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Biodegradation and Decolorization of Malachite Green and Congo Red Textile Dyes by Newly Isolated *Bacillus subtilis* strain GKRS01 from Textile Effluents

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Abstract

Water contamination from the textile industry is a major problem worldwide. Malachite Green (MG) and Congo Red (CR) dyes are commonly used in textile industries and are poisonous, mutagenic, and carcinogenic. This work intended to isolate and molecularly characterize possible MG and CR dye degrading bacteria from textile effluents. *Bacillus subtilis* strain GKRS01 (NCBI accession no. OK076699) can decolorize MG and CR dyes. Additionally, 16S rRNA sequencing validated the isolated strains' genetic differences. *B. subtilis* strain GKRS01 showed 80.64% and 94.19% decolorization of CR and MG, respectively on 100mg/1000ml at pH 7.0 and 37° C under shaking condition. The degraded products were examined across the wavenumber range of 4000–400 cm^{-1} by FTIR (PerkinElmer's Spectrum Two™ IR spectrometer). FTIR study demonstrated that *B. subtilis* strain GKRS01 decomposed hazardous Malachite Green and Congo Red dyes into non-toxic end products. The isolated *B. subtilis* strain GKRS01 showed promise in treating waste water contaminated with malachite green and congo red textile dyes.

Keywords: Malachite green, Congo Red, Dye decolorization, Biodegradation, *Bacillus subtilis*

Introduction

Rapid industrialisation prompted the development and usage of a variety of chemicals and dyes in daily life.[1] Dyes are extensively utilised in the printing, colour, textile, rubber product, and paper industries, as well as in pharmaceuticals. The chemical structure of synthetic dyes is so complex that they are extremely resistant to degradation.[2] In 1856, synthetic dye was developed.[3] Azo dyes (malachite green and congo red) are synthetic chemical compounds distinguished by their azo linkages (N=N).[4] Over 10,000 different textile dyes are available commercially on a global scale, with an estimated annual output of $7x10^5$ metric tonnes. Textile dyeing takes a lot of dye and water. The textile sector annually releases 30,000 to 150,000 tonnes of dyes into bodies of water, resulting in serious contamination.[5-6] Decolorization or detoxification of harmful azo dyes has become a global concern to safeguard the environment.[7] Toxic substances in dye effluent enter aquatic species, travel up the food chain, and eventually reach humans, causing a variety of physiological diseases such as hypertension, occasional fever, kidney damage, and cramping.[8-9]

Synthetic chemicals, unlike natural chemicals, are resistant to recycling and persist in the environment. Reverse osmosis, flocculation, coagulation, ion exchange, advanced oxidation, activated carbon adsorption, photocatalysis, photo-Fenton, electrochemical oxidation, Fenton process and filtering have all been used to breakdown azo dye from coloured textile waste. However, since these treatments are prohibitively costly and produce amine residues including sludge as a byproduct of breakdown, frequent ingestion of such untreated or inadequately treated harmful waters results in human carcinogenesis.[10-11] Scientists are working on new ways to decompose these synthetic compounds in order to protect the environment.[12]

Biotechnological techniques were used to degrade various textile dyes, and it was discovered that diverse bacteria removed up to 70% of the dye.[13] Microorganisms are critical in decolorizing and detoxifying textile dyes. Currently, a variety of approaches for the control of textile dye effluents have been developed; however, eco-friendly biological treatment methods have showed superior outcomes at a lower cost than physio chemical methods.[14] Organisms have been found to be capable of reducing dyes, including *Eubacterium* spp., *Bacteroides* spp., *Clostridium* spp., *Streptococcus faecalis*, *Proteus vulgaris*, and *Bacillus* spp.[15] In this study, 16S rDNA sequencing was used to identify the bacterial strain. The isolated bacteria were utilised to decolorize azo dyes Malachite Green (MG) and Congo Red (CR) at concentrations of 100mg/1000ml. Using FTIR methods, we also examined the

process of MG and CR biodegradation and the related metabolites/by-products. The purpose of this study is to demonstrate the dye degradation efficiency of *Bacillus subtilis* strain GKRS01 isolated from textile effluent.

