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Biofilm Formation by the Hexavalent Chromium Removing Strain *Streptococcus salivarius*: *in Vitro* Approach on Abiotic Surfaces

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ABSTRACT: In this study, a strain of lactic acid bacteria *Streptococcus salivarius* was studied for its capacity to remove hexavalent chromium (Cr (VI)) from a liquid medium and to form biofilm. Both properties are useful for using the strain in bioremediation of metal-contaminated effluents. For biofilm formation capacity, three methods were used: the tube method (TM), the Congo red agar method (CRA) and adherence to polystyrene tissue culture plate method (TCP). *S. salivarius*, showed a positive-biofilm and a correlation between the three methods was noted. The bacterial surface hydrophobicity was studied using the microbial adhesion to solvents method (MATS). On AISI-316 L stainless steel, the strain with a hydrophobic surface showed a good adhesion on this support after 18 h incubation. The colonization of the supports and the biofilms formation by the bacterial cell was observed using scanning electron microscopy (SEM). The minimum inhibitory concentration (MIC) of Cr(VI) on *S. salivarius* was determined on MRS broth, it was relatively high and equal to 400mg/l. In addition, it displayed a remarkable capacity to reduce Cr(VI) concentration on the liquid medium containing initially 50 mg/l of Cr(VI) ; the percent removal rate was equal to approximately 42% after 72 h of incubation at 37 °C. In addition to its GRAS status, the obtained results suggested that *S. salivarius* could be successfully used in Cr(VI) bioremediation.

Keywords: bioaccumulation, Biofilm, biosorption, hexavalent chromium, *Streptococcus salivarius.*

INTRODUCTION

Heavy metals are among the most dangerous environmental pollutants. Hexavalent chromium ions found in industrial wastewater or environmental ecosystems are one of the most toxic and carcinogenic substances leading to serious problems. Heavy metal ions enter the food chain via wastewater, where they accumulate due to their non-biodegradable characteristic, they also tend to accumulate in human organs

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(Zhoo and Hayens, 2010; Bilgiç and Çimen, 2019). Only a small part of industrial wastewaters is treated (less than 10%), most of them, however, remain untreated and serious pollution complications therefore occurred (Rizzi et al., 2017). New approaches requiring environmentally friendly biological resources have been proposed and several studies have been carried out in this direction using plants or microorganisms for the adsorption of heavy metals (Fosso-Kankeu and Mulaba-Bofubiandi, 2013).

Lactic acid bacteria, which are often associated with food products, especially fermented products, have a great "cleaning" potential, according to Kinoshita et al. (2013), they can effectively prevent the absorption of heavy metals in the digestive tract and their diffusion to the organism.

Some authors like Bhakta et al. (2012) and Schut et al. (2011) have studied the mechanism of biosorption of heavy metals by lactic acid bacteria. It was suggested that the presence of the extracellular polysaccharide and the S-layers in bacteria can provide several adsorption and scavenging sites for metal ions due to the presence of many anionic functional groups (e.g: sulfhydrils, carboxyls, hydroxides, sulfonates, amide and amine groups) thus immobilizing the toxic elements and inducing local detoxification **(**Etesami, 2018). The same author has reported that biofilms, usually composed of extracellular polymeric substances, also adsorb heavy metals.

On the other hand, microbial biofilms play a crucial role in a variety of disciplines, including biotechnology, immunology, biofouling and biodeterioration (Fleming and Wingender, 2001). Biofilm formation is a dynamic process and different mechanisms are involved in its attachment and growth. Depending on the biofilm type and microorganisms involved, 90% of the particulate fraction of the biofilm can be extracellular polymeric substances (EPS), they have been recognized to be involved in adhesion to surfaces and biofilm formation and to cell adhesion/ recognition mechanisms (Staudt et al., 2004; Caggianiello et al., 2016). EPS are biopolymers such as polysaccharides, proteins, glycoproteins, lipids, and phospholipids. EPS play a major role in the formation and maintenance of the biofilm structure (Neu et al., 2001). Formation of biofilms by some species of lactic acid bacteria has been reported, they, therefore, play positive roles. They can be used as flora to colonize surfaces in contact with food and prevent the introduction of pathogens (Leriche and Carpentier, 2000; Arena et al., 2017), or used as efficient metal biosorbents when fixed to biotic or abiotic materials in bioremediation processes (Rafaat et al., 2016).

