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Biotransformation of chromium (Cr-VI to Cr III) from tannery effluent using bacteria and fungi

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Abstract

Chromium, a priority pollutant is well known for its mutagenicity, carcinogenicity and teratogenicity in humans, animals and plants. Extensive use of chromium in industries such as leather tanning, stainless-steel production, electroplating and wood preservatives have resulted in extensive chromium contaminated soil and ground water. Microorganisms are involved in the removal of toxic wastes, both in the natural environment and in controlled treatment systems. The present study was undertaken to optimize the chromium reduction capabilities of microbes isolated from the tannery effluents of Dindigul district. About 68 bacterial and 23 fungal isolates were identified based on their microscopic and macroscopic features. The screening was done on minimal salt media incorporated with potassium dichromate. The *Bacillus amyloliquefaciens* CRB36 strain showed the maximum chromium reduction activity when compared to other bacterial and fungal species isolated in this study. The optimization of chromium was achieved when starch and peptone were supplied as carbon and nitrogen sources at an optimum pH of 7.0 with an incubation temperature of 30°C. Bioremediation is the most promising, eco-friendly and cost effective technology widely used now a days for detoxification of toxic industrial pollutants in soil and water.

Keywords: Chromium, Tannery effluent, Aspergillus, Bacillus, Bioremediation.

Introduction

Environmental pollution especially of water bodies is one of the major problems of the world and it is increasing day by day due to urbanization and industrialization. Waste water released from various industries is the major concerns for environmentalists nowadays (Evelyne *et al.*, 2014). Industrial effluents contain various toxic metals, harmful volatile compounds, and several organic and inorganic compounds. The long-term consequences of exposure to polluted water can cause fatal diseases like cancer, delayed nervous responses, mutagenic changes, neurological disorders etc (Megharaj *et al.*, 2003). The discharge of these toxic effluents, there has been a major loss in the ecological, social and economic perspective (Deepali., 2011).

Chromium is an essential micronutrient required for the growth of many microorganisms for the maintenance of normal glucose, cholesterol and fatty acid metabolism (Thacker *et al.*, 2005). Chromium is found in all phases of the environment, including air, water and soil. Naturally occurring in soil, Cr ranges from 10 to 50 mg/ kg depending on the parental material. In fresh water, Cr concentrations generally

range from 0.1 to 117 μ g /L, whereas values for seawater can range from 0.2 to 50 μ g/L (Pechova *et al.*, 2007). Chromium is a naturally occurring element found in many foods and drinking water, thus it makes its way into the body mainly from dietary intake. In addition to that, intake of chromium results from airborne dusts and mists, and cigarette smoke as well as from industrial and occupational exposures (Katz *et al.*, 1994).

The industries discharges many toxic pollutants like sulfides, phenolic compounds, magnesium, sodium, potassium, chromium and other mineral salts, dyes and solvents. In India alone about 2000–3000 tone of chromium escapes into the environment annually from tannery industries, with chromium concentrations ranging between 2000 and 5000 mg/l in the aqueous effluent compared to the recommended permissible discharge limits of 2 mg/l [2]. Tannery industry is one of the oldest cottage industries in India which has taken a predominant place in the country's economy (Mohanta *et al.*, 2010).

Several reports have indicated biological reduction of hexavalent chromium by microorganisms, both aerobes and anaerobes. Many researchers have been done so far to identify chromium tolerant and chromium resistant microorganisms. Several bacteria and fungi were studied for identifying their chromium VI reducing ability (Deepali., 2011; Jayalakshmi *et al.*, 2013).

Resistance is defined as the ability of a microorganism to survive toxic effects of metal exposure by means of a detoxification mechanism produced in direct response to the metal species concerned. Tolerance is defined as "the ability of a microorganism to survive metal toxicity by means of intrinsic properties or by environmental modification of toxicity (Romanenko et al., 1997). Some mechanisms for tolerating heavy metals are strategies such as exclusion by a permeability barrier. intraand extra-cellular sequestration, active transport efflux pumps, enzymatic methods and a reduction in the sensitivity of targeted cellular organelles to metal ions (Bruins et al., 2000).

