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HIGH BIODEGRADATION LEVELS OF 4,5,6-TRICHLOROGUAIACOL BY *BACILLUS* SP. ISOLATED FROM CELLULOSE PULP MILL EFFLUENT

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ABSTRACT

An aerobic Gram positive spore-forming bacterium was isolated from cellulose pulp mill effluent. This microorganism, identified as *Bacillus* sp. and named IS13, was able to rapidly degrade the organic chlorinated compound 4,5,6-trichloroguaiacol (4,5,6-TCG) from a culture containing 50 mg/l, which corresponds to about $3x10^4$ times the concentration found in the original effluent. The biodegradation of this compound, usually found in cellulose pulp mill effluents, was evaluated by spectrophotometry and gas chromatography analysis. During 4,5,6-TCG decreasing, the lack of by-products had shown by such analysis lead to verify the possibility of either adsorption or absorption of 4,5,6-TCG by the cells, instead of real biodegradation. There were no traces of 4,5,6-TCG after lysozyme and SDS cell disruption. Vigorous extraction was applied before spectrophotometry analysis and there was no release of residual 4,5,6-TCG. Plasmid isolation was attempted by using different protocols. The best results were reached by CTAB method, but no plasmid DNA was found in *Bacillus* sp. IS13. The results suggest that genes located at the bacterial chromosome might mediate the high decrease of 4,5,6-TCG. The importance of this work is that, in being a natural ocurring microorganism, *Bacillus* sp. IS13, can be used as inoculum in plant effluents to best organochlorinated compounds biodegradation.

Key Words: biodegradation, 4,5,6-trichloroguaiacol, aerobic bacteria and DNA.

INTRODUCTION

In the last few decades, the production and use of organochlorinated compounds has increased substantially. Although these compounds are responsible for many environmental problems, they are still heavily utilised due to their industrial importance (21).

Some microorganisms have adapted to living on areas highly contaminated with natural or xenobiotic organochlorinated compounds (14). Frequently, these organisms possess the capability to metabolize such substances to utilising them as a source of carbon and nitrogen (3,19).

The bacterial cells are characterized by large metabolic and physiological machinery which allow microorganisms to inhabit hostile ecological niches (20). The employment of such properties to solve environmental problems can be a possible alternative in effluent treatment.

The cellulose bleaching process is responsible for the production of many chlorinated compounds that are unavoidably spilled in the environment. Many of such substances are toxic and very difficult to microbial metabolization. 4,5,6- trichloroguaiacol is one of these compounds, which are frequently present in cellulose pulp mill effluent, and is correlated with several flora and fauna problems. Therefore, 4,5,6- trichloroguaiacol was chosen as a model compound to study the biodegradation process of chloro_aromatic substances.

Biodegrading bacterial metabolism is directly involved with enzyme production either coded by genes located in plasmids or chromosomes (5). Many biodegradation-related plasmids are able to codify complete xenobiotic biodegrading pathways or at least part of them (8,17). Several bacterial isolates from different locations have shown to harbor similar catabolic plasmids, suggesting an intense genetic transfer among bacteria in nature (7). On the other hand, chromosome-encoded genes may also be involved with biodegradation. The presence of such genes in the bacterial chromosome can be justified by the natural occurrence of organochlorinated compounds (4). Throughout the evolution, microorganisms had to survive with this kind of environmental pressure, thus having to develop biological self-defense strategies. The utilization of recombinant DNA technics may be applied in the search for genes involved with biotransformation process, and the understanding of the functional mechanisms of these DNA elements is very important if industry is willing to apply such capabilities to solve pollution problems.

The aims of this research is to identify and study microorganisms capable of biodegrade organochlorinated compounds present in a local industry and verify the existence or not of plasmids possibly involved with this function.

MATERIALS AND METHODS

Bacterial strains

Bacillus sp. IS13 was isolated from a local cellulose pulp mill effluent. The identification was performed based in the following characteristics: spore-forming capability, Gram staining, and aerobic growth. The control bacterium, *Bacillus subtilis*, MC1 was obtained from the Microbiology Department of Federal University of Rio Grande do Sul, Brazil.

