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Comparative analysis of nickel removal by *Penicillium corylophilum* in sporulated and vegetative form

Análise comparativa da remoção de níquel por ***Penicillium corylophilum*** *em forma esporulada e vegetativa*

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ABSTRACT

The objective of this work was to evaluate the capacity of biosorption of heavy metals by the fungus *Penicillium corylophilum* (CCFIOC 4297). Additionally, this paper presents a comparative study of the removal of nickel ions, in synthetic medium, by the fungus *P. corylophilum* in vegetative and sporulated forms. For this purpose, the influence of seven parameters (sugar, time, nickel, pH, biomass, $(NH_4)_2HPO_4$ and $MgSO_4$) was evaluated using the Plackett & Burmann experiment design tool. Then, the fungus was tested for its ability to biosorption in two different ways: in vegetative and sporulated forms. The results showed great biosorption ability of this fungal species and little influence of the tested parameters. The tests for comparative purposes showed a very similar kinetic profile between sporulated and vegetative forms, in which, the fungus sporulated form showed a better performance.

Keywords: Biosorption, Heavy Metals, Nickel, *P. corylophilum*.

RESUMO

O objetivo deste trabalho foi avaliar a capacidade de biossorção de metais pesados por *Penicillium corylophilum* (CCFIOC 4297). Adicionalmente, foi reavaliado um estudo comparativo da remoção de íons de níquel, em meio sintético, por *P. corylophilum* nas formas vegetativa e esporulada. Para tanto, foi avaliada a influência de sete parâmetros (açúcar, tempo, níquel, pH, biomassa, $(NH_4)_2HPO_4$ e $MgSO_4$) utilizando o padrão para delineamento de experimentos Plackett & Burmann. Em seguida, o fungo foi testado quanto à sua capacidade de biossorção de níquel nas formas vegetativa e esporulada. Os resultados mostraram grande capacidade de biossorção dessa espécie fúngica e pouca influência dos parâmetros testados. Os testes para fins comparativos mostraram um perfil cinético muito semelhante entre as formas esporuladas e vegetativas, nas quais a forma esporulada do fungo apresentou melhor desempenho.

Palavras-chave: Biossorção, Metais Pesados, Níquel, *P. corylophilum*.

INTRODUCTION

There are several wastes generated by industries, whose disposal occurs, most often, inappropriately. This is a hazardous issue since many of these wastes contain alarming levels of recalcitrant organic substances and / or heavy metals ions that constitute a threat to the health of living beings by the high degree of toxicity, as well as its bioaccumulation in the food chain by contaminating aquatic and terrestrial environments.

The contamination of aquatic and terrestrial systems by toxic metals ions is a serious environmental problem nowadays (DAS, 2012). Certain metals can cause serious problems for their high biological half-life, which causes them to remain active in the environment for a long period, besides having high capacity to accumulate in the human body (JIMENEZ, 2004; KORF, 2008).

According to Volesky (VOLESKY, 2001), the metals ions that represent an environmental risk are, in descending order of toxicity: cadmium, lead, mercury, chromium, cobalt, copper, nickel, zinc and aluminum. The first three are the most harmful to both man and the environment; whereas copper, chromium, zinc and nickel affect humans by direct contact since they are used in manufacturing many accessories.

Among these metals, nickel, the target of this study, is a major cause of allergic processes, by dermatitis, which generates local lesions in contact with products containing it in its constitution. Therefore, there is often, for example, redness around the neck of those who wear accessories made of nickel or allergy in the earlobe caused by jewelry containing nickel in its composition (SANTOS, 2011).

Therefore, the global concern about the indiscriminate use of toxic chemicals and their improper disposal into the environment has been growing over time. These facts led to the creation of various standards established in Laws and Decrees, which aim to control the issue of waste in the environment. Specifically for heavy metals ions, the main emission sources are, among others, the metallurgical activities (CHAOUI, 1997), gases released by burning fossil fuels (GIMENO-GARCIA, 1996), manufacturing and disposal of batteries (SANTOS, 2011), application of fertilizers containing pollutants, and use of pesticides (GALLI, 1996).