Bacteria that can convert toxic hexavalent chromium to non toxic trivalent chromium had been identified earlier (Jayalakshmi *et al.*, 2013; Qian *et al.*, 2013; Manojkumar., 2011]. Fungus acts as bioabsorptive material to remove hexavalent chromium (Noorjahan., 2014; Olufunmi., 2013). Biosorption mechanism is done by two methods- metabolism dependent and nonmetabolism dependent. In non- metabolism dependent mechanism chromium gets bound to the functional groups on the surface and get absorbed. The present study was carried out with the research aim the Chromium VI reduction ability of bacteria and fungi isolated and screened from tannery effluents to choose between and find a suitable, efficient and costeffective biological treatment for leather industry waste water.

Materials and Methods

Sample Collection:

The effluent samples were collected from the tannery industries, Begambur, Dindigul in Tamilnadu. Sterile containers were used for collection and they were transported to the laboratory within 2-4 hours and analyzed for the isolation of microorganisms or stored at 4^{0} C for further analysis.

Tannery Effluent Physicochemical Properties:

The collected tannery effluent was analyzed for physicochemical properties like colour, odor, turbidity, pH, total suspended solids, total dissolved salts, chemical oxygen demand (COD), biological oxygen demand (BOD) and chromium [15].

Isolation and Identification of Chromium Resistant Bacteria and fungi:

The chromium resistant microorganisms were isolated from the tannery effluent from treatment plant at Dindigul and effluent contaminated site soil samples, through culture technique. The pooled effluent sample 1 ml was serially diluted in sterile saline to obtain the serial 5- fold dilution. For bacterial isolation, aliquots of 0.1 ml were withdrawn from 10^{-2} to 10^{-1} ⁵dilutedsample suspension and inoculated on to the nutrient agar and Minimal Salt Medium (MSM) plates (amended with $K_2Cr_2O_7$ as a source of Cr (VI)) following spread plate technique. Plating were done in triplicates. The inoculated plates were incubated at 37°C for 24 hrs. Nutrient slants were prepared for sub culturing of the colonies that are obtained in nutrient agar plates and stored at 4°C until needed for further experiments. Identification of selected bacterial isolates were carried out by the routine bacteriological method like Colony morphology and Preliminary tests like Gram Staining, endospore staining, capsule staining, motility test, catalase, oxidase test and performing biochemical tests like IMViC, triple sugar iron test and growth in selective media.

For fungal isolation, 1 gm of soil sample was serially diluted and $K_2Cr_2O_7$ incorporated to the Sabouraud Dextrose Agar and incubated for 3 days at 28°C. The different fungi isolated were identified on the basis of macroscopic and microscopic morphology. The identified fungal pure cultures were preserved at 4°C after growing on Potato dextrose agar (PDA) slant.

Optimization of Hexavalent Chromium Reduction Process at Various Physical and Chemical Parameters:

In this present study the chemical parameters such as carbon source (glucose, sucrose, starch and cellulose), Nitrogen source (peptone, yeast extract and beef extract), and physical parameters such as pH (6,7and8), temperature (30°C, 35°C and 40°C) were selected. The minimal salt medium was prepared and made to differ in the above parameters. The pH adjustment was made using concentrated 1N HCl or 1N NaOH. The carbon sources and Nitrogen sources were added at 1% concentration.

Chromium (VI) Assay by Spectrophotometric DPC Method:

Concentration of Cr (VI) was carried out using spectrophotometer method followed by APHA.[1, 18]. 0.2 N sulphuric acid was added to the filtrate solution obtained and 0.25 ml of orthophosphoric acid was added. Then, 2 ml of diphenylcarbazide solution, which was prepared by dissolving 250 mg 1,5diphenylcarbazide in 50ml of acetone, was added and mixed thoroughly. Various concentration from stock solution were prepared (0.5,1,1.5,2,2.5,3,3.5,4 mg/ml) and Cr (VI) in the sample was assayed by adding 125µl of the DPC reagent to 1ml of chromium samples, mixed gently and kept at room temperature for 20 minutes after which produced a violet color. The absorbance at 540 nm wavelength was measured by Double beam UV Visible spectrophotometer.

The absorbance of the color produced was measured at 540 nm using a spectrophotometer. A standard value was plotted. Cr (VI) concentration in the sample was calculated from a standard curve using $K_2Cr_2O_7$.

