Research Article

Novel Technique for Degradation of Silver Thiosulfate Present in Wastewater of the Post- Harvest Treatment of Ethylene-Sensitive Flowers

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Abstract

Silver thiosulfate (STS) used in the post-harvest treatment of ethylene sensitive flowers is a potent producer of environmental damage because the silver cation (Ag^+) remains in the soil and groundwater for long periods and can migrate to drinking water systems and cause health problems in humans. This study presents a novel treatment of wastewater contaminated with STS that includes three steps: i) oxidation of STS by bacteria isolated from sulfur springs of the Ecuadorian highlands which were added to artificial wastewater or wastewater containing STS from floriculture. The results indicate that approximately 60% of thiosulfate is oxidized to sulfate and 10% is absorbed by the bacteria at acidic pH, ii) biosorption of silver cation with fungi pellets of *Cladosporium cladosporioides* fungi. The adsorption tests show that the maximum adsorption capacity was 16 mg/g pellet using pellets prepared with fungi of 11 days of growth at pH 6, iii) regeneration of fungi pellets using 4N nitric acid solutions. With this acidic regenerant, it was recovered 69% of silver accumulated within the fungi pellets and the adsorption capacity of the regenerated fungi decreased to 15.4 mg Ag^+/g in the second adsorption cycle.

Keywords: Silver thiosulfate; Ethylene-sensitive flowers; *Cladosporium cladosporioides*

Introduction

Flowers in general are highly perishable although they maintain their physiological functions even after harvest. The onset and progression of wilting is related in many cases with the production of ethylene. Treatments for controlling the ethylene production in the flower, spread of pathogens, water and breathing balance, color retention, and the opening of flower buds and its further development are vital for preserving flowers [1]. In Ecuador, for extending the flower life, it is used silver thiosulfate (STS), a chemical compound that slows down aging of flower by inhibiting the production of ethylene. Unfortunately, the STS is a potent producer of environmental damage as a result many countries have banned its use. The effect of silver thiosulfate in the environment is mainly due to the permanence of the metal in soil and groundwater for long periods and the potential migration to drinking water systems and eventually being ingested by the population [2].

There exist a number of conventional technologies to remove dissolved silver in industrial wastewater including ion exchange, electrolysis or chemical recovery. These technologies can be cost-effective for large scale applications and for high silver concentrations (>100 mg/L) [3]. Conversely, bioremoval of metals from aqueous solutions, including silver, is a highly efficient technique for capturing dissolved metals from water at low concentrations. Furthermore, this technique is environmentally friendly and economic [4-7]. Different types of microorganisms have been used for the removal of heavy metals from aqueous solutions including bacteria [8], fungi [9] and algae [10]. One of the bacterial species that obtains its energy from inorganic sulfur compounds is the *Thiobacillus thioparus*. This microorganism can aerobically oxidize thiosulfates [10]. On the other hand, granules of *Cladosporioides Cladosporium* fungi adsorb lead and cadmium from aqueous extracts with high efficiency [11].

In the flower-producing countries there are few alternatives to remove the STS from wastewater. Companies that market the chemical compound have tried chemical precipitation of the metal as an option to remove the silver from wastewater of floriculture and complete the silver separation passing the supernatant of the precipitation process through filters of diesel vehicles until they get saturated. This technique does not solve the environmental problem because silver is transferred only from the liquid phase to the solid phase. As being inevitable the use of STS in the flower industry, this study proposes a process for treating floriculture wastewater contaminated with silver thiosulfate. The process includes: i) oxidation of STS using bacteria for releasing the silver cation, ii) biosorption of the silver cation utilizing *Cladosporium cladosporioides* pelletized fungi, and iii) regeneration of fungi pellets with solution of nitric acid to reuse them in another adsorption cycle.

Materials and Methods

Wastewater from post- harvest treatment of ethylene-sensitive flowers

Samples collection of industrial wastewater containing STS was carried out in plastic bottles of 4L on farms of Esmeralda Group - Hilsea Investments - Quinche - Pichincha Province, Ecuador. Bottles were labeled and transported to the laboratory for further analysis. To avoid precipitation of silver, the samples were stored in a dark area at room temperature. In Table 1, it is summarized composition of post-harvest wastewater.

Samples	pH	Temperature, $^\circ$C	SO_4^{2-}, mg/L	Ag^+, mg/L
Lillium	7.28	21.9	65	0.04
Summer flowers	3.33	22.4	53.64	93.04
Composed waters	6.31	20.2	324.56	0.34

Table 1: Composition of post-harvest wastewater.