MATERIALS AND METHODS

Dyes and Chemicals

Analytical grades of the dyes congo red and malachite green were obtained from Jaipur, India for use in this research. MG is triphenylmethane dye derived from {4-[(4-dimethylaminophenyl)-phenylmethylidene]-dimethyl-azanium chloride}, whereas CR is the sodium salt of benzidinediazo-bis-1-naphtylamine- 4-sulfonic acid.

Identification of Dye Degrading Bacteria

The 200 mg L^{-1} broth MSM supplemented with glucose (0.2 % w/v) was taken into conical flasks (500 mL), and spiked with malachite green and congo red dye (100 mg L^{-1}) as the sole source of carbon and nitrogen. The mixture was inoculated with 10 mL of wastewater and 1.0 g soil. The flasks were incubated at 37°C for 7 days on orbital shaker with 120 rpm. After 3rd and 7th day of incubation, cell suspensions from each flask were plated onto Nutrient Agar (NA) medium and incubated at 37°C for 24 hours. The pure culture of bacterial strain was maintained for dye degradation essay.

The dye-degrading bacterial strain was evaluated using standard biochemical methods. Gram staining was conducted first, followed by biochemical assay for several parameters (catalase, oxidase, indole test, citrate utilisation, methyl red, Voges Proskauer, triple sugar iron agar, and urease) using a 24 hours old culture of each bacterial strain. After 24 hours of incubation at 37°C, the observed colour change was used to determine whether the result was positive or negative. Bergey's Manual of Systematic Bacteriology (2005) was used to identify the unknown bacterial strain at the genus level and to confirm the presence of varying biochemical test findings for strain. The bacterial strain was also identified molecularly using 16S rDNA sequencing.

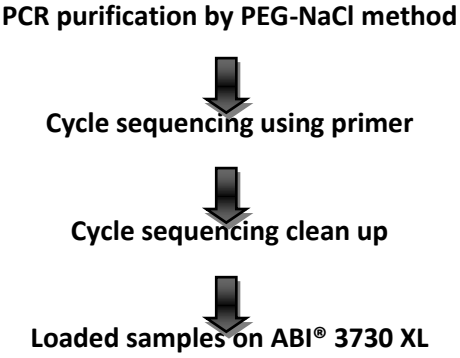
Molecular identification was also carried out at the sequencing facility of National Centre for Microbial Resource (NCMR), National Centre for Cell Science, Pune. At the facility, genomic DNA was isolated by the standard phenol/chloroform extraction method[16], followed by PCR amplification of the 16S rRNA gene using universal primers 16F27 [5'-CCA GAG TTT GAT CMT GGC TCA G-3'] and 16R1492 [5'-TAC GGY TAC CTT GTT ACG ACT T-3']. The amplified 16S rRNA gene PCR product was purified by PEG-NaCl precipitation and directly sequenced on an ABI® 3730XL automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA) as per manufacturer's instructions. Essentially, sequencing was carried out from both ends using additional internal primers so that each position was read at least twice. Assembly was carried out using Lasergene package followed by identification using the EzBioCloud database.[17]

General sequencing protocol

DNA isolation (PCR Template preparation) by Phenol-Chloroform method

PCR amplification by using 16S rRNA region primers

Check the amplification on agarose gel



The evolutionary history was inferred using Neighbor-Joining.[18] The evolutionary distances between sites are given in base substitutions per site using the Maximum Composite Likelihood[19] approach. MEGA11 was used to analyse evolution.[20]

Screening of dye degrading microorganisms

The dye degradation experiment was carried out in nutritional broth enriched with 100 mg/l Malachite green and Congo Red; the broth was then inoculated with *Bacillus subtilis* strain GKRS01 and incubated at 37°C for 7 days on an orbital shaker at 120 rpm. As controls, unsupplemented flasks were employed.

