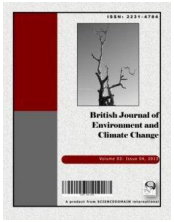

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Microalgae Assisted Bioremediation of Landfill Leachate Using a Biocoil Reactor: Evaluation of Operational Conditions Using Taguchi Experimental Design

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

The utilization of organic matter present in low-value effluents, such as landfill leachate, for cultivation of microorganisms exhibit an opportunity for low-cost cell growth while reducing the pollutants in the residue. The feasibility of reducing the organic load and toxic leachate through microalgal cultivation, using *Chlorella* sp., was investigated using a biocoil reactor. Operating conditions, as temperature, residence time, and illumination cycle were evaluated as control factors, and the responses in reduction of organic matter, turbidity, and metals present in the leachate were assessed. Statistical experimental design and analysis were performed using a Taguchi L4 array, and results show removal rates of TOC in 60%, COD in 68%, turbidity and boron contents in 98%, and a complete removal of iron.

Keywords: Microalgae; leachate; biocoil; Taguchi.

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1. INTRODUCTION

Microalgae have been traditionally used as food material and as feedstock to obtain a number of valuable products for application in cosmetics, pharmacy and nutrition sectors, as pigments, high value fatty acids, carotenoids, antioxidants and proteins [1,2]. In the last years, research about these microorganisms have been intensified, mainly considering their use for biofuels production. Alternatives of biorefineries to obtain biodiesel, bioethanol and other fuels and chemicals from microalgae have been evaluated [3].

Although most of the research evaluated using the bioprocessing capability of microalgae relies on its photoautotrophic capacity, there has been a current trend in utilizing mixotrophic-related growth on carbon-rich effluents [4]. In this particular mode of growth, both, organic compounds and CO_2 are the carbon sources, and it is proved to achieve higher biomass productivity when compared to merely autotrophic systems [5]. A number of different culture media has been described and significant research has been done on utilizing wastewater streams for microalgal growth [6-8].

Among the possibilities to compose a growth medium to culture microalgae, an interesting option is landfill leachate, a dark colored aqueous effluent formed by the percolation of water through landfills. In these systems, water interacts with waste in an anaerobic environment, transporting organic matter and inorganic compounds. It is often characterized as a toxic effluent with difficult degradation [9,10]. Landfill leachate is an issue of high concern in the academia and in the society as a whole, mainly considering the massive quantities generated and the possibility of transferring contaminants to groundwater, other ecosystems and human communities [11,12]. Thus, a proposed solution to reduce its toxicity by using it to compose the medium for microalgae stands out as a desirable alternative.

Due to many possible compositions of landfills, leachate composition is variable and depends on a wide variety of factors that include rainfall conditions, age of the landfill operation and its structure, and environmental, cultural, and dump management conditions [13]. Christensen et al. [14] classified organic compounds in leachate into two categories: the first corresponding to dissolved organic matter, humic macromolecules, fatty acids, fulvic acids, in addition to lignin; the second corresponding to xenobiotic organic compounds, which are aromatic hydrocarbons and halogenated phenolic compounds, alcohols, aldehydes, ketones and light organic acids, along with other substances with characteristics toxic to the environment. One main concern found in treating leachate with phototrophic microorganisms is related to its dark color and high turbidity. This limitation reduces light penetration in the leachate and consequently becomes a critical factor for cell growth. Therefore, in order to industrially cultivate microalgae in landfill leachate, the adequate choice of a photobioreactor is fundamental. Photobioreactors are constructed of optically transparent materials such as glass and plastics derived from acrylic, allowing efficient light penetration [3]. Among the most common types found in literature, three main categories stand out: flat-plate, bubble column, and traditional aerobic fermenters [3]. There are also some studies with reactors designed specifically for microalgae, such as the biocoil reactor. The development of a pilot plant called "Biocoil" was held in the UK and in Australia in the 1980's [15]. One of the characteristics presented by the biocoil design is an enhancement of the area/volume ratio, which, in the case of microalgae, provides better light administration.