Untreated Effluent Treatment by Selected Bacterial and Fungal Strains:

The untreated effluents should be toxic to the human, plant and also animals. Even presence of 5-10 ppm chromium concentration will affect the oat plants growth and 48% reduce the seed germination. As per chromium limitation standards there should be only 0.1 to 2 mg/L in water sources both for human usage of for agriculture. The plants have only up to 0.02-2ppm of their dry mass. So, in this study untreated effluent was treated by using identified indigenous effective bacteria and fungi under bioremediation process.

In order to study the microbial mechanism behind chromium reduction the procedure as described below was followed.

After two weeks incubation period, the 2 ml effluents of 10 ml inoculum size treatment batch were centrifuged to pellet out the cells. The microbial cell pellet were again suspended in distilled water and the suspension was subjected to gentle mechanical agitation for 15 minutes and then centrifuged to perform the Chromium VI assay in the supernatant. The assay result will indicate the presence of adsorbed chromium to microbial surface, the microbial pellets were homogenized and diluted in 2 ml distilled water and assayed for chromium VI presence intracellular within microbes.

Molecular identification:

To confirm the identities of the isolates, PCR amplification and sequencing of the 16S rRNA gene were performed. The 16S rRNA genes were PCR-amplified from the genomic DNA using the bacterial universal primer set of F (5'-AGAGTTTGATCCTGG CTCAG-3') and R (5'-GGCTACCTTGTTACGACTT-3'), which were also used for sequencing. The thermal cycling program was as follows: initial denaturation at 95°C for 5 min, which was followed by 30 cycles of denaturation at 95°C for 30 sec, extension at 72°C for 90 sec, and a final extension at 72°C for 10 min. The amplified PCR products were analyzed by 0.8% (w/v) agarose gel.

The acquired sequences were used for a gene homology search, with the 16S rDNA sequences available in the public databases from BLAST and were identified to the generic level. Using the CLUSTAL-X Multiple Sequence Alignment Program, the 16S rDNA sequences of the isolated strains were aligned with sequences of related organisms obtained from GenBank (Joo *et al.*, 2007).

Results

Characterization of Tannery Effluent:

Sample Analysis:

In every step of tanning process a considerable amount of waste water is released. The waste water was found to contain salts, fat, protein and preservatives for chromium salts, soaking, lime, ammonia and sulphides for fleshing, trimming and bating and polyphenolic compounds for tanning and solvent chemicals with metals for wet finishing.

Isolation and Identification of Microorganisms from Effluent:

The number of colonies in each plate differed according to the serial dilution. The numbers of colonies grown in higher dilution plates were low when compared to the lower dilutions. The 68 isolates were identified in effluent sample growing up to 500ppm of chromium concentration plates. The bacterial isolates were named as CRB-1 to CRB-68(CRB - Chromium Resistant Bacteria). Based on colony morphology the colonies were differentiated, selected and subcultured. Using Biochemical characterization following strains were identified: Bacillus spp, Micrococcus spp, Staphylococcus aureus, Escherichia coli, Enterobacter spp.and Pseudomonas aeruginosa. The 23 fungal colonies were isolated in Sabouraud Dextrose Agar. The fungal strains were named as CRF-1 to CRF-23 (CRF -Chromium Resistant Fungi). All the fungal colonies obtained were grouped based on the colony

morphological. The fungal isolates were identified based on the colony microscopic and macroscopic observation. The identified fungal isolates were *Aspergillus fumigatus, Aspergillus niger, Penicillium* spp., *Mucor* and *Fusarium* spp.

Screening of Cr (VI) resistant Bacteria and Fungi:

Cr (VI) resistant bacteria were isolated on the basis of growth in Cr (VI) enriched medium. The bacterial counts in chromium amended nutrient agar plates were observed and the results were recorded. Strains CRB-23, CRB-36, CRB- 49 and CRB- 63 showed maximum resistance to chromium up to 500mg/L and strains CRB-7, CRB-19, CRB-23, CRB-32, CRB-36, CRB-49, CRB-54, CRB- 61 and CRB-63 showed maximum resistance to chromium up to 400mg/L respectively.

