deposited in the collection of photosynthetic microorganisms for Agroenergy Research at Embrapa (Brasília, DF, Brazil) under access number Embrapa LBA#31 (IAN193.096).

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Received December 13, 2016; **Accepted** January 06, 2017; **Published** January 09, 2017

Citation: Mezzari MP, Prandini JM, Kich JD, Da Silva MLB (2017) Elimination of Antibiotic Multi-Resistant Salmonella Typhimurium from Swine Wastewater by Microalgae-Induced Antibacterial Mechanisms. J Bioremediat Biodegrad 8: 379. doi: 10.4172/2155-6199.1000379

Salmonella enterica serovar Typhimurium was collected from a local river stream (Concórdia, SC, Brazil), characterized as a multi-resistant bacteria [1] and stored at the Embrapa's microorganism bank collection under accession # 12301.

Analytical methods

Samples were analyzed twice a day for pH, temperature (pH–mV, Hanna Instruments, Inc.), dissolved oxygen (DO) (Lutron DO-5519). Microalgae were quantified by optical density at 570 nm (Hach DR/2000). A satisfactory correlation (r^2=0.97) between DW (gravimetric measuremets) and OD_{570} (mg-DW L^{-1}=536.2 × OD_{570nm}-36.89) was obtained. The specific growth rate μ (day^{-1}) was calculated by fitting the microalgae dry weight obtained from samples within the first 24 hours of microalgae growth to an exponential function of (lnX-lnX_0/t), where t was the time between the two measurements, and X and X_0 (mg-DW L^{-1}) were concentrations of biomass at t (24 hours) and t_0, respectively.

S. Typhimurium quantification – plate culture and qPCR

S. Typhimurium inoculum was collected from an overnight culture plate, sub-cultured into nutrient broth (50 ml) and incubated in a shaker (100 rpm, 37°C, 24 h). Density of cell suspension was assessed with McFarland turbidity standard No. 05 (1-2 × 10^8 CFU mL^{-1}). Samples from PBRs were diluted (10^0-10^{-5}) in saline media (0.85%) and spread onto Chromogenic XTL4 agar plates (100 µL) to enumerate *S.* Typhimurium colonies. Plates (duplicates from PBRs) were incubated overnight and results were reported as CFU mL^{-1}.

Samples from PBRs were collected daily and diluted ten times in buffered peptone water for incubation at 37°C for 6 h – pre-enrichment step [15] prior DNA extraction (MoBio UltraClean Microbial DNA kit). Template genomic standard curves (10^{-1} to 10^{-10}) were also subjected to the pre-enrichment step. *hilA*, a *Salmonella* gene required for pathogenicity invasion, was quantified by qPCR using specific primers [16]. Each 20 µL qPCR reaction mixture contained 2 × qPCR-SYBR-Green mix (Ludwig Biotec, Brazil), 500 nM forward and reverse primers (Prodimol Biotecnologia®, Brazil), sterile DNAse-free water (Ludwig Biotec, Brazil) and 16 µg of DNA template. qPCR reactions were performed (7500 Applied Biosystems, The Netherlands) in a two-step thermal cycling procedure (95°C, 10 min; followed by 40 cycles of 95°C for 15 s and 62°C for 30 s). Quantitation of *S.* Typhimurium was performed by interpolation from DNA template standard curves.

Statistical analysis

Tukey's Pairwise Comparison test was used to determine significant difference between two data sets at 95% confidence level (p<0.05). All data were subjected to one-way analysis of variance (ANOVA) using OriginPro 8© OriginLab Corporation.