There are numerous ways to eliminate or reduce contaminants in the environment, among them, the process of biosorption is one of the most interesting from an economic point of view when compared to other forms of treatment using physical-chemical processes such as flocculation and electrolysis (PINTO et al, 2003).

The biosorption is based on the ability of some biological materials, such as microorganisms (bacteria, fungi and microalgae), macroscopic plant (algae, grasses and aquatic plants) and specific parts or tissues of plants, agricultural or industrial waste (bark, mulch, seeds) have to accumulate heavy metals from water pollutants through physical and chemical processes of absorption / uptake (PINTO, 2003).

Filamentous fungi, such as *Penicillium corylophilum*, and yeasts have shown, over the years, excellent biosorption characteristics. These microorganisms have, among other advantages, high speed reproduction, and synthesis of high amount of cellular material (biomass), and can be manipulated genetically, what can intensify its biosorption power. Additionally, considering the intensive use of fungi in industrial bioprocesses, it's possible to use the fungal biomass as biosorbent agents, contributing to the sustainability of the planet (KAPOOR & VIRARAGHAVAN, 1995).

Thus, the aim of this study is to verify the biosorption ability of the filamentous fungus *P. corylophilum* regarding the removal of nickel, and whether the form in which it is used (sporulated or vegetative) has influenced this removal.

This paper is the first of two articles on the biosorption capacity of the fungal strain *Penicillium corylophillum* (CCFIOC 4297). In a future article, we will present the study regarding the removal of the metals nickel, copper, chromium, and zinc by the fungus in sporulated form in competitive and non-competitive systems.

MATERIALS AND METHODS

Microorganisms

The strain of *Penicillium corylophilum* CCFIOC 4297 was obtained from the Culture Collection of Filamentous Fungi (CCFF) of the Oswaldo Cruz Institute/Fiocruz, Rio de Janeiro, Brazil, where it was kept in lyophilized form. It is noteworthy that *P. corylophilum* (CCFIOC 4297) is derived from a screening of contaminated oil samples, conducted by Lemos (LEMOS, 2004) and deposited in the bank of strains of the Oswaldo Cruz Institute. Stock cultures of the fungal strain were maintained in the laboratory by monthly subcultures in tubes containing the medium Potato Dextrose Agar (PDA) inclined, as recommended by the Culture Collection of CCFF. After a seven-day incubation in an incubator at 30°C, the culture was preserved in a refrigerator at about 4°C.

Preparation of Inoculum

Roux bottles were used in order to increase the volume of conidia. After a seven-day incubation in an incubator at 30±1°C, the conidia of three bottles were transferred to Erlenmeyer flasks of 250 ml capacity containing 100 ml of sterile water. Then, it was-disposed in a rotary shaker (Controlled Environmental Incubator Shaker, New Brunswick Scientific Co, EUA), at150 rpm e 30±1°C for 10 min., in order to disperse the conidia.

Aliquots of this suspension were removed for counting of conidia in a Neubauer Chamber. The average of three separate counts was used to calculate the volume of suspension to be used in the assays biosorption. This procedure aimed to standardize the initial concentrations of conidia in each specific test, according to the experimental P&B planning (Table 1).

For obtaining the strain in vegetative form (pellets), conidia were inoculated in Sabouraud culture medium, and were kept in a shaker at 30°C and 150 rpm. The total contact time was three days.

Preparation of Nickel Solution

A concentrated solution of nickel chloride hexahydrate ($NiCl_2.6H_2O$) containing 160 g/L reagent was prepared to elaborate the solutions used in the experimental biosorption. The tests were carried out in Erlenmeyer flasks of 250 ml capacity containing 100 ml of the aqueous solution, consisting of several salt concentrations, according to the experimental P&B planning (Table 1).