The nitrogen concentration in leachate is high (2000-5000 mg L^{-1}) and the presence of components as phosphorus and metals are interesting for cultivation of some species of microalgae [16-18]. Rai et al. [19] indicates that the algae from genus *Chlorella* can be a good choice for biological treatment of waste, due to their high resiliency in non-favorable growth media, with high concentration of inhibitors, such as high concentration of nitrogen, and heavy metals present. Researchers lately have been exploring reduction in toxicity of wastewater using species of *Chlorella* [16,20]. This article addresses the utilization of Chlorella sp. in landfill leachate, which is a highly non-favorable medium growth for traditional bioprocessing studies involving other types of aerobic organisms, like microalgae and fungi.

The present work aims to investigate the growth of *Chlorella sp*. in landfill leachate medium using a biocoil photobioreactor. This exploratory study aims to evaluate physical factors (temperature, light cycle, and residence time) involved in the operation of the proposed biocoil reactor. The

main objective is, thus, to evaluate the feasibility potential for a low-cost bioreactor to aid in leachate wastewater treatment.

2. MATERIALS AND METHODS

2.1 Microalgal Strain and Culture Medium

The marine microalgae *Chlorella sp.* used in this work was isolated from Cabo Frio (Rio de Janeiro, Brazil) and was provided by the "Aidar & Kutner" Microorganism Bank (BMA&K) from the Oceanographic Institute at the University of Sao Paulo. Stock culture was stored in a medium based on Guillard f/2.

2.2 Leachate Sampling and Storage

Leachate used in this study was obtained from a commercial landfill in Cachoeira Paulista (State of São Paulo, Brazil). After sample collection, the effluent was stored at 4ºC to minimize alterations of its physical-chemical properties [21]. Medium used in the experiments in the reactor was composed by leachate diluted ten times in water. Some properties and information about the composition of the diluted leachate are shown in Table 1.

Table 1. Turbity, COD, TOC and concentration of boron and metals in the diluted leachate

Component	Value
Turbidity (NTU)	9.19
COD (mg O_2 L^{-1})	251.8
TOC (mg L^{-1})	92.7
Ag (mg L^{-1})	0.025
B (mg L^{-1})	3.38
Cd (mg L^{-1})	0.11
Cu (mg L^{-1})	0.87
Fe (mg L^{-1})	19.615
Pb (mg L^{-1})	0.28

2.3 Biocoil Reactor and Cell Cultivation

The photobioreactor used in this study was adapted from Biocoil design described by Robinson et al. [20], being composed of two parts: a 20-L tank connected to a 24-L lighted tubular section. A total of 6 100-W fluorescent lamps was used in the prototype. In the system, schematically represented in Fig. 1, air was provided by an air compressor (1), and sterilized using a 0.22-µm filter, being directed to the tank (2). A sparger (3) was included to induce the formation of small air bubbles, providing a fixed aeration rate of 0.56 vvm (volume of air per volume of medium). A ½" (1.27 cm) - diameter pipe connected the aeration tank to a centrifugal pump (4), which promoted an up flow recirculation of medium into the illuminated section of the reactor, composed by a 100-m ¾" (1.91 cm)-diameter PVC pipe.

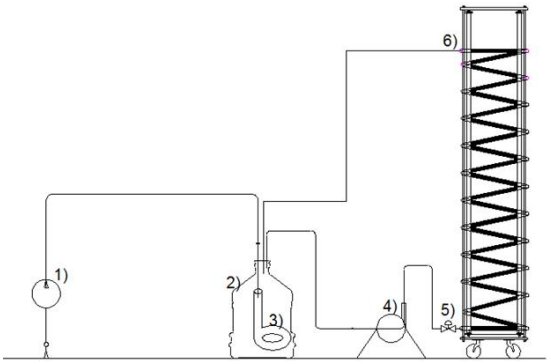

Fig. 1. Schematic representation of the biocoil reactor used in this study, composed by air compressor (1), growth tank (2), sparger (3), centrifugal pump (4), ball-type valve (5) and illuminated section (6)

Leachate was diluted 10 times with water, autoclaved for 30 min at 121°C and used as substrate in a mixotrophic cultivation of the microalgae. Stock solution of microalgae was added to obtain an initial cell concentration of $0.7*10^6$ cells mL^{-1}. Residence time was controlled by a ball-type valve (5).