Results and Discussion

Scenedesmus spp. growth

Scenedesmus spp. are known for their environmental ubiquity and predominance in waste stabilization ponds and high-rate algal ponds [17]. In the present study, microalgae growth rates of 96.5 ± 1.7 and 82.3 ± 3.4 mg-DW L^{-1} d^{-1} were reached in PBRs with and without pH adjustments, respectively. Specific growth rates (μ) were also calculated during the exponential growth phase of *Scenedesmus* spp. The mean value of μ in the interval from 0 to 24 h for PBRs with and without pH adjustments were 0.97 and 0.92 day^{-1}, respectively. These values were within the higher growth rates reported for *Scenedesmus* sp. in swine wastewaters [10,17]. The biomass increase measured in both PBRs treatments were very similar (p<0.05), with maximum microalgal concentration of 265 ± 3.9 mg-DW L^{-1} observed at 72 h (Figure 1). These experimental results are within typical values of maximal biomass production (0.2-1.0 g-DW L^{-1}) reported for *Scenedesmus* spp. in domestic and swine wastewaters from mixotrophic growth [17]. Interestingly, *Scenedesmus* spp. is a promising microalgal species that can thrive in wastewaters with great performance on biomass production and lipid accumulation than other high-lipid-content microalgae [18]. Although not the scope of the present investigation, the obtained *Scenedesmus* spp. growth performance and biomass production are comparable to other studies focused on microalgal biomass to bioenergy conversion [19,20]. Thus, for a swine wastewater treatment plant that uses tertiary phycoremediation treatment to remove pathogens and nutrients, microalgae biomass production could also aid in valuable biofuel products.

Phycoremediation effects on S. Typhimurium

Salmonella spp. are able to thrive in different environments, survive several weeks in dry environments and several months in water, thus proliferation of *S.* Typhimurium in swine wastewater digestate was

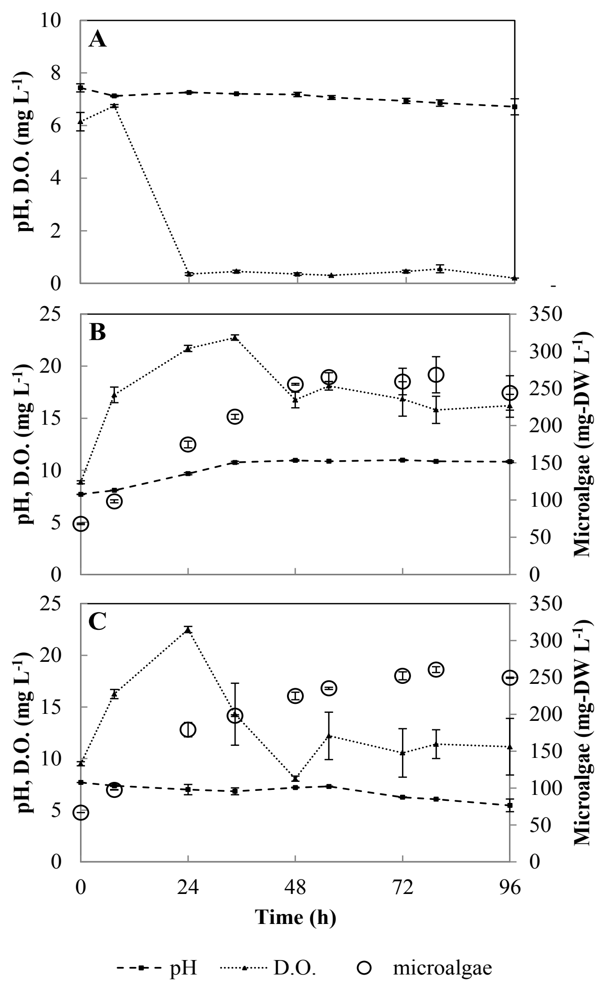

Figure 1: Dissolved oxygen (mg L^{-1}), pH and microalgae biomass concentration (mg-DW L^{-1}) profile in mixotrophic photobioreactors (PBRs) without microalgae (A), with microalgae (B) and with microalgae under pH control conditions (C). Data are presented as mean ± standard deviation (n=2).

somewhat expected and later confirmed in the biological reactors without microalgae (Figure 2). In these reactors, *S.* Typhimurium increased from 7.8 ± 1.9 × 10^2 CFU mL^{-1} (t=0 h) to a maximum of 3.4 ± 0.8 × 10^4 CFU mL^{-1} (t=72 h).