Collection, isolation and incubation of microorganisms

Native bacteria were isolated from different parts of the Ecuadorian highlands; two strains were recovered from sulfurus waters of the Tungurahua volcano (Tungurahua 1, Tungurahua 2), another from the Sillunchi sector and the fourth one from the area called La Calera in Machachi (Calera). Aliquots were taken from each collected sample and from the original pure-culture strain of autotrophic *Thiobacillus thioparus* (American Type Culture Collection: ATCC 23645) and seeded in solid ATCC® Medium 290: S6 medium for *Thiobacilli* [12]. Water samples were directly spread in the sterilized petri dishes while the sediment and soil samples were agitated in a vortex with sterile water before adding to the sterilized petri dishes. Inoculated boxes were maintained at 30°C and pH 7 for five days [13]. Each formed colony was inoculated into tubes with ATCC® 290S6 solid medium and incubated under agitation at 150 rpm in an orbital shaker Heidolph®, Model 1010 at room temperature for six days. Then, cultures were tested for Gram stain, motility, nitrate reduction and oxygen requirements [14]. On the other hand, fungi were collected in boxes containing PDA medium + chloramphenicol [15]. The boxes were kept for five days in a dark area at room temperature. The morphological identification of the strain (*Cladosporium cladosporioides*) was performed microscopically and by direct observation [16,17]. Colonies showing round shape, olive green on the front and black on back were taken. Next, samples were spread using a bacteriological loop in tubes with PDA medium and incubated at room temperature for eight days. The strain identified as *C. cladosporioides* was maintained in tubes with PDA medium + chloramphenicol [15].

Formation of pelletized fungi

For the formation of fungi pellets, it was used two culture media i) Czapeck [18] and ii) medium for *C. cladosporioides* [19]. The isolated strain was inoculated into each culture medium and incubated at 28 ± 2°C with stirring at 150 rpm to achieve the formation of spherical structures of approximately 3 mm in diameter after 11 to 17 d of incubation. Subsequently, the media containing the spherical structures were filtered and weighed.

Silver thiosulfate oxidation using synthetic water and industrial wastewater

Laboratory tests were conducted in batch type mini reactors using several volumenes of artificial wastewater (with STS + 10 mg/L of citric acid) and wastewater from post-harvest treatment of flowers (with STS + biocide + citric acid). Samples were inoculated with different volumes of the strains isolated from Sillunchi, Calera, Tungurahua1 and *T. thioparus* ATCC 23645. Tests of thiosulfate oxidation were conducted at room temperature, pH 6.5 for the artificial wastewater and lower than 5.0 for the floriculture wastewater, agitation of 40 rpm during four, five, six and seven days. In all tests a blank sample was used [20]. Samples from the reactors were then centrifuged at 3000 rpm for 30 min to remove insoluble particles and bacteria. In the supernatant the content of thiosulfate, silver and sulfate were analyzed.

Adsorption of silver with pelletized fungi

Volumes of 100 and 200 mL of aqueous solutions containing from 5 to 500 mg/L of silver at pH of 6 were placed in contact with 0.25 and 0.5 g of pelletized fungi for three days with an agitation of 40 rpm. The metal equilibrium concentration within the fungi was calculated with Equation 1:

$$q_e = \frac{V(C_i - C_e)}{m} \qquad (1)$$

where q_{eq} is the equilibrium concentration of silver in the pelletized fungi (mg/g), V volume of solution (mL), C_i the initial concentration of silver in solution (mg/L), C_e liquid phase concentration at equilibrium (mg/L), m biosorbent mass (g). With the calculated data of q_e and the measured values of C_e, adsorption isotherms were drawn using linearized models of Langmuir and Freundlich, Equations 2 and 3, respectively.

$$\frac{C_e}{q_e} = \frac{1}{bQ_{max}} + \frac{1}{Q_{max}}C_e \qquad (2)$$

$$\ln q_e = \ln K + \frac{1}{n}\ln C_e \qquad (3)$$

In Equation 2, b is the Langmuir constant, Q_{max} maximum sorption capacity (mg/g) and in Equation 3, ln K, is the interception with the y-axis that provides the capacity of adsorbent and 1/n the slope that gives the sorption intensity.