The aim of this work was, firstly to detect and evaluate the potential of *S. salivarius* to form biofilms on various solid surface and secondly to test its ability to remove hexavalent chromium from the growth medium.

MATERIAL AND METHODS

The strain of *S. salivarius* St.sa used in this study, previously isolated from milk was kindly provided by Prof. T. Idoui from the laboratory of Biotechnology, Environment and Health/University Mohamed Seddik Benyahia of Jijel. It was grown in de Man, Rogosa and Sharpe (MRS, MERCK, Germany) agar and broth and incubated at 37 °C/24 h prior to use.

Biofilm formation of *S. salivarius* was evaluated by TM according to the protocol described by Christensen et al. (1982) with some modifications. Nine ml of bacterial culture (10^8 CFU/ml) in Trypticase-Soy Broth (TSB, DIFCO, France) were poured into polystyrene tubes (PS) and incubated at 37 °C for 24 h. The tubes were decanted and washed with Tryptone Salt solution (TS, pH 7.0) and dried. Dried tubes were stained with crystal violet (0.1%) (SIGMA,

France). Excess stain was removed and tubes were washed with distilled water. Tubes were than dried in inverted position and observed for biofilm.

The ability of bacteria to produce slime, was tested on solid medium CRA, (52g Brain Heart Infusion (BHI) agar, 25 g glucose, 25 g sucrose, 0.8 g of congo red, 1000 ml of distilled water) as described by Sedláček et al. (2010). All the ingredients were from MERCK (France). The medium is inoculated with the bacterial culture. After 48 h incubation at 37 °C, agar medium was observed.

The quantitative method of adherence to polystyrene TCP proposed by O'Toole et al. (1998) was used with some modifications. Briefly, 100 µl of culture (10^8CFU ml^{-1}) in TSB-YE (TSB supplemented with 0.6% Yeast Extract, Difco, France) were added to the wells of sterile flat bottom 96-well polystyrene tissue culture plates (Nunc®, polystyrène, France) and incubated for 48 h at 25, 30 and 37 °C. Cultures were decanted and wells were washed with distilled water in order to remove non adherent cells. 150 µl of 0.1% crystal violet (SIGMA, France) were added to the wells for 30 min. The stained biofilms were rinsed with distilled water and extracted with 200 µl of 95% ethanol (SIGMA, France). 125 µl of the content of each well are then transferred to a new sterile microplate. The amount of biofilm was quantified by measuring the OD at 570 nm using a microplate reader.

Microbial-adhesion to solvents (MATS) is based on comparing microbial cell affinity to polar and nonpolar solvent (Bellon-Fontaine et al., 1996). The polar solvent can be an electron acceptor or an electron donor. The following pairs of solvents were used: (i) chloroform an acidic solvent (electron acceptor), hexadecane, a nonpolar, *n*-alkane, and (ii) ethyl acetate, a basic solvent (strong electron donor), and decane, a nonpolar *n*-alkane. The four solvents used were from SIGMA (France). Culture of *S. salivarius* in TSB (DIFCO) was harvested by centrifugation at 8,000 x *g* at 4 °C for 20 min and then washed twice with 150 mM NaCl solution pH 6.8 (SIGMA, France) and resuspended, in the same solution, to give an absorbance of 0.8 (A_0). 0.4 ml of solvent was added to 2.4 ml of cell suspension. The two-phase system was mixed to form an emulsion by vortexing for 60 s and allowed to separate for 15 min. The absorbance at 600nm (A_1) was measured with a UV-visible spectrophotometer (JENWAY 6105 UV/VIS spectrometer, USA). The affinity was calculated using the following formula:

% Affinity = $[1- (A_1/A_0)] \times 100$,

where A_0 is the absorbance of the aqueous cell suspension before mixing and A_1 is the absorbance after mixing.

The stainless steel surface used for biofilm experiments was AISI 316 L (20×10 mm). Before adherence assays, the coupons were washed as described by Bellon- Fontaine and Cerf (1990) with some modifications. They were washed using a 10-min immersion in an alkaline detergent 2% (v/v) RBS 35 solution, then rinsed by immersion in tap water (50 °C) for 25 min. Five further 1-min immersions in distilled water were performed, then they were autoclaved (120 °C/20 min) and dried in a laminar air flow hood. Adherence assays were performed using a modification of the procedure of Chavant et al. (2002). Bacterial cells were harvested (8000×g/20 min, 4 °C) and washed in TS solution then resuspended in the same solution (10^8 CFU/ml). Ten millilitres of the bacterial suspension was poured into a Petri plate containing a stainless steel coupon and incubated at 25, 30 and 37 °C for 3 h. The coupons were then washed with TS solution, and the remaining cells were either re-incubated for 18 h with 10 ml TSB-YE or detached from the inert surfaces by vortexing, for 2 min, in 20 ml of the same solution in the presence of sterile glass beads. Viable adherent cells were counted after cultivation on TSA (Difco).