Chemicals

4,5,6-trichloroguaiacol (4,5,6-TCG) was obtained from Hélix Biotech Co., Canada. 3,4,5,6-tetrachloroguaiacol (3,4,5,6-TeCG) was gently provided by Dra. Maria do Carmo Ruaro Peralba of The Chemistry Institute of The Federal University of Rio Grande do Sul. All other chemicals used were of analytical grade.

Culture conditions

The cultures were grown in 250 ml Erlenmeyers flasks containing 150 ml of LB medium, pH 7.0 supplemented with 50 mg/l of 4,5,6-TCG (maximum water dissolved concentration of 4,5,6-TCG reached). They were incubated in rotatory shaker at 150 rpm and 37ºC. The organochlorinated compound was dissolved previously in distillated water, pH 5.5, by shaking at 150 rpm and 37oC for 48 hours.

CFU determination

Bacillus subtilis MC1 (control bacterium) and *Bacillus* IS13 were cultured overnight at 37ºC in LB broth. The cultures were dilluted with destillated water and plated on pure LB and LB supplemented with 50 mg/l of 4,5,6-TCG. The CFU/ml was determinated by colony formation.

Biodegradation analysis

Decrease of levels of 4,5,6-TCG was analysed by spectrophotometry (spectrophotometer Shimadzu UV-160A) and gas chromatography (HP 5890 chromatograph equipped with a PONA column 50 m x 0.2 mm x 0.5 µm, and FID detector). The gas chromatography analysis were performed utilising N_2 as a

carrier gas with a flow of 1.5 cm^3/min., and the temperature program was 185^oC isothermally for 30 minutes. The quantitative analysis was made by using tetrachloroguaiacol as internal standard. The results of both methodologies were compared.

Spectrophotometry analysis. Samples of 1 ml were collected from a culture of *Bacillus* sp. strain IS13 at 0, 3, 6, 12, 20 and 24 hours of incubation. To the sample, 2 ml of hexane were added and shaked in vortex for 60 cycles of 1 second each. 1 ml of the hexane layer was dispensed into a plastic spectrophotometer cuvette and analysed at 296 nm. Samples were also extracted with acetone, dichloromethane and petrolleum ether, but the best results were obtained with hexane.

Gas chromatography. Samples of 20 ml from the same culture described as above were extracted during 5 min. with 20 ml of hexane. The hexane phase was collected and completely dried by N_2 stream. The

4,5,6-TCG extracted was re-dissolved in 0.1 ml of hexane. The samples were derivatized with diazomethane. A sample of 50 µl from a solution of 10 mg/ml of 3,4,5,6-tetrachloroguaiacol was used as standard control. A 1 µl was injected in the CG. For these experiments samples were collected after biomass evaluation by optical density (600 nm).

Cellular lysis. Cellular lysis and vigorous shaking were carried out to verify absorption and adsorption of 4,5,6-TCG respectively, as follows.

Adsorption test. 3 samples of 1 ml each of a 24 hours culture were collected and added with 2 ml of hexane. Extraction was performed applying 2 minutes of intermittent shaking on vortex with maximum agitation power. Then, 1 ml of hexane layer was collected and analysed in a spectrophotometer at 296 nm wave length. The results were compared with standard extractions (shaking 60 times /1 second pulse).

Absorption test. 3 samples of 1 ml of a 24 hours culture were dispensed into Eppendorf tubes and centrifuged at 8000 g by 2 minutes. The supernatant was extracted with 2 ml of hexane and analysed in a spectrophotometer at 296 nm wave length. The cellular phase was ressuspended with 1 ml of distilled water and centrifuged at 8000 g. To the pellet were added 0.2 ml of STET buffer (8% w/v sucrose, 0.1% v/v Triton X-100, 50 mM EDTA, 50 mM Tris-HCl pH 8.0). Cellular lysis was carried out by 5 minutes incubation at room temperature after addition of 20 µl of lysozyme solution (10 mg/ml). Cell suspensions were boiled for 45 seconds and extracted with 2 ml of hexane by shaking in vortex 60 times of 1 second pulse. Cellular lysis was also performed by alkaline lysis with SDS (12) following extraction and analytical procedures as described above.