Similarly, 23 fungi isolates were screened by the ability of chromium concentration broth ($K_2Cr_2O_7$) amended with the Sabouraud Dextrose agar. The fungal strains were grown on the 500 ppm of chromium concentration. Strains CRF-12, CRF-17 and CRF-21 showed maximum resistance to chromium up to 500mg/l and strains CRF-3, CRF-8, CRF-12, CRF-17 and CRF-21 showed maximum resistance to chromium up to 400mg/l respectively.

Standardization of Chromium(VI)

Chromium (VI) was determined by UV spectrophotometer in 540 nm. And the OD (Optical Density) values were plotted for Standards of Cr (VI) prepared using Potassium dichromate.



Figure 1 Standardization of Chromium (VI).

Optimization of Physical and Chemical Parameters for Bacteria and Fungi:

Effect of various carbon sources:

The results of 2ppm and 4ppm treatments did not show any significant statistical difference between the independent variables namely sugars. The results of 6ppm treatments with statistically significant differences were considered for interpretation. The chromium (VI) concentration reduction was carried out by bioremediation process by using various carbon sources. Among the four carbon sources namely starch, glucose, dextrose and sucrose, it was noted that *Bacillus amyloliquefaciens* best utilized starch and *Aspergillus fumigates* utilized sucrose for obtaining maximum Cr (VI) reduction. But the maximum concentration reduction of Cr (VI) was done by *Aspergillus fumigatus* (45%) when compared to the *Bacillus amyloliquefaciens* (31%).

Optimization of Physico Chemical Parameters of Bacillus amyloliquefaciens



Effect of various carbon sources:

Effect of various nitrogen sources:

The chromium (VI) reduction was carried out by bioremediation process by using various carbon sources. Among the four nitrogen sources Beef extract, Malt extract, peptone and yeast extract. Peptone was best utilized by *Bacillus amyloliquefaciens* and yeast extract was best utilized by *Aspergillus fumigatus* with the maximum Cr (VI) reduction. But the maximum reduction of Cr(VI) was done by *Aspergillus fumigatus* (35%)when compared to the *Bacillus amyloliquefaciens* (25%).



Effect of various Nitrogen sources: Bacillus amyloliquefaciens

Effect of various Temperature:

Among the three T^o, 35^oC and 30^oC were best utilized by both *Bacillus amyloliquefaciens* and *Aspergillus* *fumigatus* with the maximum Cr (VI) reduction. But the maximum reduction of Cr (VI) was done by *Aspergillus fumigatus* 75% when compared to the *Bacillus amyloliquefaciens* 55%.

Effect of various Temperature - Bacillus amyloliquefaciens



Effect of various Temperature - Aspergillus fumigatus



Effect of various pH:

The chromium (VI) reduction was carried out by bioremediation process by using various pH. Among the three pHs, 6, 7 and 8the latter two were best utilized by both pH7 *Bacillus amyloliquefaciens* and

pH 6 was preferred by *Aspergillus fumigatus* for maximum Cr (VI) reduction. But the maximum reduction of Cr (VI) was done by *Aspergillus fumigatus* when compared to the *Bacillus amyloliquefaciens*

Effect of various pH - Bacillus amyloliquefaciens



Effect of various pH - Aspergillus fumigatus



The optimized parameters were applied during mass cultivation of bacterial and fungal strains in appropriate medium. After overnight cultivation the cells were harvested using centrifugation and suspended in 20 ml of sterile distilled water and used as inoculums. The number of cells was estimated using viable plate counts. In the bacterial inoculum, total count of bacteria were found to be 20×10^3 cells/ml. Total colony forming unit of fungi were found to be 1000 number in the overnight grown culture used as fungal inoculum.





Figure 7 Untreated effluent sample treated by using Bacterial biomass

1ml inoculums/100 ml effluent; 5ml inoculums/100 ml effluent; 10 ml inoculums/100 ml effluent



Figure 8 Untreated effluent sample treated by using fungal biomass

1ml inoculums/100 ml effluent; 5ml inoculums/100 ml effluent; 10 ml inoculums/100 ml effluent

Untreated effluent with chromium of 180 ppm was used as the chromium rich growth substrate for bacteria. The initial Cr (VI) concentration of 180 mg/ml was reduced to 0, 10 and 54 mg/ml (100%, 94% and 64%) on 14^{th} day with the initial bacterial inoculums load of 3×10^4 (30,000), 1.5×10^4 (15,000) and 3×10^3 (3,000) CFU/ ml of untreated effluent respectively.