Biofilm of *S. salivarius* was prepared as described previously by Chavant et al. (2002) and incubated for 7 days with removal medium each 24 h**.** Sessile cells were fixed on the support with a solution of 1% glutaraldehyde (SIGMA, France) for 1 h, washed 3 times with TS for 3 min then cells were dehydrated using a graded ethanol series (50, 70, 90 and 100%, three times for 10 min each) and finally kept in sterile Petri plates. SEM technique allows us to observe the structure of biofilms and the production or not of exopolymers by *S. salivarius*. The used microscope is an environmental one.

The method used to determine the MIC of Cr(VI) is the liquid medium dilution method described by Guo et al. (2010), corresponding to a modified protocol of Hassen et al. (1998). First, a stock solution for Cr(VI) at 1000 mg/L was prepared using $K_2Cr_2O_7$. The test consists of preparing an increasing concentration gradient of Cr(VI) (ranging from 0 mg/l to 500 mg/l), a first series of test tubes with a total volume of 10 ml, containing MRS broth and the metal was prepared to which is added 200µl of the LAB strain (OD_{600nm}=1.0) previously activated. On the other hand, a second set of representative blank tubes for each concentration of metal was also prepared. The tubes were then incubated with shaking at 37 °C for 24 h. Bacterial growth was monitored by measuring the absorbance at 600 nm at t0 and t24. The MIC is the lowest concentration (expressed in mg/l) capable of inhibiting the growth of the bacterium.

Sterile 250 ml Erlenmeyer flask containing a final volume of 100 ml of MRS with a concentration of 50 mg/L of Cr(VI), was inoculated with 2% of the bacterial culture (OD_{600nm} 1.0) previously activated (Shakoori et al., 2000). Three other flasks were also prepared: the first is exempt of bacterial culture, to assess the impact of abiotic factors on the fate of chromium. The second is devoid of Cr, to estimate the growth pattern without the metal, and the third contained an autoclaved biomass at the same final concentration together with the hexavalent chromium, to assess the possible biosorption effect on cell debris. The Erlenmeyer flasks were incubated at 37 °C with shaking for a period of 72 h.

To monitor the concentration of chromium in time, samples of 10 ml are collected at times t0, t24, t48 and t72. The samples were centrifuged for 20 min at 6000 rpm to obtain cell-free supernatants (Bhattacharya and Gupta, 2013). In parallel, other samples were taken every 24 hours to monitor the growth of *S. salivarius*. Determination of Cr(VI) concentration was carried out according to the standard colorimetric method of Greenberg et al. (1985). The Cr(VI) of the test sample reacts with a complexing agent, 1,5-diphenycarbazide (DPC) in acid pH. Thus, after 10 to 15 minutes, the complex formed gives the purplish pink color whose intensity is proportional to the hexavalent chromium concentration and is measured spectrophotometrically at 540 nm (Ncibi et al., 2008). The rate of chromium reduction is calculated using the following formula (Ozturk et al., 2012).

$$R\% = [(C_0-C_t) / C_0] \times 100$$

where C_0 is the initial concentration of Cr(VI), C_t is the concentration of Cr(VI) in time t and t is the time of incubation.

All data in this study represented the mean of three experimental replicates. Statistical comparisons among the different results obtained by the different tests were performed by One-way analysis of variance using XL-STAT version 2009. A significance level of 0.05 was used.