Preparation of $(NH_4)_2HPO_4$ and $MgSO_4$ Solutions

For assessment tests on the effect of the sources of nitrogen, phosphorus and sulfur, individual concentrated solutions of $(NH_4)_2HPO_4$ (200 g/L) and $MgSO_4.7H_2O$ (200 g/L) were prepared. Then, they were sterilized separately by filtration on Millipore membrane with 0.22 mm of porosity, collected in sterile bottles and kept in a refrigerator at approximately 5°C. All tests were performed in Erlenmeyer flasks of 250 ml capacity containing 100 ml of the aqueous solution, consisting of several salt concentrations, according to the experimental P&B planning (Table 1) (FARIAS & LEMOS, 2008).

Sugar concentration and pH determination

In tests performed with sucrose, according to the experimental design, initial and final concentrations of the carbon source were determined by the method of Somogyi-Nelson (NELSON, 1944).

Measurements of pH were made immediately after the test. Analyses were performed with digital potentiometer (QUIMIS, model Q400AS).

Experimental stages

Tests conducted in the first stage of this work were based on experimental design P&B12 (Plackett & Burman 12) (RODRIGUES & LEMMA, 2009). Seven variables (Table 1) were evaluated in order to verify the influence - significant or not - of each one. This design consisted of 12 trials with three central points (Table 2). The tests were performed randomly. The response variables were the final concentrations of nickel, the pH and the concentration of sugar consumed.

Table 1. Levels of the tested variables in experimental design Plackett & Burman12

+1	*CP*	*-1*	*Parameter*
1,5	1	0,5	$(NH_4)_2HPO_4$ (g)
1,5	1	0,5	$MgSO_4$ (g)
10	5	0	Sucrose (g)
24	12	1	Time (h)
30	20	10	Nickel (mg)
6	4	2	pH
10^7	5 x 10^6	10^6	Biomass (conidia/mL)

All tests were performed in a bench scale in Erlenmeyer flasks of 250 ml capacity containing 100 ml of the aqueous solution consisting of different concentrations of nickel, $(NH_4)_2HPO_4$, $MgSO_4.7H_2O$, and sucrose. Once inoculated, the solutions were incubated in a rotary shaker at 150 rpm and 30°C for different time periods.

For comparative tests between vegetative and spore forms, from the results obtained in the previous steps, inoculum was standardized to an initial concentration of 10^6 conidia /ml or pellets produced from the previous cultivation of 10^6 conidia /ml in 100 mL of Sabouraud's broth. Before the use, the pellets were separated from the broth by centrifugation, guaranteed aseptic technique, and washed with sterile distilled water. The other conditions were: 30 mg / l of nickel; pH 6.0; and no addition of nutrients. The total contact time was 24h, with withdrawn at intervals of 15 min., 30 min., 1h, 6h, 12h and 24h.

After the time for each test condition, the whole volume contained in each flask was vacuum filtered through a Millipore membrane in order to separate the biomass. Aliquots of filtered solutions, free of biomass, were collected and sent for analysis of the final nickel concentration by the method of Atomic Absorption Spectrometry (AAS) in the Department of Mineral Analyses (COAM) located at the facilities of the Centre for Mineral Technology, CETEM / MCT.

The biomass retained on the membrane was measured by the technique of dry weight. With the data obtained, it was possible to calculate the value of "q", i.e., the amount of adsorbed metal per gram of biomass by Equation 1 (MOREIRA, 2007; CALFA, 2007). Thus, it was possible to verify the removal of the metal in mg/g.

$$q = V \, x \, Ci - Cf / M \quad \text{(Eq. 1)}$$

where:
q – Amount of adsorbed metal per gram of biomass (mg/g)
V – Volume of the solution containing the metal (L)
Ci – Initial concentration of metal (mg/l)
Cf – Final concentration of metal (mg/l)
M – Mass of biomass (g)

RESULTS

Analysis of the influence of the parameters

In general, the *P. corylophilum* has shown excellent potential for removal of nickel, as can be seen in Table 2. The analysis of the table permits to say that the *P. corylophilum* lineage CCFIOC 4297 can remove nickel, although the rate of biosorption has varied according to the test conditions. Except for test 11, wherein the removal of nickel was null, the metal removal varied significantly, achieving higher rates in the tests 9 and 10.