Regeneration of pelletized fungi

The regeneration of fungi was conducted using a regenerating solution of 4N HNO_3. The process was performed using 100 mL of the regenerant solution and 0.25 g of silver concentrated fungi in plastic mini-reactors. These reactors were placed on a rotary shaker at 40 rpm of agitation for 24 h. To quantify the amount of silver recovered during regeneration, the supernant was filtered and analyzed for Ag^+ using atomic absorption spectrometry.
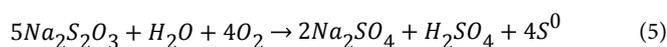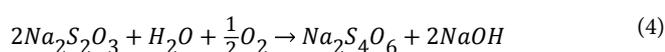
Chemical analyses

Thiosulfate concentration was determined by iodometric method. For this test, water samples with silver thiosulfate were titrated with 10 mL of potassium iodide (10% v/v), 15 mL of sulfuric acid (2N), 25 mL of potassium dichromate (0.1N) and 1% of starch indicator. 0.1N of sodium thiosulfate was used as the standard. The indicator was added when the solution change from bricked-red to strawed-yellow. After adding the indicator the solution turned blue and the further change into transparent was interpreted as the end of the titration [20,21]. The sulfate concentration was measured using the method of 4500-SO_4^{2-} of the Standard Methods [22]. Silver cation concentration was analyzed using an atomic absorption spectrometer (Perkin Elmer AA800 at λ = 338.3 nm) [22]. Before measuring the silver concentration, the equipment was calibrated using four standards: 2.5, 5.0, 8.0 and 10.0 mg Ag^+/L and it was needed to pretreat samples and standards with nitric acid at 5%.

Results and Discussion

Bacterial growth

During the growth in the liquid selective medium, bacterial strains isolated from sulphurous waters produced different compounds compared with those generated by the *Bacillus Thiobaci-thioparus* ATCC 23645 strain. The degradation of thiosulfate with native bacterial strains produced sodium dithionate and sodium hydroxide. These compounds produce an increase in pH (Equation 4) and turn blue the color of the culture medium. While products of thiosulfate degradation using *T. thioparus* ATCC 23645 strain produce acidification of the medium due to the formation of sulfuric acid and the color of the medium turns yellow (Equation 5).

$$2Na_2S_2O_3 + H_2O + \frac{1}{2}O_2 \rightarrow Na_2S_4O_6 + 2NaOH \tag{4}$$

$$5Na_2S_2O_3 + H_2O + 4O_2 \rightarrow 2Na_2SO_4 + H_2SO_4 + 4S^0 \tag{5}$$

Furthermore, liquid media became cloudy during bacterial growth. The medium containing strain ATCC 23645 became more turbid so these bacteria grew in greater amounts compared to the native bacterial strains. This particularity can be attributed to the energy source used for metabolism of the microorganisms. In the case of the *T. thioparus* strain, thiosulfate is the only source of energy while in the native bacterial strains, the transformation of thiosulphate is complementary, it is rather an alternative mechanism used in the nutrition of bacteria and is not essential for their metabolism [23].

Oxidation of silver thiosulfate

Figures 1 and 2 show that in tests with artificial wastewater, *T. thioparus* ATCC 23645 bacteria exhibit better efficiency in the oxidation of thiosulfate to sulfate compared to native strains (Tungurahua 1 and Calera). Sulfates produced in the oxidation of thiosulfate by *T. thioparus* strain have a ratio of 6 to 1 and 8 to 1 in comparison with the native strains with and without pH adjustment, respectively. Note however in studies with wastewater from the post-harvest process, oxidation of thiosulfate was 63% with Tungurahua 1 strain and 67% with *T. thioparus*. In previous research has been reported that oxidation of thiosulfate with *T. thioparus* MCM B-39, reaches an efficiency over 99% and no thiosulfate were detected in samples of photofilm processing wastewater after completion of the treatment [20,24]. The high efficiency achieved in the oxidation may be the result of the absence of biocide, since the residual water of photodevelopment contains ammonium thiosulfate, sodium acetate, sodium sulfite, glacial acetic acid and boric acid and no biocides [25]. Biocides are known to kill bacteria or inhibit their growth. Donegan et al. reported that two biocides (copper hydroxide and streptomycin sulfate) caused significant reductions in bacterial populations after 14 d of application [26]. Thus populations of *Thiobacillus thioparus* ATCC 23645, Tungurahua 1 and Calera may have been affected by the action of the biocide dissolved in wastewater of the floriculture and resulting in a reduction of silver thiosulfate oxidation. In this research no active bacteria count was performed during treatment to quantify whether they increase or decrease.
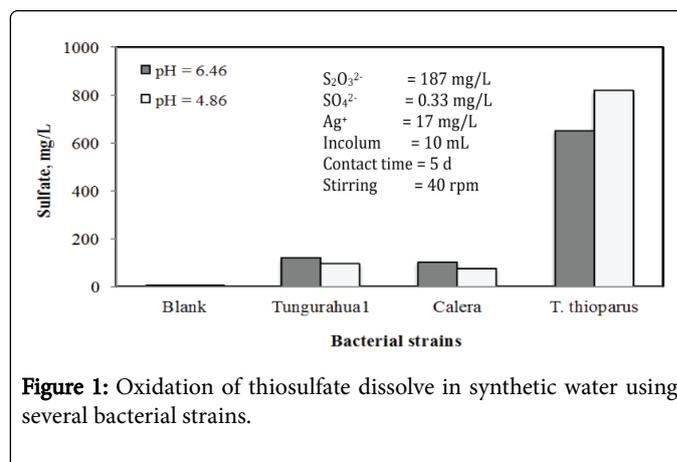