Decolorization assay

The degree of colour reduction was determined using an absorbance measurement at λ_{max} = 497 nm using a UV/VIS spectrophotometer. After 15 minutes of culture centrifugation at 5000 rpm, the supernatants were tested for absorbance changes. The biodegradation test findings were represented as a percentage of decolorization, which was calculated using the following formulation:[21]

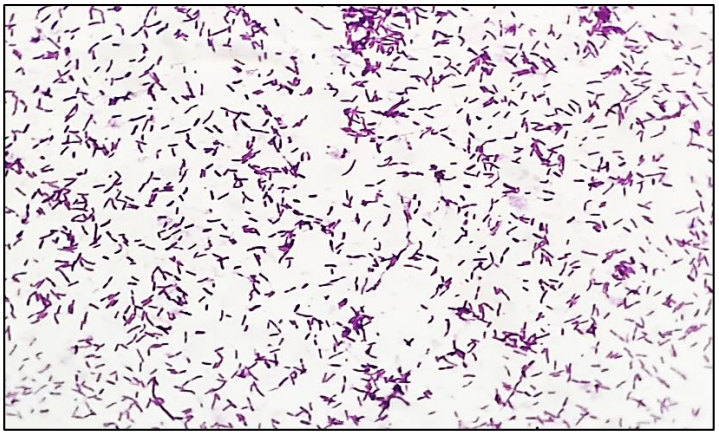
$$\boldsymbol{Decolorization}\ (\%) = \frac{\mathbf{(A0-A1)}}{\mathbf{A0}}\ \boldsymbol{X}\ \mathbf{100} \text{.....(i)}$$

A0: dye absorbance before decolorization and, A1: dye absorbance after decolorization.

The biodegraded malachite green and congo red were characterised by FTIR using PerkinElmer's Spectrum Two™ IR spectrometer and compared to a control dye in the 400–4,000 cm^{-1} mid-IR region with a 16-scan speed. The samples (Control and degraded) were mixed with spectroscopically pure KBr in the ratio of 5:95, pellets were fixed in sample holder, and the analyses were carried out. Changes in the percentage of transmission at different wavelengths were reported.

RESULTS AND DISCUSSION

The purpose of this work was to isolate and molecularly characterise dye degrading bacteria from textile effluents. The isolated dye degrading bacteria were identified based on morphological, biochemical (Photograph 1; Tables 1) and molecular characterization using the standard protocol. The sequence was further used for BLAST analysis from NCBI database, which showed 99% similarity with *Bacillus* spp. And identified as *Bacillus subtilis* strain GKRS01 with NCBI accession no. OK076699. Azo dyes are extensively employed in a broad variety of industries. Numerous bacteria have been demonstrated to cleave these azo dyes reductively. The search for microorganisms capable of degrading the azo dye began in 1970, with the identification of three strains: *Bacillus subtilis, Aeromonas hydrophila,* and *Bacillus cereus.*[22]



Photograph 1: Gram-stained microscopic photographs of dye degrading bacteria under 100 x magnifications

Table 1: Morphological and Biochemical Characteristics shown by *Bacillus subtilis*

Test	Result
Gram's Staining	+
Shape	Rod
Motility	Motile
Catalase	+
Indole Production	-
Methyl Red	+
Voges-Prausker	+
Oxidase	-
Citrate utilization	+

In the phylogenetic tree (Figure 1), constructed by MEGA11, a relationship between bacterial strains was shown.