Periodic samples were taken from the top of the illuminated part of the recirculation pipe system (6) and cell concentrations were estimated by optical density. Cell separation was performed using induced coagulation by $Al_2(SO_4)_3$ at a concentration of 1.0 g L^{-1}, as described by Papazi et al. [22]. Then, the supernatant phase was filtrated using 0.45 µm filters and frozen at -20°C until to be analyzed with relation to turbidity, COD, TOC, boron and metals concentration.

2.4 Experimental Design

Experiments were carried out according to a Taguchi experimental design and the evaluated variables were temperature, light/dark cycle time and residence time. An L_4 array was chosen, and it consists on an arrangement of four experiments with up to three factors at two levels (high and low). The codification of the variables and levels is presented in Table 2.

Table 2. Uncoded experimental conditions of Taguchi L_4 array

Code	Factor	Levels	
		1	2
A	Temperature (°C)	20	30
B	Light cycle (Light : dark, in hours)	12:12	24:00
C	Residence time (min)	5	12

The temperature was controlled using an air-conditioning/heater system, light cycle and light administration were controlled using a timer connected to the lamps in the reactor. The recirculation flow was controlled by a ball-type valve adapted at the entrance of the illuminated section of the reactor.

The response factors analyzed were COD (Chemical Oxygen Demand), TOC (Total Organic Carbon), Turbidity, and concentration of boron and metals (Ag, Pb, Zn, Fe, Cd, Cu). Statistical analysis was performed using Minitab 17 Statistical Software, and as response factors were codified as reduction factors. Reduction factor of a certain response variable was calculated as a function of the value of this response at time 0 ($factor_0$), and at the end of the microalgal cultivation, after cell separation ($factor_f$), as seen in Equation 1.

$$Reduction\ rate\ (factor) = 1 - \frac{factor_f}{factor_0} \quad (1)$$

2.5 Analytical Methods

Microalgal growth was monitored by optical density (OD) measurements at 690 nm, OD values were related to the number of cells per volume by a standard curve previously established, aided by a Neubauer chamber [6].

Nephelometric turbidity of the effluent samples was measured in a bench turbidimeter (TECNOPON 1000, MsTecnopon, Piracicaba, Brazil). COD, TOC, boron and metals were analyzed following standard procedures defined by APHA [21].

3. RESULTS AND DISCUSSION

Results from Taguchi L_4 array are presented in Table 3 (Appendix).

3.1 Cell Growth

Experiments 3 and 4 achieved similar growth profiles, reaching a steady concentration of cells after the first 24 h. Experiments 1 and 2, which were performed at a higher temperature, acquired a stationary profile between 48 h and 72 h (Fig. 2).

3.2 Statistical Analysis

Each experimental observation provides one degree of freedom to each factor at the analysis of variance (ANOVA), according to Taguchi method. Degree of freedom can be assigned, in this case, as the number of observations that can be varied independently of each other. The utilization of a L_4 array with 3 experiments provides no degree of freedom for the residual error, which is the basis of calculation for the F statistics, and therefore, the significance of certain source of error, or experimental factor. Thus, the statistical analysis of the experiments, in each analysis of variance, had the experimental factor with the least sum of squares used as a source of residual error in the experiments, due to its least statistical significance [23].