Figure 1: Oxidation of thiosulfate dissolve in synthetic water using several bacterial strains.
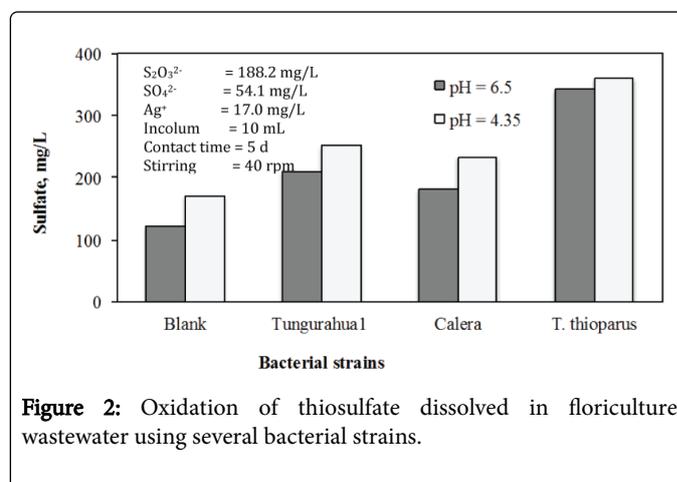

Figure 2: Oxidation of thiosulfate dissolved in floriculture wastewater using several bacterial strains.

Influence of silver concentration in the oxidation of thiosulfate and bioaccumulation in bacteria

In Figure 3 it is observed that up to concentrations of 72 mg/L of silver, the production of sulfates increases but for higher concentrations, sulphates formation decreases. Around 50% reduction is seen at 120 mg/L of silver. Earlier studies have reported the toxic action of some heavy metals. Poirier et al. (2008) in a physiological and biochemical study of *Pseudomonas fluorecens*, reported a significant disturbance in the bacterial growth when treating a sample contaminated with 25 mg/L Zn, 20 mg/L Cu, and 10 mg/L Cd. Metals appear to have a toxic effect on the bacterial strains as their concentration increases [27]. Another researcher reports that when living cells are used in a metal removal system, toxicity can lead to the poisoning or inactivation [28]. It is suggested that silver can be transported through the cell wall as silver thiosulfate by the mechanism of sulphate/thiosulphate transport [29], as the metal forms a series of very stable hydrophilic complexes with thiosulfate [AgS_2O^{3-}, $Ag(S_2O_3)_2^{3-}$: $logK_1 = 8.82$, $log\beta_2 = 13.50$] [30]. Earlier investigations show that the sulfate and thiosulfate share a common transport system in bacteria [31,32] and algae [33,34]. Once silver thiosulfate complexes are fragmented by bacteria, silver can also be accumulated on the microorganisms. Figure 4 shows a diagram displaying the mechanisms that facilitate the transport of silver into the cell. Past research reveals that in the absence of thiosulphate and with different concentrations of sulfate, algae accumulate the cation, probably via the transport system