RESULTS & DISCUSSION

Based on Christensen et al. (1982) observations, the formation of a visible film lining the wall and the bottom of the tube, indicated a positive biofilm formation, the score ranges from strong,

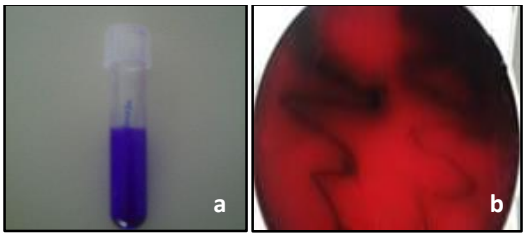

Fig. 1. Biofilm and slime production by *S. salivarius* as confirmed by the tube method (a) and the Congo red agar method (b).

moderate to weak depending on the intensity of the dye (crystal violet); however, biofilm formation is considered negative when a ring is formed at the liquid interface. The test showed that *S. salivarius* was biofilm positive and considered a strong biofilm producer (Fig. 1a).

To be considered as strong slime producers, colonies should be colored in black with a dry crystalline consistency, while weak slime-producing colonies are stained in pink with occasional darkening at the centers. Moderate slime-producing colonies showed a darkening with the absence of a dry crystalline morphology (Mathur et al., 2006). This result showed that *S. salivarius* displayed a strong production potential of slime by the formation of black colonies on CRA (Fig. 1b).

A quantitative evaluation of the biofilm formation by *S. salivarius* was performed in 96-well microplates. Absorbance values at 570 nm after incubation at different temperatures (25, 30 and 37 °C) and staining with crystal violet were measured. These values were considered an index of bacterial adhesion to the surface and consequently their ability to form biofilms. The results revealed that *S. salivarius* was able to adhere and consequently to form biofilms under the tested conditions (Fig. 2). Following the classification of Stepanovic et al. (2000) using the absorbance (A) of the sterile broth as a control (Ac): non-adherent (non-biofilm producer), $A \leq 0.06$ (Ac); weakly adherent (weak biofilm producer), $0.12 \geq A > 0.06$; moderately adherent (moderate biofilm producer), $0.24 \geq A > 0.12$; and strongly adherent (strong biofilm producer), $A > 0.24$. *S. salivarius* was a strong biofilm producer at 25 °C (A=0.28 nm) and moderate biofilm producer at 30 and 37 °C (A=0.18 nm). Statistical analysis showed significant differences ($P<0.05$) on the adherence ability of the strains when cultivated at 25 °C compared to 30 or 37 °C, but no difference ($P>0.05$) was noted between the values recorded at 30 and 37 °C. These results led us to conclude on the high impact of growth temperature (25 vs. 30 °C and 25 vs. 37 °C) on the adherence ability and the disparity among the strains.

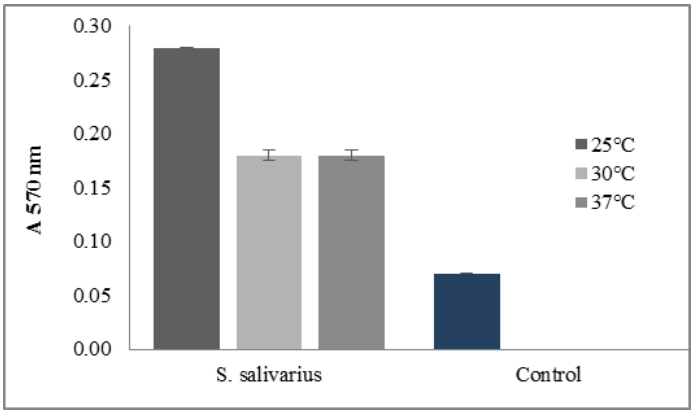

Fig. 2. *S. salivarius* biofilm formation evaluated by tissue culture plate method.

S. salivarius has a hydrophobic surface; its affinity was higher to hexadecane (87.70%) and decane (92%) (nonpolar solvents) as shown in Fig. 3. The determination of the electron donor/acceptor character is based on the comparison of the affinity of the bacterium for polar and nonpolar solvents (couple of solvents). The difference between the percentage chloroform affinity and that of hexadecane makes it possible to evaluate the electron donor character; if this difference is positive, the character of the strain studied is considered basic (Bellon-Fontaine et al., 1996). The difference between the percentage of affinity to ethyl acetate and that of decane makes it possible to evaluate the acceptor character of electrons of the microbial cells; if this difference is positive, the character of the strain studied is considered acidic (Bellon-Fontaine et al., 1996). The result showed that *S. salivarius* has an acidic character.

S. salivarius showed an adherence level of 7 log CFU/ml after 18 h. No significant differences ($P>0.05$) were recorded on the adhesion ability of the strain at the three tested temperatures (25, 30 and 37 °C). As shown in Fig. 4, we can observe that *S. salivarius* adheres to stainless steel AISI 316 L and seems to excrete exopolymers that enshrine bacteria creating small clusters on the surface.