A fact that was corroborated by the analysis of Figure 1, where we see the value of "q" of each test. In the said figure, the test 9 showed the best results, demonstrating the removal of 3.60 mg/g, followed by test 10 with a value of 2.21 mg/g.

Table 2. Matrix of planning Plackett & Burman12 for evaluation of seven parameters in the removal of nickel by strain *P. corylophilum* CCFIOC 4297

Test	*Parameters*							*Responses*		
	$(NH_4)_2HPO_4$ (g/L)	**$MgSO_4$ (g/L)**	**Sucrose (g/L)**	**Time (h)**	**Nickel*¹ (mg/L)**	**Initial pH**	**Biomass (esp./mL)**	**Nickel*² (mg/L)**	**sucrose consu mption (g/L)**	**final pH**
1	1,5	0,5	0	24	10	2	10^7	5,5	-	2,5
2	1,5	1,5	10	1	10	2	10^6	7,9	0,7	2,2
3	0,5	1,5	0	24	30	2	10^6	24,1	-	2,1
4	1,5	0,5	10	24	10	6	10^6	9,5	0,8	5,5
5	1,5	1,5	10	1	30	2	10^7	24,4	1,7	1,9
6	1,5	1,5	0	24	30	6	10^6	25,5	-	6,0
7	0,5	1,5	10	24	10	6	10^7	8,8	3,4	5,5
8	0,5	0,5	10	24	30	2	10^7	22,1	0,1	1,9
9	0,5	0,5	10	1	30	6	10^6	12,6	1,1	5,9
10	1,5	0,5	0	1	30	6	10^7	13,2	-	6,0
11	0,5	1,5	0	1	10	6	10^7	*³	-	6,1
12	0,5	0,5	0	1	10	2	10^6	5,0	-	1,4
13	1	1	5	12	20	4	$5x10^6$	14,2	1,6	3,2
14	1	1	5	12	20	4	$5x10^6$	13,8	1,4	3,1
15	1	1	5	12	20	4	$5x10^6$	15,2	1,3	3,2

*¹initial concentration; *²Final concentration in solution; *³ no removal.

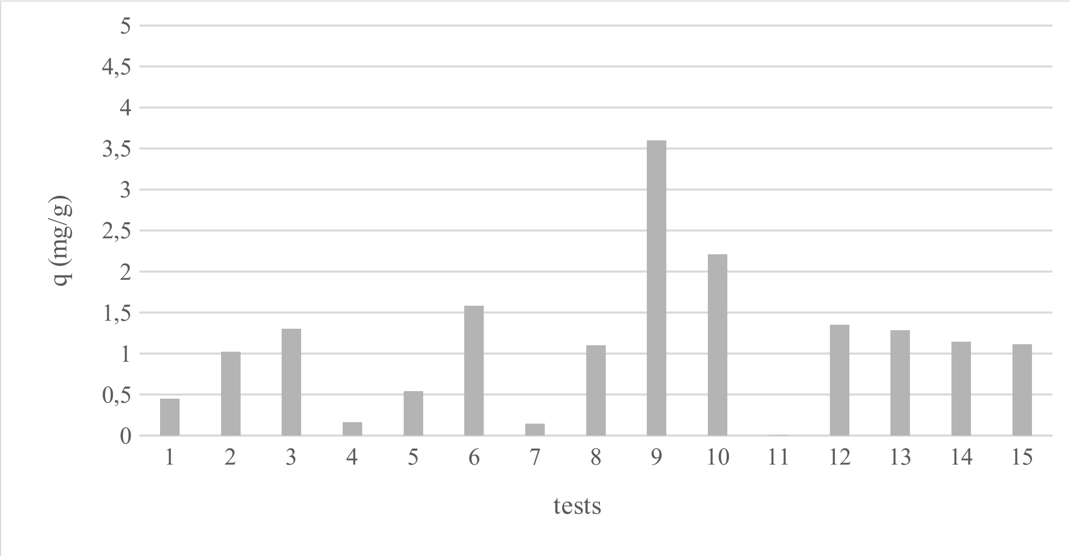

Figure 1. Removal of nickel according to the P&B design

In Figure 2, we note that the metal removal was independent of substrate consumption, indicating the metal uptake by the fungus was not conditioned on metabolic activity, may occur therefore, without sugar consumption. The consumption of this substrate ranged from 1% to 30.4% (Test 7).