of Cu^+ [35-37], as this transporter is not affected by changes in the concentrations of thiosulfate or sulfate. In Figure 5, it is seen a decrease of silver in the liquid phase (78%) when the aqueous solution is contacted with Tungurahua 1 and Calera strains. These data confirm the fact that microorganisms and their products can be efficient bioaccumulators of soluble metals and particulates, especially when they are diluted [28]. Also, it was notorious the formation of silver precipitates by action of light, presence of sulfides and the increase of pH. The precipitation of silver sulfide (Ag_2S) was evident as observed the formation of black films on the reactor walls. Although concentrations of reactants were low, precipitation of silver sulfide was effective because their solubility in water is very low ($K_{sp}=1.6\times10^{-49}$ at $20^{\circ}C$). However, it should be noted that the protonation of sulfur can cause an increase in solubility, process that is sensitive to basic pH because H2S is a weak acid ($Ka_1=9.1\times10^{-8}$ and $Ka_2=1.2\times10^{-15}$). Besides increasing the pH above 6, silver is precipitated as silver hydroxide ($K_{sp}=1.52\times10^{-8}$ at $20^{\circ}C$) [38].
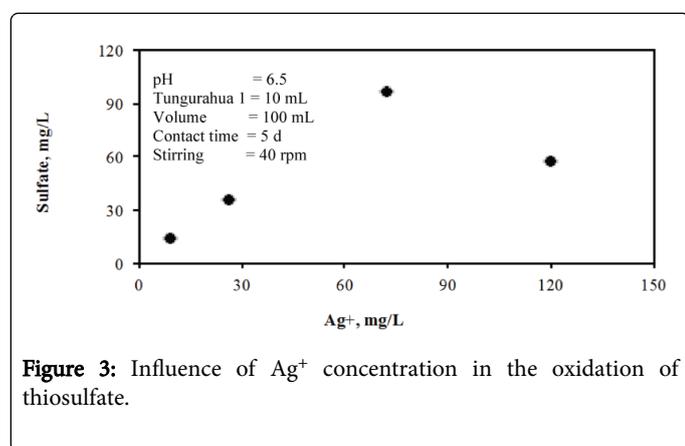

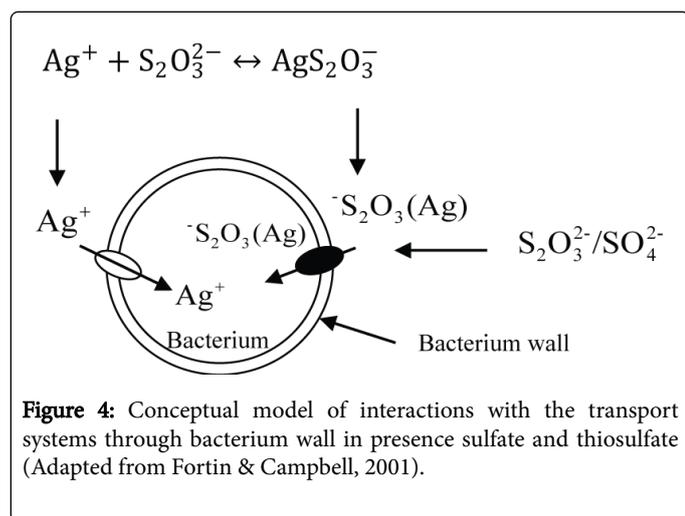**Figure 3:** Influence of Ag^+ concentration in the oxidation of thiosulfate.

Figure 4: Conceptual model of interactions with the transport systems through bacterium wall in presence sulfate and thiosulfate (Adapted from Fortin & Campbell, 2001).
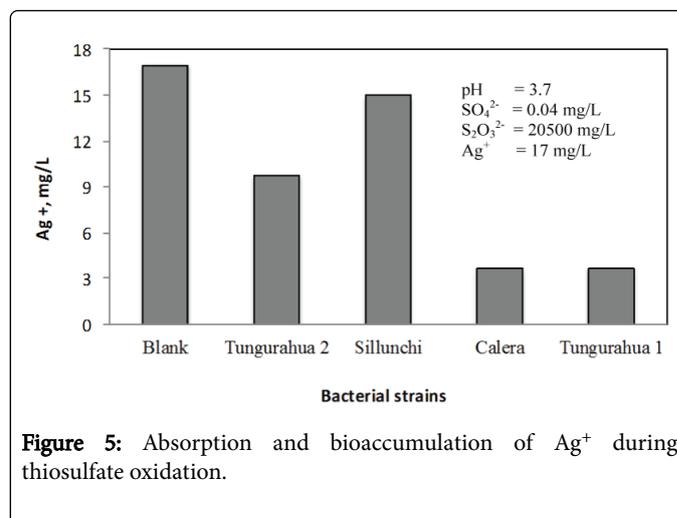

Figure 5: Absorption and bioaccumulation of Ag^+ during thiosulfate oxidation.