ANOVA is presented on Table 4, where MS is the mean of squares (equation 2), DF is the degree of freedom, and F is the ratio between the mean of squares and the mean of squares error. F test is used to evaluate the significance of each factor on the response variable (equation 3) [24].

$$MS = \frac{\sum_{i=1}^{n}(y_i - \overline{y})^2}{DF} \quad (2)$$

$$F = \frac{MS_{effect}}{MS_{error}} \quad (3)$$

3.3 Turbidity Reduction

The analysis on turbidity reduction was performed based on the initial turbidity of the diluted leachate of 9.19 NTU (Nephelimetric Turbidity Unit). From a qualitative view statistical analysis of results (Supplementary material, Fig. S1), it is expected that for a more efficient reduction in turbidity, the experiments should be performed with temperature and light cycle at

their low levels (20°C, 12 h: 12 h, respectively) and the circulation time, variable related to the average residence time in the lit tube, at the high level, 12 min. However, from the ANOVA (Table 4), it is seen that the temperature factor is the one that contributes least to the effect of turbidity reduction, as its quadratic sum is the smallest of all (0.0121<0.0289<0.0841).

The reduction in turbidity is related to the reduction in interfering particulate suspended matter in the effluent. The prototype biocoil photobioreactor showed promising results for the reduction of turbidity. Interestingly, the temperature did not become of great significance in the results. Extrapolating this idea, the operation of the photobioreactor in open-air conditions would not present significant differences for treatments at 20°C or 30°C; which is a typical temperature range throughout the year in many tropical and sub-tropical cities. The critical factor to the reduction of turbidity was the circulation time variable, which is related to residence time of the medium in the lit section of the reactor. For further reduction of turbidity, the bioreactor should be operated with a longer light irradiation time, which is related to fact of cells are being able to metabolize or adsorb compounds present in the chromophoric nature of leachate [25].

3.4 COD and TOC Removal

The qualitative analysis for COD removal (Supplementary material, Fig. S2), indicates that the temperature and light cycle variables must be operated at their high levels (30 ° C and 24 h: 0 h, respectively), and the variable 'circulation time', at its low level (5 min). ANOVA of the results (Table 4) shows, from the quadratic mean of the factors that the temperature was the most significant factor, and the circulation time, the least.

The qualitative analysis for TOC removal (Supplementary material, Fig. S3), indicates that the temperature and light cycle variables must be operated at a high level (30°C and 24 h: 0 h, respectively) and the circulation time variable, at the low level (5 min). ANOVA (Table 4) shows that the temperature was the most significant factor and 'circulation time', the lowest. Analyzing the results obtained for the reduction of the organic load, measured both as COD and TOC, it is notable that both the results have a statistical correlation [26].