The chromium reduction study was carried out using selected fungal strain in pooled untreated effluent with initial Cr (VI) concentration of 180 mg/ml. The chromium was reduced to 2, 6 and 36 mg/ml (96%, 96% and 80%) on 14^{th} day with the fungal inoculums load of 1×10^4 , 5×10^3 and 1×10^3 CFU/ ml of untreated effluent respectively. It was noted that Chromium concentration reduction percentage was microorganism dose dependent. It was also noted that in 10ml inoculum of both treatments though 100%

reduction was achieved, in fungal treatment an increasing trend in chromium concentration was observed in a couple of days.

Sequence analysis:

The 16S rRNA sequence obtained from PCR amplification of the gene that encodes the ribosomal RNA universal primers F using (5'-AGAGTTTGATCCTGG CTCAG-3') and R (5'-GGCTACCTTGTTACGACTT-3') were aligned to those available in the NCBI database using BLAST Multiple &CLUSTAL-X Sequence Alignment Program for comparison with other sequence and assigned the Genbank accession number for this strainCRB36 isKM282103. Isolates CRB36 was closely associated with the members of the diverse Bacillus spectrum showing sequence similarities of 99% with Bacillus amyloliquefaciens.

Phylogenetic tree analysis of Bacterial Strain



Discussion

The toxic chromium (VI) is highly mobile soluble in water and gets its way into the water bodies being released in the large amount from the tanning industries, dye industries and chrome plating effluents. The chemical treatment methods for chromium removals may not be desired from eco-friendly point of view and also that not being cost effective. The chromium (VI) content was about 180 m/l which is more than 300 times greater than the permissible limit described. Hence the present study a complete cost effective biological method was used. There had been literature showing that bacteria and fungi can be used to bring down the chromium (VI) concentration.

The effluent released from tannery industry was turbid, brown in colour and had an offensive odour. The colour of the effluent might be due to the presence of biodegradable and non-biodegradable high molecular weight organic compounds and high amount of inorganic chemicals like sodium and chromium used during the processing and the odour may be due to putrefaction of the organic residues from the processed skin and hides. The yellowish brown colour might be hindering the penetration of sunlight causing depletion in the rate of oxidation process (Zahid *et al.*, 2006).

Noorjahan 2014 had reported that the analysis of the physico chemical parameters of untreated industrial effluent was carried out. The results of the study revealed that colour of the untreated industrial effluent were blackish in colour with offensive odour. pH of the tannery effluent was found to be alkaline. The presence of high level of TSS and TDS may be due to the insoluble organic insoluble organic and inorganic present in the effluent. The present study various indigenous bacteria and fungi colonies were used to isolate from the pooled effluent before undergoing treatment process. The effluent was dark brown in colour with offensive odor. The pH is 10.5, TSS is 2300, and TDS is 12,900. Such a high tolerance of these microbial strains to elevated Cr concentration is conferred under natural condition due to horizontal dispersion of genetic information, which depends upon the physicochemical characteristics of the site of isolation.

The most suitable temperature for Cr resistant bacteria isolate was found to be 37 °C. Bacterial Cr (VI) reduction was found to be maximum at 25 to 30 °C for five bacterial isolates and higher temperature (above 37° C) severely retarded Cr (VI) bioreduction (Benedict *et al.*2008). In a similar study, the optimum pH for the growth of Cr resistant bacteria was reported as pH 7 to 7.8. But Cr forms are soluble over a wide range of pH and generally mobile in soil-water systems.

In my study, total count of bacteria were found to be 20×10^3 cells/ml. Total colony forming unit of fungi were found to be 1000 no's. The culture result showed that there was much reduction in the microbial counts than in sewage effluence indicating that presence of high concentration of chromium was inhibitory to the microbial growth. The chromium concentration was about 180 mg/l in the effluent.

Sharma and Srivastava 2013 had reported that, the pH of 8 was best suited for the metal uptake denoting the chromium VI removal as $Cr_2O_7^{-2}$ and CrO_4^{-1} . The temperature played an important role in the biosorption process. The optimum temperature was at 25- $28\pm2^{\circ}$ C. My study, the pH optimization for chromium (VI) reduction of the bacterial strain *amyloliquefaciens* and Bacillus fungal strain Aspergillus fumigatus done using minimal salt broth revealed that fungi required a lower pH value of 6 when compared to bacterial growth pH of neutral. The temperature optimization study for chromium (VI) reduction results were revealed that fungi required a

lower temperature of 30° C when compared to bacterial requirement of 35° C.