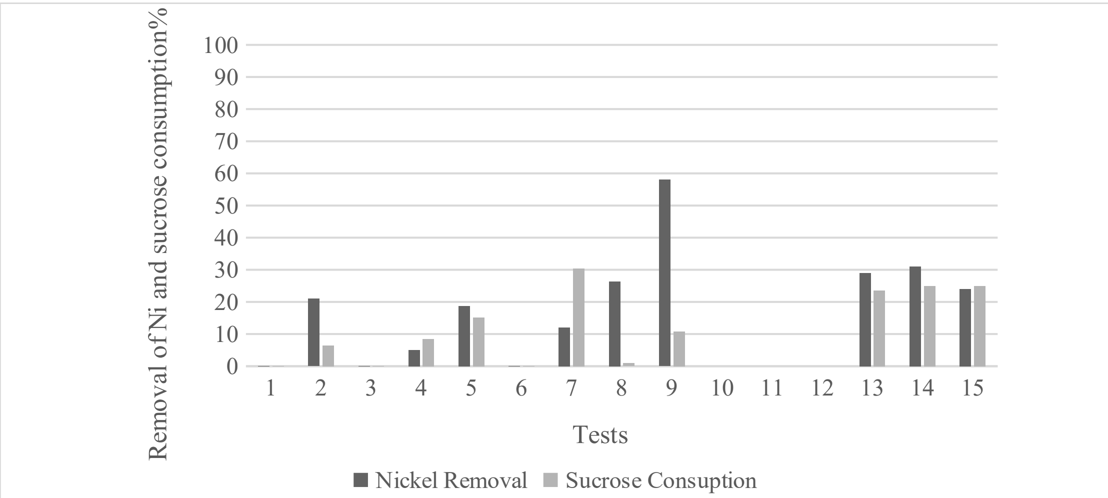

Figure 2. Percentage removal of nickel and consumption of sucrose in the tests as P&B design

In Figure 3 the values of the variation of the pH for each of the fifteen trials were plotted. Positive and negative variations ranging from 4.5% to 28.5% can be observed. The largest variations occurred when the initial pH was adjusted to 4, while for the pH 6 minor changes were observed. It is noteworthy that the best removals were achieved in tests 9 and 10, where the initial pH was 6.

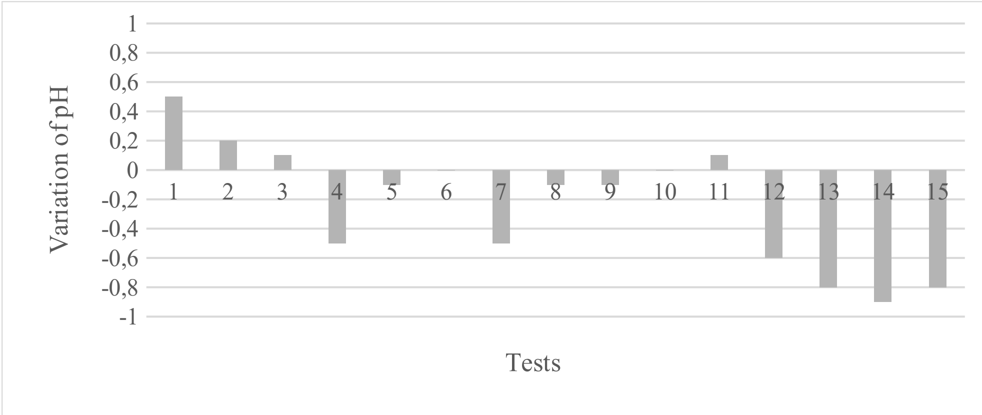

Figure 3. Variation of pH in tests carried out by planning P&B12

The evaluation of the influence of the studied parameters is supported by the visual analysis of the Pareto chart, which in turn should lead to select those relevant to the process improvement (Figure 4). The dotted cross line indicates the parameters with statistical significance at p = 0.09, i.e., for the 90% level of confidence.