Variation of pH during the biological oxidation

Studies with artificial wastewater (STS + 10 mg L^{-1} citric acid) without pH adjustment (pH = 4.86), show that the strains balance pH of the aqueous solutions to an ideal value for their growth (Figure 6). It should be noted that native strains increase the pH to 7.6 and *T. thioparus* ATCC strain lowers it to 4.2. Though native strains grow in a pH range between 3 and 7, in this study, it appears that during their metabolic activity the pH increases. Raising the pH can be attributed to protonation of the amino groups present in bacteria [5,39] and also due to the decomposition of thiosulfate [40]. However, this deprotonation mechanism is contrary to chemolithotroph metabolism, because strains that oxidize sulfur reduced compounds commonly generate protons and consequently pH decreases. Hence the oxidation of reduced sulfur compounds generally produces acidification of the liquid medium. For this unusual behavior, the native strains may be new species, which have been adapted to the medium and have a chemolithotrophic metabolism of different group of sulfur bacteria. The taxonomic characterization of native bacteria by the 16S rDNA sequences (data no shown) has suggested that they belong to the genus *Pseudomonas* which degrade thiosulfate [41-44].
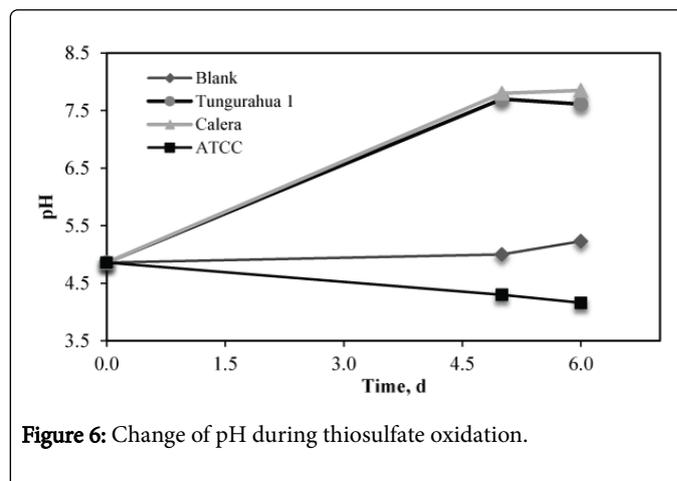

Figure 6: Change of pH during thiosulfate oxidation.

Influence of pH on silver adsorption

Previous studies with bioadsorbentes report that pH plays a very important role on the adsorption capacity of silver [45]. Figure 7 shows that the adsorption of the metal is affected by the pH of the wastewater. The pK of chitin, the main component of the *C. cladosporioides* fungus, is 6.0 [46] thus in the pH range from 1 to 5, chitin is predominantly neutral and there is no electrostatic interaction with the Ag$^+$ cation. As a result adsorption is limited. In contrast, at alkaline pH, silver is precipitated as metal sulfide thereby reducing the availability of the metal in the aqueous phase. Lin et al. 2005 reported the effect of pH on the adsorption capacity of Ag$^+$ for the *Lactobacillus spp.* A09 strain when this microorganism was contacted with silver nitrate for 24 h [45]. These authors point out that at pH>5, the adsorption is increased because the negatively charged carboxyl groups of the cell walls interact with Ag$^+$. Other researchers report that *C. cladosporioides* fungal biomass mixed with keratinous material of natural origin remove 100 mg gold/g of biomass and the maximal biosorption of the precious metal (80%) occurs under acidic conditions (pH 1-5) [47]. Additionally, Lasko and Hurst (1999) reported that the capture the silver cations from aqueous solutions by chitosan occurs in the pH range of 4 to 8 [5]. In the present study, it is verified that the increase on the adsorption of silver by *C. cladosporioides* fungi takes place at pH = 6 (7.5 mg Ag$^+$/g) (Figure 8).
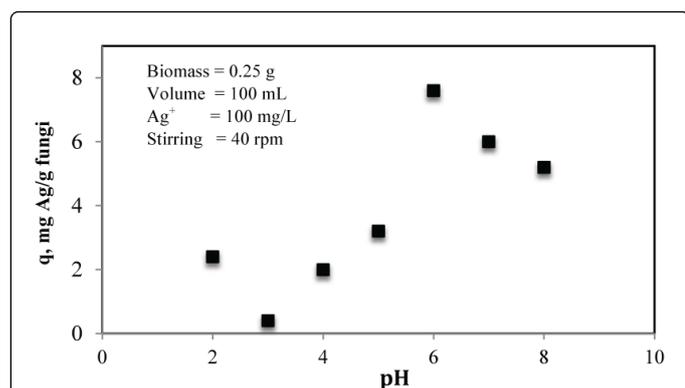