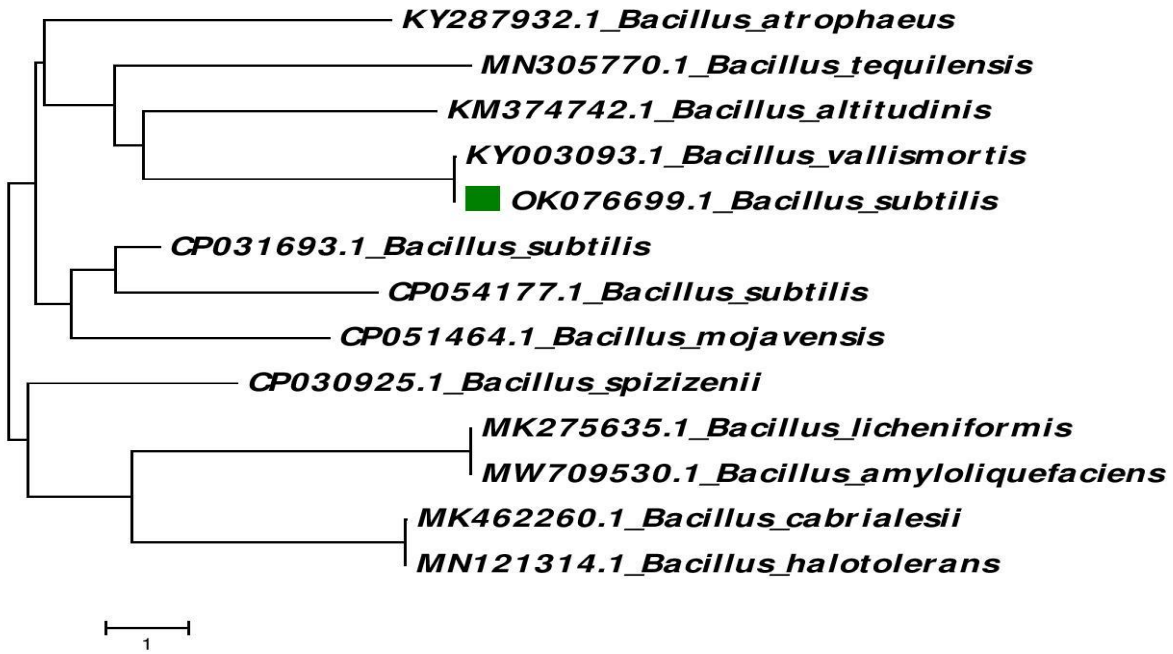


Figure 1: The phylogenetic tree depicts the evolutionary connection between the isolated bacterial strain identified using 16S r-DNA sequencing

Since waste of textile industries is full of the mixture of various dyes, so the ability of the *B. subtilis* strain GKRS01 was studied to decolorize the malachite green and congo red. The rapid decolorization was observed within 72 hours includes congo red and malachite green by 80.64% and 94.19% decolorization respectively in 100mg/1000ml concentration at pH 7.0 and Temperature 37° C under shaking condition. Kumar and Sawhney[23] discovered that *Bacillus subtilis* (RA 29) decolorized Congo red 95.67% at 37°C. Ashri[24] reported that a bacterial consortium decolorized 98% of the Congo Red dye at a concentration of 100 mg/L at a pH of 7 and a temperature of 37°C. Isik and Sponza[25] revealed the decolorization efficiencies for Congo Red degradation over 9 days of the incubation time introduced into nutrient broth with *E. coli* under anaerobic conditions. The greatest decolorization was discovered 98% under anaerobic settings whereas 30% and 39% colour removal efficiencies were attained under aerobic and microaerophilic conditions, respectively.

FTIR spectrum of control malachite green and congo red showed number of peaks in the fingerprint region (1,500–500 cm^{-1}) (Figure 2a and 3a).