DNA plasmid extractions

DNA plasmid extraction was attempted by six different methodologies: Kado and Liu (12); Anderson and Mckay (2); Del Sal (7) with some modifications, alkaline lysis (16) and boiling lysis (16); and pulsed field eletroforesis (6, 11) with some modifications. The best results were obtained by CTAB

method which was performed as follows. *Bacillus* sp. IS13 was inoculated in LB medium containing 50 mg/l of 4,5,6-TCG and grown overnight. Bacterial cells were centrifuged at 8000 g by 2 minutes. The supernatant was discharged and the pellet ressuspended in 0.2 ml of STET buffer. The suspension was incubated at room temperature for 5 minutes after addition of 4 µl of lysozyme (50 mg/ml). Tubes were boiled for 45 seconds and centrifuged for 10 minutes. A toothpick was used to remove lysed cellular debris and 8 µl of CTAB (cetyl trimethyl ammonium bromine 5%w/v) (Sigma Co.) was added before centrifuging at room temperature for 5 minutes. The pellet was ressuspended in 0.3 ml of NaCl 1.2 M and ressuspended with a micro pipette. 2 µl of RNAase (20 mg/ml) and 2 µl of Proteinase K (20 mg/ml) were added and incubated at room temperature during 20 minutes. Next, samples were incubated at 65ºC for 90 minutes and cooled again to room temperature. One or two extractions with 1:1 phenol-chloroform (phenol pH 8.0 / chloroform v/v) were carried out. The solution was re-precipitated by centrifuging by 10 minutes after addition of 0.75 ml of ethanol. The pellet was rinsed with 70 % ethanol, dried under vacuum and ressuspended in 20 µl of distilled deionized water.

RESULTS AND DISCUSSION

4,5,6-TCG Consumption

The *Bacillus* sp. IS13 was isolated from cellulose pulp mill effluent while *Bacillus subtilis* MC1 (control bacterium) came from nosocomial samples. These bacteria share very similar morphology, cell wall and growth characteristics. Both *Bacillus* sp., strain IS13 and *B. subtilis* MC1, were cultured in LB medium supplemented with 50 mg/l of 4,5,6-TCG, but only *Bacillus* IS13 was able to degrade this chemical. The biomass concentration reached in such medium was 1.7×10^8 (*Bacillus* sp. IS13) and 7.9×10^7 CFU/ml (*B. subtilis* MC1) after 24 hours of culture. The concentration of 4,5,6-TCG in LB medium remained unaltered in the control without bacterial cells after 7 days of incubation at 37^oC (Fig. 2). The bacterium control (*B. subtilis* MC1) did not show the same behavior of the isolated (*Bacillus* sp. IS13) when cultured with 4,5,6-TCG. Since 4,5,6-TCG concentration was not modified by cellular absorption (no 4,5,6-TCG was found in lysed cells), either cellular adsorption (no 4,5,6-TCG was found after vigorous cellular agitation) or evaporation, the high decrease of the 4,5,6-TCG concentration was interpreted as being a consequence of *Bacillus* sp. IS13 metabolism. The experimental results demonstrated that 50 mg/ml of 4,5,6-TCG do not sustain perceptive biomass increament (results not shown) as a unique carbon source. Based on culture analysis, the microorganism *Bacillus sp.* IS13 showed the capacity to degrade near all 4,5,6-TCG by co-metabolism with LB medium components from a culture with 50 mg/l in less than 12 hours as demonstrated by spectrophotometry at 296 nm (Fig. 1) and by gas chromatography (Fig. 2). Alkaline and acid extractions (14) were performed to identify biodegradation by-products as anysoles, veratroles, catechols and seringols. After gas chromatography analysis no by-products were detected, suggesting chemical instability or rapid biodegradation of such compounds. Similar results were obtained by Häggblom *et al.* (10) in the biodegradation of 3,4,6/3,5,6-TCG and 4,5,6-TCG by *Rhodococcus* sp. and *Mycobacterium* sp. In their work, they were also unable to detect metabolites from