Figure 7: Influence of solution pH on the silver adsorption by fungi.
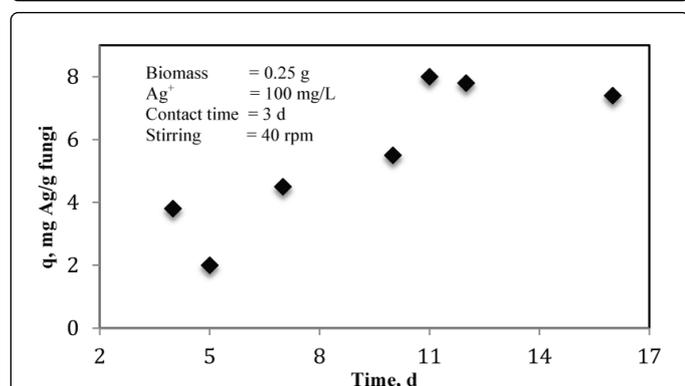

Figure 8: Influence of *C. cladosporioides* fungi harvesting time on silver adsorption by fungi.

Adsorption isotherms

Once the optimal time for the formation of the pelletized fungi was estimated (11 days) and the pH at which the adsorption of Ag$^+$ is higher (pH=6), isothermal tests were conducted to estimate the adsorption capacity of the pelletized fungi.

Figure 9 displays the increase of adsorption capacity of fungi up to 100 mg/L with the increase of silver concentration in the liquid phase. At concentration higher than the former, metal uptake remains constant, indicating saturation of the reactive sites on the fungi pellets. Values of q_e (calculated with Equation 1) and C_e (measured) are used to represent the adsorption isotherms according to Langmuir and Freundlich models (Equations 2 and 3). The best fit for silver adsorption is Langmuir model (Figure 10). Values of constants estimated by curve fitting are: Q_{max}=25.5 mg/g fungi, b=0.058. Previous studies on adsorption using *C. cladosporioides* fungi reported constant values for Langmuir isotherms, b=0.91 and Q_{max}=3.08 mg/g [20]. It is clearly observed that pelletized fungi used in this investigation adsorb more metal. This difference can be attributed to the contact time used in our tests. Authors of the past study used only 30 minutes while in our investigation the contact time to reach equilibrium was three days.
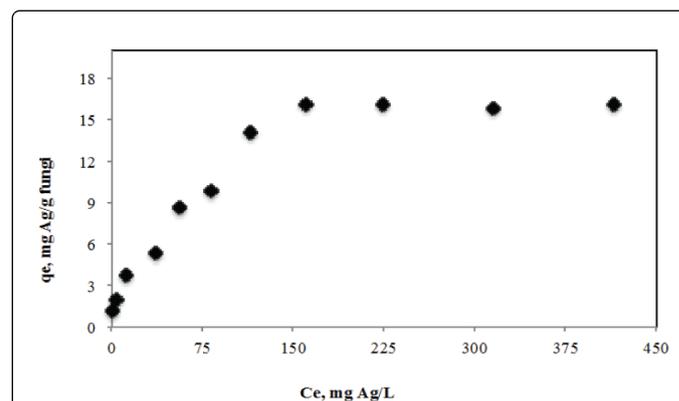

Figure 9: Isotherm for adsorption of silver using *C. cladosporioides* fungi.
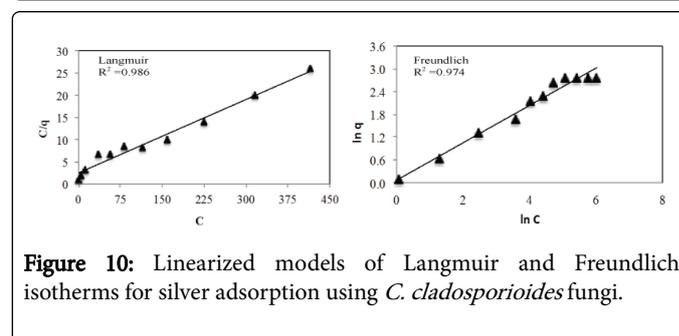

Figure 10: Linearized models of Langmuir and Freundlich isotherms for silver adsorption using *C. cladosporioides* fungi.