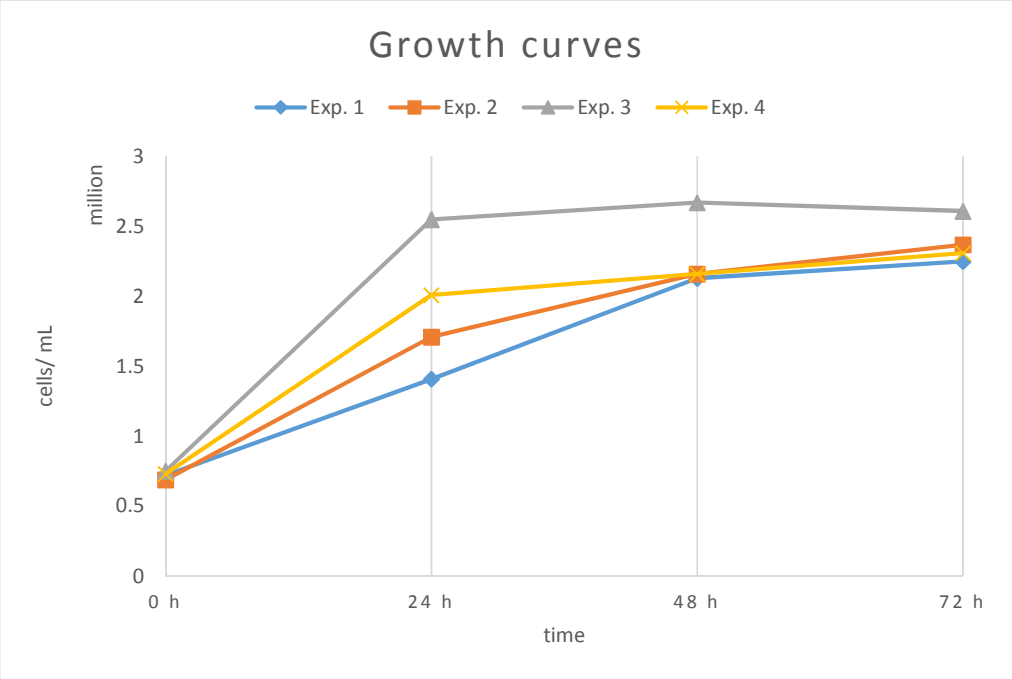

Fig. 2. Cell growth curves obtained in the culture of *Chlorella* sp. according to the conditions established in the L_4 experiments

Table 4. Analysis of variance of the results

Source of error - turbidity	**DF**	**MS**	**F**
Temperature	1	0.0121	2.39
Light cycle	1	0.0289	6.95
Circulation time	1	0.0841	*
Total	3		
Source of error - COD	**DF**	**MS**	**F**
Temperature	1	0.0511	29.61
Light cycle	1	0.0379	21.98
Circulation time	1	0.0017	*
Total	3		
Source of error - TOC	**DF**	**MS**	**F**
Temperature	1	0.0511	29.61
Light cycle	1	0.0379	21.98
Circulation time	1	0.0017	*
Total	3		
Source of error - Ag	**DF**	**MS**	**F**
Temperature	1	0.0100	25.00
Light cycle	1	0.0004	25.00
Circulation time	1	0.0004	*
Total	3		
Source of error - B	**DF**	**MS**	**F**
Temperature	1	0.0049	5.44
Light cycle	1	0.0009	1.78
Circulation time	1	0.0016	*
Total	3		

DF: Degree of freedom; MS: Mean of squares; F: F-statistics

Although the reduction of COD content was greater than the reduction in TOC content, it is seen that temperature and light cycle factors in both tests appear as first and second for statistical significance. Moreover, both factors have the best results, i.e., higher reduction rates when operated at similar level (higher in both cases).

A possible conclusion for this is that although the experiment 4 was not the one with the highest cell counting, temperature and the light cycle present therein were able to make cells assimilate the organic compounds present in the leachate more rapidly. Evidences from these exploratory studies show that although the consumption of the organic load was higher in this experiment; the reduction level of toxins was also higher; which may have led to a limitation on cell growth.

This can lead to indicate that the *Chlorella* sp. species metabolism is a combination of heterotrophic and photoautotrophic pathways, since the largest light cycle was able to induce a further reduction of the organic load, assuming that the reduction of the organic load is the directly related to microalgal metabolism.

The experiment 3 was performed on a light cycle of 12 h: 12 h. It is known that the phototrophic dark phase of growth is responsible for a slower cell growth rate, as compared with the light phase [27]. As the average residence time found to be statistically with lower significance than the other analyzed factors, experiments 3 and 4 (those with temperature at 30°C, high level), had a significant difference due to the light cycle factor. Although the experiment 3 showed a cell counting greater than experiment 4, the reduction of the organic load was well below that of the experiment 4. Therefore, this finding may be the basis for future tests as to the significance of the light cycle in the treatment of leachate in mixotrophic pathway, especially in dark effluents.