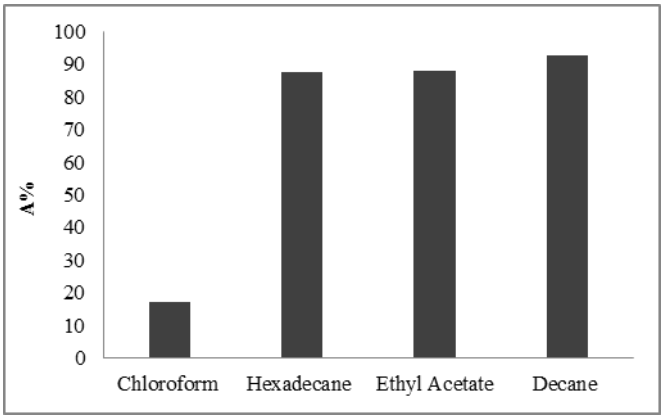

Fig. 3. Affinity (A%) of *S. salivarius* to solvents, chloroform, hexadecane, ethyl acetate and decane

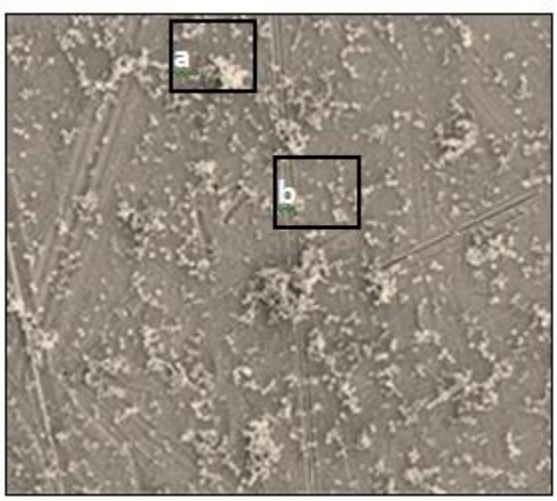

Fig. 4. Scanning electron micrographs showing *S. salivarius* biofilm on stainless steels obtained after 7 days. a: Cells embedded in exopolymeric matrix and b: Cells aggregates.

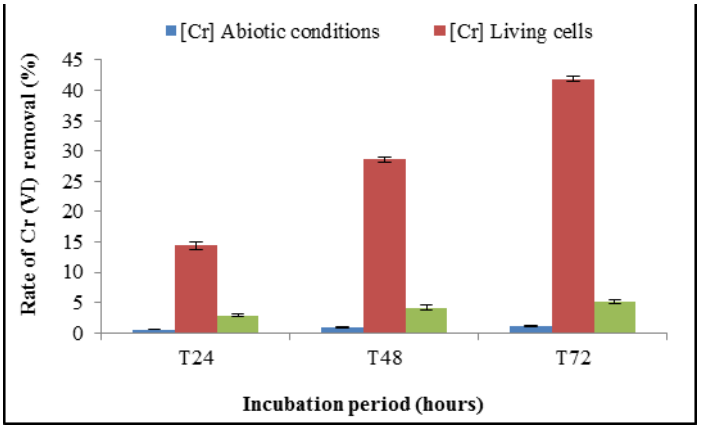

Fig. 5. Percent Cr(VI) removal by living and dead *S. salivarius* cells (Initial Cr(VI) concentration was 50 mg/l).

For MIC determination, it was found that the lowest concentration of Cr(VI) in which no growth of *S. salivarius* could be observed is equal to 400 mg/l.

Fig. 5 shows the variation of Cr(VI) reduction rate in different conditions, the obtained results indicated that the extracellular Cr(VI) concentration decreased considerably in the flask containing living cells, where the reduction rate is high and reached its maximum 41.89 ± 0.41% after 72 h, whereas with the dead biomass the percent reduction is lower (5.13 ± 0.32%), furthermore, in abiotic conditions, which does not contain bacterial cells, the percent reduction was 1.19 ± 0.09% after 72 h of incubation.

In this study we demonstrated that *S. salivarius* was able to adhere and to produce biofilms on abiotic surfaces like plastic and stainless steel as it was confirmed by SEM. In fact, the number of adherent cells is not important since the presence of a single cell on a substratum, such as stainless steel, can proliferate to form biofilms (Lewis et al., 1987). According to Whitfield and Roberts (1999) the bacteria in biofilms can produce polysaccharides; these compounds may be either unattached as slime or linked to the cell surface. Based on these, the results of this study have shown that *S. salivarius* was a good producer of slime.