TCG biodegradation assays. Dichloroanysoles and dichlroveratroles have been reported as by-products of the biodegradation of chlorinated phenols and guaiacols, respectively (15). The difficulty to recovery such metabolites could be explained by volatility (9) or transformation in less toxic and rapid biodegradable compounds. The 3,4,6/3,5,6-TCG and 4,5,6-TCG biodegradation rates in such work were approximately 2.27 mg/48h. In other experiments of the same authors (10), 3,4,6/3,5,6-TCG was biodegradated at levels approximately of 0.0006 mg/h. Neilson *et al.* (13) described an anaerobic consortium that could reduce 100 µg/l of 4,5,6-TCG in 24 hours of culture. Allard *et al.* (1), working with the biotransformation of di- and trichlorocatechols by an anaerobic bacteria consortium, demonstrated reductions ranging from 60 to 100 µg/l of these compounds in culture periods varying from 8 to 50 days. Somewhat higher degrees of biotransformation were observed for *Rhodococcus* sp. and *Acinetobacter* sp. which were able to biotransform about 100 µg/l in 10 hours of 2,6-dibromophenol by O-methylation. Results in the present work contrast with several previous researches by the fact that *Bacillus* sp. IS13 was able to decreases much higher concentrations (50 mg/l) of 4,5,6-TCG, in a short period of time (less than 12 hours). Such span of time corresponds to the exponential growth phase of the tested bacterium (Fig. 1) and suggests a correlation between biomass increament and the decrease of 4,5,6-TCG. Bacterial control (*Bacillus subtilis*) was able to survive but not to reduce the 4,5,6-TCG concentration under same culture conditions. Some other researchers have been identifying very efficient microorganisms, which were able to biodegrade chloro aromatic compounds, others than 4,5,6-TCG. Zaitsev *et al.* (23) isolated a strain of *Rhodococcus opacus* GM-14 that utilizes 4-chlorophenol and 3-chlorophenol at concentrations of 250 mg/l and 100 mg/l respectively. Wu *et al.* (22) studied an anaerobic consortium isolated from sludge granules able to degrade 40 to 60 mg/l of pentachlorophenol per day. Although different bacteria have been identified as related to biodegradation, the genus *Bacillus* is one of the most frequently. Such genus is known to possess a very efficient capacity to adapt itself to new environments due to its broad range of enzymatic metabolism (18). Such metabolic versatility can be utilized to break down the structure of toxic molecules allowing microbial survival even in the presence of uncommon compounds. Moreover, it is known that the higher capability of degradation showed by *Bacillus* sp. IS13 could be induced by the long exposure of these microorganisms to effluents containing many different kinds of organochlorinated compounds. Such selective pressure could have activated genetic routes responsible for transformation of 4,5,6-TCG.