Removal of pathogens from phycoremediation in ponds are usually attributed to a combination of long retention times, UV irradiation, high temperature, increased dissolved oxygen (DO) concentrations and high pH [21]. Among these factors, elevated DO and pH levels, resultant from optimal microalgal photosynthetic activities, have greater effects on pathogen inhibition and inactivation [22]. While it is possible to artificially increase DO and pH by aeration and chemicals addition, respectively, these practices are usually economically unfeasible and can be challenging to dosage at field scale. In this regard, phycoremediation could be alternatively considered as a disinfection step in wastewater facility. S. Typhimurium was completely removed from PBRs within 48 hours (Figure 2). In pH adjusted PBRs, *S.* Typhimurium concentrations did not vary (p=0.12) throughout the entire experiment time frame and remained constant at 7.5 ± 1.4 × 10^2 CFU mL^{-1}. Negligible difference in DO (p>0.05) concentrations were observed between PBRs treatments (Figure 1), thus discharging the probability of bacteria elimination by DO. The measured DO concentrations were above solubility levels (i.e., 8.3 mg L^{-1} at 1 atm and 25°C) (Figure 1). Oxygen saturation is normally found in ponds or photobioreactors due to high microalgae growth rate and associated photosynthetic activities [10,17,21]. The high pH values measured in the PBRs without pH adjustment seemed to play a major role on the inactivation of *S.* Typhimurium during Scenedesmus spp. growth.

The antibacterial properties of microalgae exsudates should not be ruled out [14]. *Scenedesmus* sp. has been previously shown to act against Gram-positive (e.g., *Staphylococcus aureus*) and Gram-negative (e.g., *Escherichia coli* and *Pseudomonas aeruginosa*) bacteria, but it was not very effective against *Salmonella* sp. [14,23]. This fact associated with the lack of *S.* Typhimurium removal from the pH controlled PBRs suggests that microalgae metabolites had insignificant contribution on bacteria removal.

Sample preenrichment and qPCR fast approach

Colony count techniques consider CFU numbers, which can consist of more than one cell count and thus lead to data overestimation [24]. Therefore, to increase accuracy in bacterial concentration, qPCR quantification method was also performed in parallel. The plating method showed significantly higher cell concentrations (between 0.8 to 1.5 log differences) than qPCR (Figure 2). However, no differences (p>0.05) in *S.* Typhimurium growth ratio (X/X_0) over time was verified between these two bacterial quantification methods.

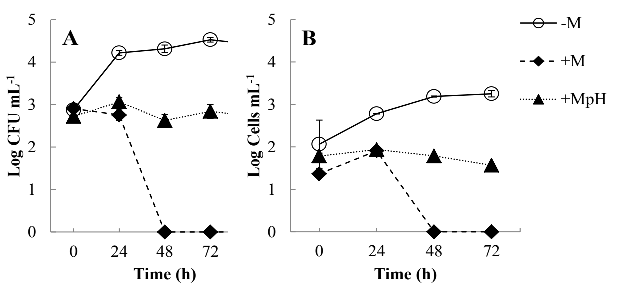

Figure 2: *Salmonella enterica* serovar Typhimurium concentration profile from PBRs without microalgae (-M), with microalgae (+M) and with microalgae and pH controlled conditions (+MpH). Bacteria were quantified by plate count (A) and qPCR (B). Error bars indicate standard deviation for replicates (n=4).

Conclusions

Phycoremediation of swine wastewater digestate was very effective to produce valuable microalgae biomass and eliminate antibiotic multi-resistant *S.* Typhimurium within 48 h. Pathogen removal was linked to the inhibitory effects of high pH (as high as ≅ 11) as a result of photosynthesis. Quantification of invasive *S.* Typhimurium in environmental complex samples was demonstrated by preenrichment followed by qPCR. Phycoremediation of swine wastewaters can be a promising treatment strategy to control the spread of antibiotic resistant bacteria in the environment. This can be particularly appealing when cogitating water reuse in animal farming and the risks associated with public health outbreaks from such practices.

Acknowledgements

The authors thank Remídio Vizzotto and Luiza Letícia Biesus from Embrapa Swine and Poultry for technical support. Authors thank financial support from Coordination for the Improvement of Higher Level or Education Personnel CAPES-EMBRAPA (#001/2011) and the Brazilian Agricultural Research Corporation – EMBRAPA (#02.12.08.004.00.05).

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