3.5 Removal of Silver and Boron

Metals that had measurable concentrations were: Ag, B, Cd, Cu, Fe, and Pb. The final concentrations of Cu and Pb were not significantly lower than their original values (0.870 mg L^{-1} to 0.860 mg L^{-1} of Cu, and 0.280 mg L^{-1} to 0.270 mg L^{-1} of Pb). Fe concentrations in all experiments reached unmeasurable values, thus it was not possible also to perform statistical analysis to its removal. Iron is one of the

constituents of the Guillard f/2 medium, used in the maintenance of the strain [28]; this may be the reason for its consumption in the experiments.

Statistical analysis for silver removal generates the results as displayed in Fig. S4 (Supplementary material). Results from ANOVA (Table 4), indicate that both light cycle and residence time factors have lower quadratic sums; leaving only the temperature factor with greater significance.

Fig. S5 presents the qualitative factor analysis for boron removal. These results, with their respective ANOVA (Table 4), indicate that the Light cycle variable has the lowest mean square.

Consequently, it is observed that the variable of greatest significance is temperature in means of removal of silver and boron. It can be concluded from the qualitative analysis, that operational levels to greater reductions in levels of both elements is high temperature (30°C) and circulation time in its low level (5 min). Statistical analysis allows the conclusion that light cycle had little influence on reducing the boron and silver contents (Supplementary Figs. S4, S5 and Table 4).

Interestingly, the boron reduction reached high values (greater than or equal to 87% in the four experiments). Due to the exploratory nature of the experiments, it was not possible to determine whether the metal was used as a nutrient or adsorbed onto the cell wall structure. The reduction of silver had lower levels, but nevertheless significant. If the silver is being adsorbed on the cell wall, probably a higher cell concentration, combined with a suitable temperature factor, would provide better results.

The capacity of *Chlorella* species in uptaking several metals by sorption on cell structure or metabolic assimilation has been widely reported in the literature, and well explained by Mehta et al. [29]. Usually the amount of metal accumulated by algae is related with the concentration of metal in water within a threshold capability.

4. CONCLUSION

Marine species have higher chances of development, as the high salinity of its habitats make a significant factor in cellular adaptation to unfavorable environments, such as leachate. The microalgae Chlorella sp. used in this work is of marine nature, and this, combined with other factors such as the reactor design and metabolic conditions, may have secured a significant cell growth and significant reduction of the organic load and turbidity of the effluent. Reactor design was critical to the success of this study, as probably most of leachate treatment, as well as cell growth was due to the high rate of light irradiation at the lit tube part of the reactor. Since leachate has a high turbidity and a naturally dark color, aerobic microbiological treatment in mixotrophic systems in environments that do not favor the light diffusion may become ineffective. Therefore, the proposal bioreactor configuration for a biological pre-treatment of the leachate, promoting cell growth, became efficient and opened possibilities for various research projects in the field, due to the removal rates of TOC in 60%, COD in 68%, turbidity in 98%, boron content in 98% and a complete removal of Fe. Furthermore, it is important to note that the total price of the reactor building was lower than USD 400.The low cost and easy operation are factors that may rise the interest of new developments in the area.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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APPENDIX

Table 3. Results obtained from Taguchi L_4 array in reduction of Turbidity, COD, TOC, Ag, and B

Run	Variables			Response variables									
				Turbidity		COD		TOC		Silver		Boron	
	A	B	C	Final (NTU)	Reduction	Final (mg O_2 L^{-1})	Reduction	Final TOC (mg L^{-1})	Reduction	Final (mg L^{-1})	Reduction	Final (mg L^{-1})	Reduction
1	1	1	1	1.85	0.8	197.75	0.21	75.8	0.18	0.022	0.12	0.4	0.88
2	1	2	2	0.71	0.92	179.85	0.29	61.6	0.34	0.023	0.08	0.45	0.87
3	2	1	2	0.16	0.98	170.71	0.32	58.7	0.37	0.02	0.2	0.31	0.91
4	2	2	1	4.41	0.52	79.63	0.68	36.8	0.6	0.02	0.2	0.06	0.98