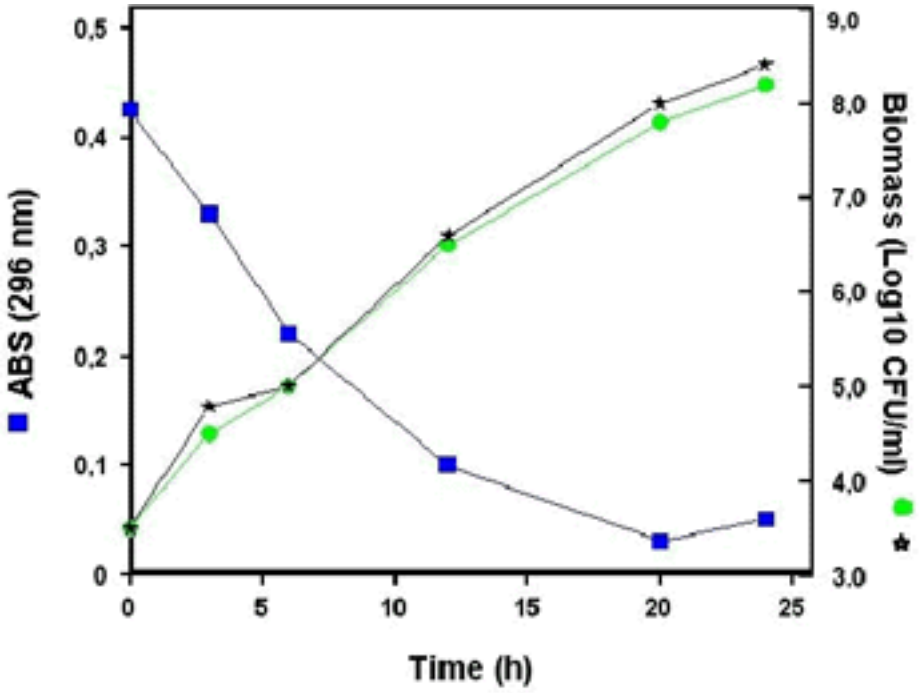

Figure 1. Spectrophotometry analysis of the decrease 4,5,6-TCG (n) and biomass increase (l) by *Bacillus sp.* IS 13 in pure LB («) and LB +50 mg/l of 4,5,6-TCG. ABS (296 nm) of 0.4=50 mg/l of 4,5,6-TCG.

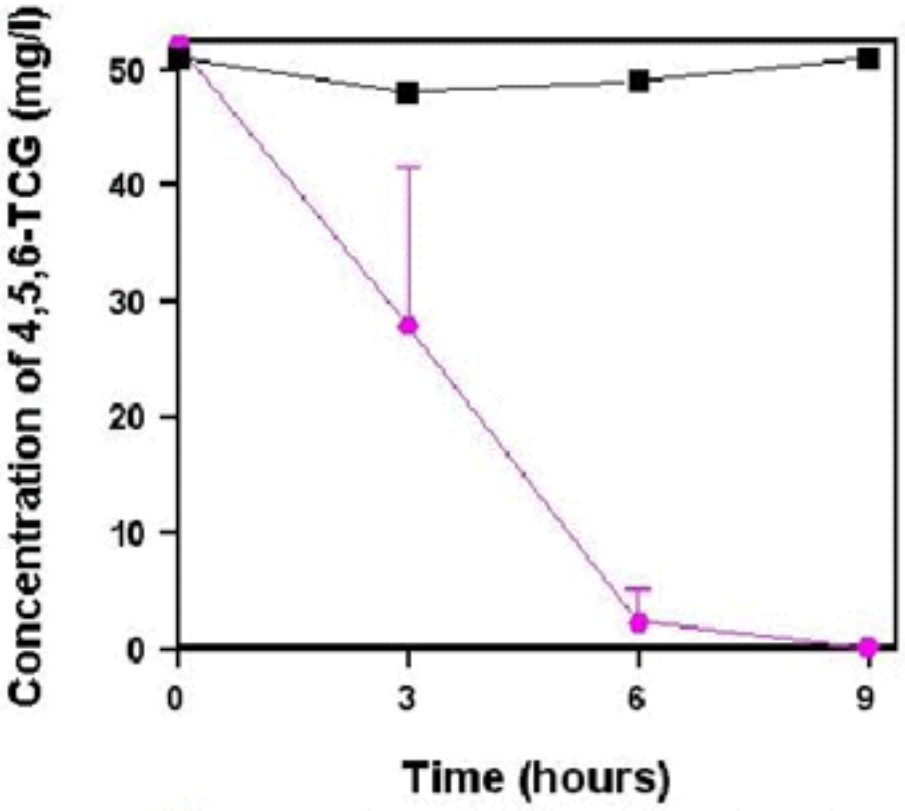

Figure 2. (l) Gas chromatography analysis of the decrease of 4,5,6-TCG by culture of *Bacillus sp.* strain IS 13 in LB +50 mg/l of 4,5,6-TCG. After 9 hours, 4,5,6-TCG were not detected. (n) Control without bacteria.