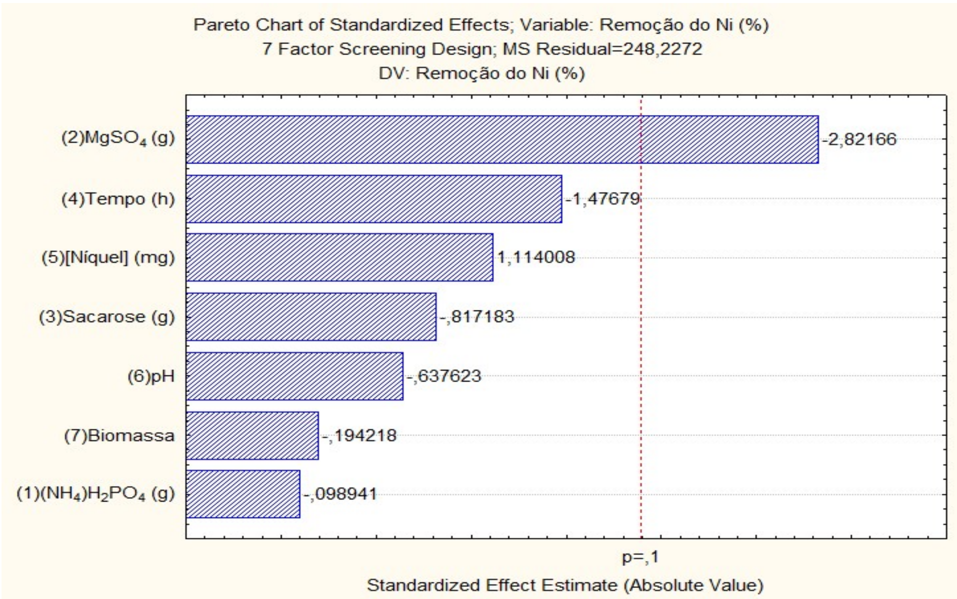

Figure 4. Pareto Chart showing the contribution of the variables studied for the removal of nickel by *P. corylophilum* CCFIOC 4297

Looking at Figure 4, we can see that the magnesium sulfate ($MgSO_4$) was the only one among the factors evaluated in the design of experiments P & B that significantly influenced the biosorption of nickel by *P. corylophilum* CCFIOC4297, with a negative effect. This indicates that biosorption could be increased if a lower concentration of magnesium sulphate was used, or , if the salt was eliminated completely from the assay.

Comparison of spore and vegetative forms

Figure 5 shows the percentage removal of nickel, in aqueous solution, by conidia and pellet during 24h of experiment. The kinetic profile was similar for both, although, comparatively, the conidia form has shown a better performance in metal removal in the monitored period, reaching 28% removal in just 15 minutes of contact, reaching a maximum removal percentage of about 57% in 12 h. Note that the contact time of only 1h the conidia were able to reduce by 54% the amount of nickel in solution, while the use of pellets reduction was 35%.