Jayalakshmi and Ramachandra 2013 his study carried out a total of 4 Chromium resistant bacteria were isolated from Tirupur Industrial Estates (TIE Park) effluent in his study. Four selected isolates according to their morphological shape were plated in media amended with 194 mg/l Cr (VI) (1Mm). Out of 4 isolates (TIE 1, TIE 2, TIE 3 and TIE 4), the maximum degradation ability was observed in TIE2 strain and was identified as *Pseudomonas sp.* For my study, 68 bacterial and 23 fungal colonies are isolated in tannery industry, Begambur, Dindigul, Tamilnadu. After 48 hrs of incubation, strains CRB-23, CRB-36, CRB- 49 and CRB- 63 and strains CRB-7, CRB-19, CRB-23, CRB-32, CRB-36, CRB-49, CRB-54, CRB-61 and CRB-63 showed maximum resistance to chromium up to 500mg/L and 400mg/L respectively. And the fungal strains CRF-12, CRF-17and CRF-21and strains CRF-3, CRF-8, CRF-12, CRF-17 and CRF-21 showed maximum resistance to chromium up to 500mg/L and 400mg/L respectively.

Cr (VI) is reduced to Cr (V) inside bacteria which are oxidized back to Cr (VI). Reactive oxygen species (ROS) are generated when Cr (VI) donates it's electron to molecular oxygen. In general, bacteria are found to employ several reductase enzymes against ROS. *Bacillus* spp. has access to different resistance mechanisms against toxic metal stress due to induced over expression of varieties of proteins (Thacker *et al.*, 2006).

The lab scale chromium (VI) reduction study was carried out using untreated effluent having 180mg/l chromium(VI) concentration of the various inoculums sizes 3000 cells/ml,15,000cells/ml and 30,000 cells/ml of bacterial strain used, it was found that the inoculums size of 3×10^4 cells/ml of untreated effluent could achieve 100% chromium(VI) reduction in 12 days incubation period with the bacterial inoculums size of 3×10^3 cells/ml of effluent up to 70% reduction was obtained at 14 days incubation period and 95 % chromium(VI) reduction was observed at 14th day with the inoculums size of 15×10^3 bacterial cells/ml of untreated effluent.

In the reduction experiment with fungi the percentage of chromium reduction was 100% at 12^{th} day with inoculums size 1×10^4 cells/ml, 90% at 14^{th} day with inoculums size of 1×10^3 cells/ml. It has to be noted that during fungal treatment the chromium (VI) concentration that reached 100% reduction with 1×10^4

CFU/ml inoculums size during 12th day of incubation slowly started to show little increased chromium (VI) concentration up to 2mg/ml at 14th day incubation period. This can be due to the release of fungal adsorbed chromium bacteria in to the effluent medium again that may be due to the disintegration of fungal mycelium. It is evidence that the mechanism of reduction of chromium (VI) concentration is different for both bacteria and fungi. Since, in bacterial treatment the 100% chromium reduction result remained constant. From this study it can be observed that both indigenous bacterial and fungal strain can be used effectively to bring down the concentration of chromium (VI) to permissible limits by using biological treatment methods alone.

Conclusion

The tannery effluent analyzed was found to contain excessively high amount of Cr-VI (180 ppm) and organic carbon content which can be hazardous if returned back to the environment untreated. The study achieved 100% present bacterial biotransformation of Chromium in tannery effluent to nontoxic forms using Bacillus spp in 12 days' time period. Though similar reduction was achieved in fungal treatment, the reversal of chromium concentration reduction in fungal treated effluent may be due to desorption phenomena where the mycelial bound chromium is released into the surrounding effluent media again. The risks of chromium VI reappearance by desorption phenomena from fungal mycelium could be avoided by applying bacterial strains for toxic chromium bioremediation process. This investigation indicates the possibility of the effective use of microbes as an eco-friendly option to overcome Chromium VI toxicity in tannery effluents.

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