Adsorption and absorption tests

The experiments demonstrated that transformation of 4,5,6-TCG was related with biomass increment supported by growth on LB medium, being the decrease of the organochlorinated compound due to co-metabolism. During biomass increment, detection of 4,5,6-TCG biodegradation by-products were not observed, neither by scanning spectrophotometry nor by gas chromatography. This fact lead to the verification of the possibility of 4,5,6-TCG adsorption or absorption by cells, instead of being metabolized Fig. 3, shows the amount of 4,5,6-TCG after several cycles of incubations and different extractions methods. Very low concentration of 4,5,6-TCG was found at intact and lysated cells after 24 hours of incubation, demonstrating no absorption of the 4,5,6-TCG. To verify adsorption possibility, vigorous agitation was applied during extraction, showing only traces of 4,5,6-TCG after the same time of incubation. Spectrophotometer analysis of pure hexane (without 4,5,6-TCG) demonstrated same values for recovered cultures after 24 hours of incubation. Such results suggest that there were no significative of 4,5,6-TCG absorbed or adsorbed on the cells, which could be interpreted as a false positive biodegradation result. Gas chromatography confirmed spectrophotometry results showing the same behavior for *Bacillus* sp. IS13 cultures growing in LB medium supplemented with 4,5,6-TCG.

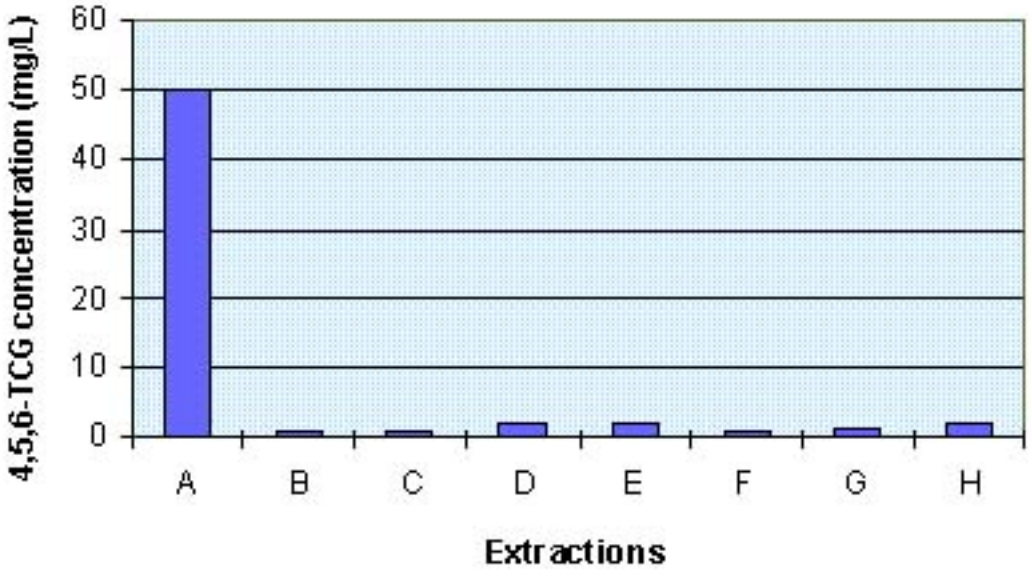

Figure 3. Absorbance at 296 nm relative to 4,5,6- TCG concentration in LB medium. A) Standard extraction of culture of *Bacillus* IS13 at 0h. B) Standard extraction of culture of *Bacillus* IS 13

after 24h of incubation. C) Vigorous extraction of culture of *Bacillus* IS13 after 24h of incubation. D) Cellular phase of culture of *Bacillus* IS13 after 24h of incubation. E) Liquid phase of culture of *Bacillus* IS13 after 24h of incubation. F) SDS lysis after 24h of incubation. G) Lysozyme lysis after 24h of incubation. H) Hexane X hexane (without 4,5,6-TCG).