Regeneration of pelletized fungi

Environmental remediation process is economically viable when adsorbent materials are reusable. To find out if pelletized fungi can be regenerated without losing their capacity for removing silver in continuous cycles adsorption-desorption, regeneration experiments were conducted using 4N HNO$_3$. Results show that silver was

recovered in about 70% of the metal on adsorbed on the fungi (Table 2). The moderate silver recovery is due to the high content of hydrogen ions (H^+) in the regenerant. It is known that the H^+ does not totally displace homovalent cations from the reactive sites [48]. Moreover, it was found that the regenerant does not significantly alter the ability of fungi to adsorb silver during the second adsorption cycle performed after the first regeneration, as the adsorption capacity reaches a concentration about 7 mg/g (Table 3). The spent regenerant was treated with a photocatalytic process to reduce silver ions to metallic silver and preventing further contamination (data not shown in this paper).

C_i mg Ag+/L	C_{eq} mg Ag^+/L	q_{AD} mg Ag^+/g	C_{reg} mg Ag^+/L	q_{DE} mg Ag^+/g	% Recovery
19.9	10.7	4.05	6.65	1.2	69.4
49.6	34.9	6.03	9.04	1.74	70.9
104.3	74.5	12.11	20.64	4.21	66.6

Table 2: Results of adsorption and desorption of cation silver.

C_i mg Ag^+/L	q_{AD} 1st mg Ag^+/g	q_{AD} 2nd mg Ag^+/g
19.49	4.05	3.76
50.92	7.03	6.95
100.56	12.11	10.91

Table 3: Silver accumulated on fungi during two cycles of adsorption.

Conclusions

The native strains effectively oxidize the silver thiosulfate to sulfate; however, they are less efficient compared with the *T. thioparus* ATCC 23645 strain. Research results show that the *T. thioparus* strain oxidizes eight times more than the native strains using artificial wastewater. In contrast, when using the wastewater of the post-harvest process, thiosulfate oxidation by *T. thioparus* strain is two or three times better compared to Tungurahua 1. The minor difference in the oxidation of thiosulfate reached in the latter test can be attributed to the presence biocide in the wastewater. Also, the concentration of dissolved silver in the water affects the production of sulfates. It is shown that sulfate concentration increases during the thiosulfate oxidation process up to 70 mg Ag^+/L. At higher concentrations there is a rather significant decrease of sulphates. Silver appears to have a toxic effect on the bacterial strains as its concentration increases. Furthermore, strains used in the oxidation of silver thiosulfate absorb and bioaccumulate some amount of the metal. With strains of Calera and Tungurahua 1 there is absorption of 78% of silver. This absorption percentage of silver suggests that native bacteria can be efficient bioaccumulators of soluble metal, especially when they are diluted. The mechanism that carries silver through the cell wall as silver thiosulfate is the transport-system sulfate/thiosulfate transport. Also, it was verified that pH of the liquid medium varies during the oxidation of silver thiosulfate. Native bacterial strains increase the pH while *T. thioparus* bacteria decrease it. The increase of pH can be associated to the adsorption of protons from water by the amino groups of chitin. Whereas *T. thioparus* strain throughout the oxidation of the reduced sulfur compounds produces protons as a result pH decreases following the given mechanism: $S_2O_3 + 2Ag^+ + H_2O \rightarrow 2SO_4^{2-} + 2Ag^+ + 2H^+$. This unusual behavior suggests that the native strains may be new species with a different group of chemolithotrophic metabolism.

The adsorption of silver on *C. cladosporioides* fungi depends on time of incubation. Fungi harvested at 11th day showed higher metal adsorption efficiency while maintaining a pH equal to 6. On the contrary, fungi harvested before 11 days exhibited efficiency less than 14% and fungi harvested after 11 days showed an adsorption of 19.6% and decreases with increasing the harvest time. The variation in the adsorption capacity can be attributed to the amount of chitin formed in the wall of fungi which varies with the incubation time. Adsorption of silver is also affected by the pH of the floriculture wastewater or artificial wastewater. In the pH range of 1 to 5, chitin is predominantly neutral and does not interact with the Ag^+ and adsorption is limited accordingly. In contrast, at alkaline pH although chitin is negatively charged, the electrostatic interaction is less because silver is precipitated as metal sulfide. In this research is observed that the higher adsorption efficiency of silver on *C. cladosporioides* fungi is achieved at pH of 6. The moderate silver recovery is due to the high concentration of hydrogen ions (H^+), cation that competes with Ag^+ for reactive sites. Also, *C. cladosporioides* fungi can be regenerated using 4N HNO_3 and silver be recovered in approximately 70% without a significant change in the capacity of the regenerated biomass.

Acknowledgements

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