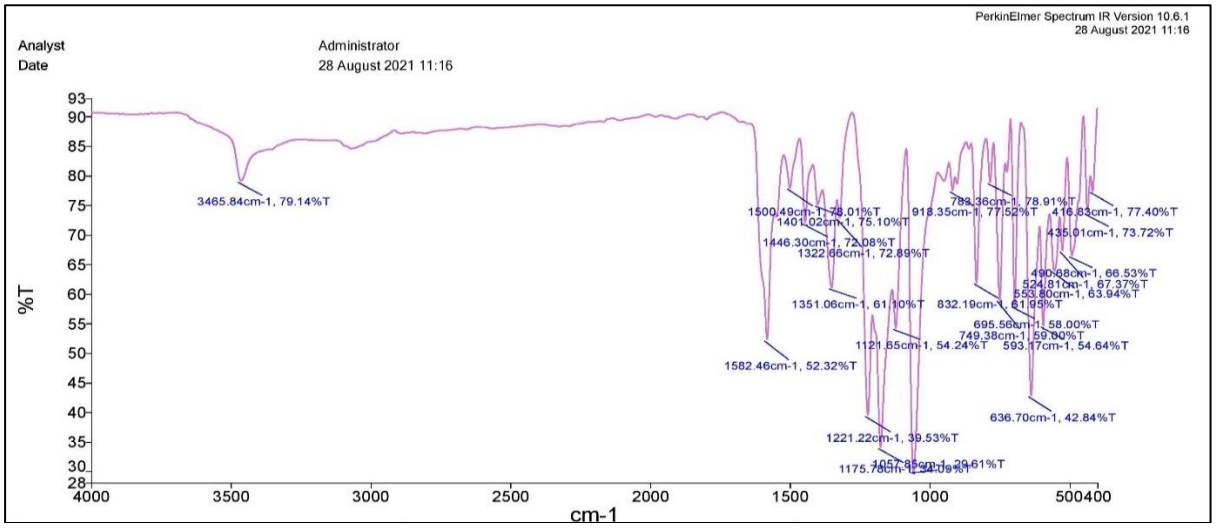


Figure 2a: FTIR analysis of Congo Red before degradation

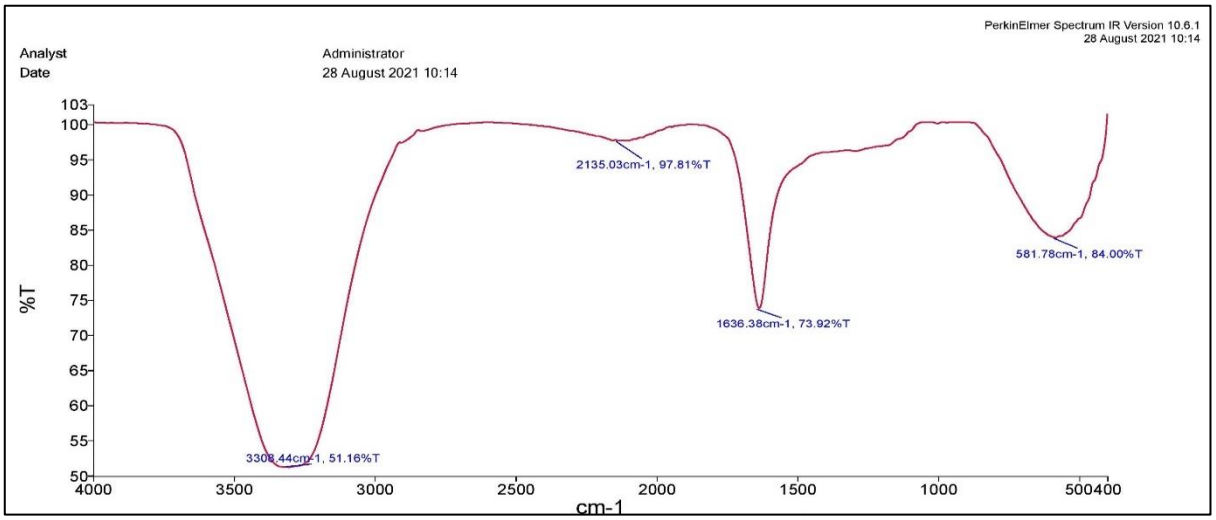


Figure 2b: FTIR analysis of Congo Red after degradation by *Bacillus subtilis* strain GKRS01