DNA plasmid extraction

Once *Bacillus* sp. IS13 was characterized as a potencial bacterium for bioremediation processes, it was decided to search for DNA plasmids that could contain genes responsible for biodegradative activity. DNA extractions were attempt more than 30 times by six different methodologies. Performing the CTAB method (7), it was possible to identify two plasmids from *Pseudomonas* sp. (results not shown) and also to extract DNA from *Bacillus* sp. strain IS13. The results showed that this strain seems to lack any plasmidial DNA structures. The absence of plasmids suggests that the biodegradation capacity of *Bacillus* sp. strain IS13 is chromosome-encoded. The chromosomic presence of genes able to transform organochlorinated compounds can be explained by the necessity of the bacterial cells to detoxify its surrounding environment in order to survive. Toxic xenobiotic and, especially, toxic natural compounds were frequently present in nature with microorganisms during evolution. This fact has always imposed a selective pressure, justifying the presence of stable biodegrading routes in the bacterial metabolism. The biodegradation potential of *Bacillus* sp. IS13 must be further verified to allow its possible utilization in bioremediation of polluted effluents and locations.

CONCLUSIONS

Bacillus sp. strain IS13 is a highly adapted bacterium with great potential to biodegrade 4,5,6-TCG and possibly other related chlorinated compounds. Such microorganism can be further studied to be utilized in the industrial effluent treatment and decontamination of natural areas. Results in this work also suggest that the genes responsible for 4,5,6-TCG biodegradation may be located in the bacterial chromosome. Such characteristics could be interesting to industrial effluent treatment due to biodegradation active stability.

RESUMO

Altos níveis de biodegradação do 4,5,6-tricloroguaiacol por *Bacillus* sp. isolado de efluente de indústria de polpa de celulose

Isolou-se uma bactéria gram positiva, esporulada a partir de efluente de fábrica de polpa de celulose. Esse microrganismo, identificado como *Bacillus* sp. e nomeado IS13, foi capaz de degradar rapidamente o composto orgânico clorado 4,5,6-tricloroguaiacol (4,5,6-TCG) presente em meio de cultura a uma concentração de 50mg/L. Essa concentração equivale a $3x10^4$ vezes mais 4,5,6-TCG que a concentração encontrada no efluente original. A biodegradação desse composto foi analisada por espectrofotometria de varredura e cromatografia gasosa. A falta de sub-produtos de degradação sugeriu a verificação da possibilidade de adsorção e absorção celular do 4,5,6-TCG ao invés de biodegradação propriamente dita. Não foram encontrados traços de 4,5,6-TCG após lise celular com lisozima e SDS e não houve desprendimento desse composto após agitação vigorosa. Logo, o desaparecimento do 4,5,6-TCG do meio de cultura analisado foi interpretado como biodegradação devido ao metabolismo do *Bacillus* sp. IS13. A partir desse microrganismo, buscou-se isolar plasmídeos utilizando diferentes protocolos. Os melhores resultados foram obtidos através do método do CTAB, porém não encontraram-se plasmídeos no isolado IS13. Os resultados sugerem que a alta taxa de degradação do 4,5,6-TCG é mediada por genes presentes no cromossomo bacteriano. A importância desse trabalho encontra-se na possibilidade de utilização do *Bacillus* sp. IS13 como inóculo em plantas de efluentes industriais, a fim de biodegradar compostos orgânicos clorados presentes nesses locais.

Palavras-chave: biodegradação, 4,5,6-tricloroguaiacol, bactéria aeróbia, DNA.

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