Microbial exopolysaccharides are comprised of either homopolysaccharides or heteropolysaccharides (Monsan et al., 2001). It has been reported that a number of lactic acid bacteria produce heteropolysaccharides. β-D-glucans are produced by *Streptococcus* spp., and fructans are produced by *Streptococcus salivarius* containing β(2→6) linked fructosyl units (Sutherland, 2001).

The hydrophobic character of lactic acid bacteria is related to the hydrophobic components present on their surface and it has been suggested that in Gram-positive bacteria, lipoteichoic acid is involved in hydrophobic interactions. Furthermore, the hydrophobicity can increase with the rate of membrane proteins (deduced from the nitrogen/carbon ratio [N/C]) present on the surface of the bacterium (Mozes et al., 1988). It has been also reported that the hydrophobic character plays an important role in the adhesion of a bacteria on a hydrophobic support (Pieniz *et al*., 2014). The acceptor character of electrons (acid) is generally attributed to acidic groups such as $R\text{-}NH^{+3}$ (Bellon-Fontaine et al., 1996; Briandet et al., 1999). Acid-base Lewis interactions (electron donor / acceptor) are considered to be strong interactions that allow the formation of a hydrogen bond between the two surfaces (bacteria and solid supports) (Burgain et al., 2014). The outstanding capacity of *S. salivarius* to form biofilm on various surfaces renders it

a candidate of choice for being used in "biofiltration" processes for remediating metal contaminated effluents.

Results of Cr(VI) MIC indicated that *S. salivarius* is highly tolerant to the toxic metal when compared to other bacteria from the literature, for example Srinath et al. (2002) found that the MIC of Cr(VI) for three *Bacillus* species was equal to 100 mg/L which is lower than our value. It is important to notice that no data on the toxicity of Cr(VI) toward LAB and particularly streptococci is available; this is why our results were only compared to other bacterial Genera.

Concerning the metal removal results, the dead cells were found to fix the metal but the rate was relatively low, this could be due to the low biomass concentration. In the same context, Priya et al. (2013), showed that biosorption of hexavalent chromium by dead biomass of bacteria, algae, molds and yeasts is mainly due to electrostatic interactions between metal ions in the culture medium and microbial cell walls. On the other hand, Schut et al. (2011), examined the ability to accumulate copper of eight *Lactobacillus* species from grapevine in two physiological states of bacteria: live (activated bacteria) and dead, after treatment by heat, they found a considerable degree of Cu (II) adsorption by dead bacteria, however, it was lower compared to biosorption by living cells.

The reduction of chromium by living biomass comprises mainly two mechanisms: the first is independent on bacterial metabolism and includes adsorption where chromium ions are bound to the cell wall components, the second, which is metabolism-dependent and by which chromium ions are transported through the cell membrane is called "bioaccumulation". In this study, living cells where capable of removing higher amounts of Cr(VI), suggesting that both mechanisms are involved in eliminating the metal ions by *S. salivarius*. The role of EPS in Cr(VI) resistance was reported in other microorganisms; Ozturk and Aslim (2008) found that exposure to elevated concentrations of Cr(VI) affected the composition of EPS produced by *Chroococcus* sp. H4, and that a correlation exists between Cr(VI) resistance and EPS concentration in some cyanobacteria. Furthermore, Rafaat et al. (2016), revealed that *S. thermophilus* was able to remove several heavy metals namely Pb, Cd and As; the rates were 40.5, 25.7 and 38.3 %, respectively, while higher values were obtained with other LABs, used separately or as consortia, in our case the percent removal of Cr(VI) is considered moderate to high.

CONCLUSION

Based on the obtained results, it could be concluded that *S. thermophilus* is a strong biofilm producer and is able to resist and remove considerable amounts of Cr(VI); both properties are highly recommended for microorganisms to be used in biofiltration of metal-contaminated effluents, however, more in-depth investigations are required to understand the involved mechanisms.

GRANT SUPPORT DETAILS

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CONFLICT OF INTEREST

The authors declare that there is not any conflict of interests regarding the publication of this manuscript.

LIFE SCIENCE REPORTING

No life science threat was practiced in this research.

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