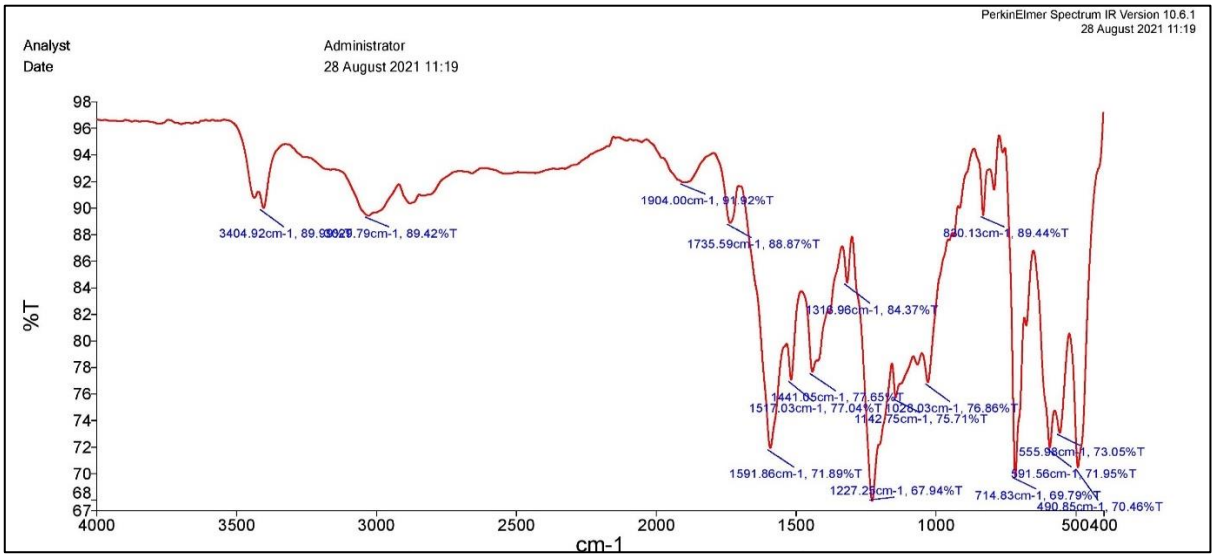


Figure 3a: FTIR analysis of Malachite Green before degradation

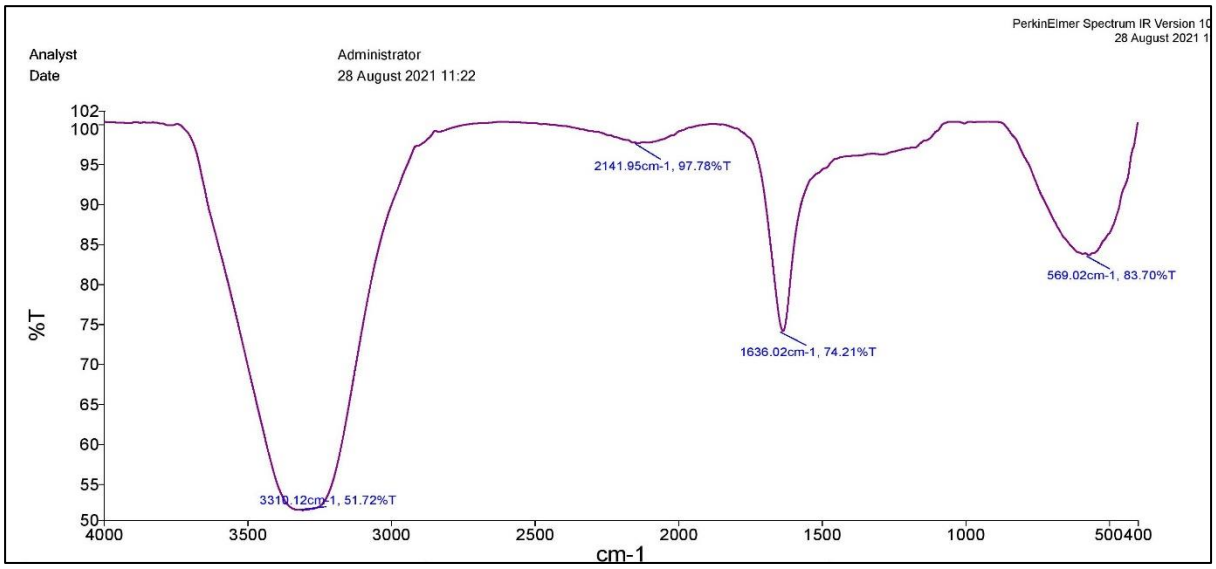


Figure 3b: FTIR analysis of Malachite Green after degradation by *Bacillus subtilis* strain GKRS01

The results of FTIR analysis revealed the reduction of the spectra peaks (Figure 2b and 3b). Appearance of some new peaks and absence of important peaks required for structural integrity of the dyes have been observed in the FTIR analysis of the metabolites produced after decolourization. Bacterial degradation of azo dyes in aerobic/anaerobic settings might occur either at intracellular or extracellular level. These enzymes gratuitously degrade azo dyes owing to their nonspecific nature.[26] These findings show that *Bacillus subtilis*'s effectiveness may be used to breakdown textile dyes comprising a variety of chromophore groups.

CONCLUSION

The potential of *Bacillus subtilis* strain GKRS01 to decolorize MG and CR, followed by degradation, was investigated. *B. subtilis* strain GKRS01 had the maximum decolorization effectiveness of 80.64% and 94.19% decolorization of congo red and malachite green, respectively in the presence of glucose as co-substrate. According to biodegradation studies of CR, the process seemed to be complete without the formation of aromatic amines as secondary pollutants. Analytical degradation investigations demonstrated that dye degradation leads in the generation of non-toxic metabolites that are naturally occurring in the environment.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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