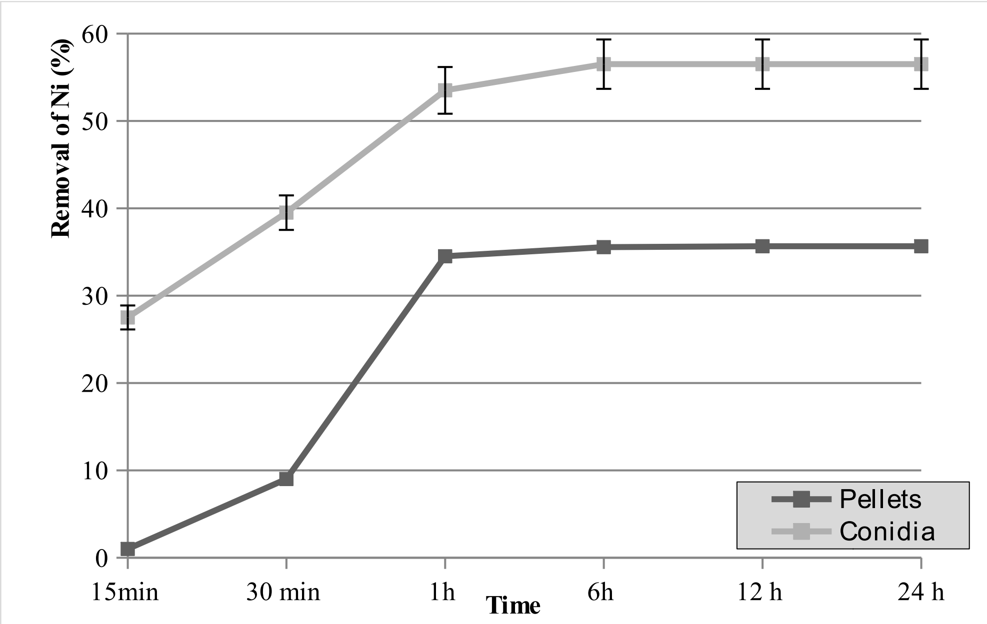

Figure 5. Removal of nickel in aqueous solution by *P. corylophilum* CCFIOC 4297, in the vegetative and sporulated forms

The results shown in Figure 5 ratified the ability of the fungal species *P. corylophilum* to uptake nickel in solution as both the sporulated and vegetative form. However, we can observe a better absorption capacity when the fungus was in the sporulated form.

DISCUSSION

On the results of table 2 and which were corroborated by the results found in figure 1, we see that some authors have also achieved similar results in the removal of heavy metals by some fungal strains, such as, for example, the work of Aksu & Donmez (2006). They found removal rates ranging from 2.0 to 3.0 mg/g in work with various metals biosorption microorganisms. Pallu (2006), working with strains of *Aspergillus sp*. found removal values up to 23.4 mg/g for the cadmium metal.

The results of figure 2, in which we evaluated the removal of nickel by the fungus and its relationship with the consumption of substrate, we see that some studies suggest that biosorption may occur without energy expenditure by the biomass and, therefore, there would be no need for the consumption of substrate for it to occur significantly (PALLU, 2006; FRANCO et al, 2004). These authors suggest that this phenomenon would be more associated with the mechanisms involving the cell wall (adsorption) than the active transport in the membrane or the metabolism of the cell.

In relation to the variation of pH some authors explain that this parameter is one of the most important parameters in the removal of heavy metals by microbial biomass process, since, besides influencing the solubility of the metals, it is also related to its speciation (PINO, 2005). Moreira (MOREIRA, 2007) observed that at low concentrations, adsorption of nickel may occur independent of pH value. And at high concentrations, the retention of the metal was highly related to the increase of pH.

Therefore, studies with filamentous fungi demonstrated the ability of conidia in the biosorption of some organic compounds. For example, Celestino *et al.* (2009), using conidia of *A. niger* for treating synthetic effluent containing BTX, obtained percentage removal of the contaminant by 83%. Pires *et al.* (2010), also using *A. niger* for the treatment of indigo dye removal reached 94% within seven days.

CONCLUSIONS

From the development of this study it can be concluded that the fungus *Penicillium corylophillum* shows excellent potential for biosorption of nickel in aqueous solution, with removal of 3,60 mg/g in test 9. Among the parameters studied, only $MgSO_4$ showed a significant effect, however negative, for a significance level of $p < 0.1$. Both conidia and pellets of the fungus can be satisfactorily employed as biosorbents, reaching the maximum removal (57%) with conidia 12h contact. Therefore, it is clear that the fungus *Penicillium corylophilum* shows potential for the remediation of areas contaminated by nickel.

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