Journal of Chemical Health Risks

www.jchr.org

JCHR (2023) 13(6), 2906-2913 | ISSN:2251-6727

Biotransformation of a Textile Azo dye Acid Yellow 25 by *Marinobacter gudaonensis AYAF-130*

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(Received: 07 October 2023 Revised: 12 November Accepted: 06 December)

KEYWORDS

Marine Bacteria, Acid Yellow 25, Decolorization, Degradation, COD reduction.

ABSTRACT:

Biotransformation is the process of altering an organic compound's form to reduce its toxicity and persistence. A marine bacterium capable of degrading the textile azo dye Acid Yellow 25which was isolated from natural environments on a nutrient medium containing high salinity and the dye. The isolate AYAF-130 was obtained and was identified as Marinobacter gudaonensis AYAF-130 based on biochemicals and phylogenetic analysis based on 16s rRNA gene sequence. The isolate was able to decolorize the dye up to 98% in a nutrient medium and up to 100% in the presence of different co-substrates. The degradation of the dye was confirmed by the GC-MS analysis. The isolate reduced the COD of the dye up to 65%.

I. Introduction

The process of changing an organic compound's form from one to another to lessen its toxicity and persistence is known as biotransformation. A wide variety of microorganisms and their byproducts, including bacteria, fungi, and enzymes, support this process. Water is a huge resource on earth. Of all the water resources on earth, only 3% of it is not salty and two-thirds of fresh water exists in the form of glaciers and ice caps. Color present in the industrial effluent gives a direct indication that the water is polluted. Hence color is the first contaminant recognised in the textile effluent and it has to be removed before discharging into large bodies [1]. Textile industries are usually located in places near the sea, mainly for easy overseas transportation. However, the toxic effluents released by the industries cause a great challenge to marine life. Textile dyes profoundly disturb the marine ecosystem, as they undergo chemical and biological changes. Their breakdown products might also be toxic to some aquatic organisms. Biodegradation causes the mineralization of dyes to simpler inorganic compounds that are not lethal to life forms [2].

The basic step in the decolorization and degradation of azo dyes is a breakdown of azo bonds, leading to the removal of color and toxicity of the dye. The dye wastewater can be treated by physicochemical, biological, and microbiological methods. The major disadvantage of physicochemical methods has been largely due to the high cost, low efficiency, limited versatility, and interference by other wastewater constituents [3,4]. Traditional wastewater treatment technologies have proven to be markedly ineffective for handling wastewater from synthetic textile dyes because of the chemical stability of these pollutants [5]. Also, these techniques generate a huge volume of sludge and cause secondary pollution due to the formation of sludge and hazardous by-products [6]. The use of microorganisms for the removal of synthetic dyes from industrial effluents offers considerable advantages; the process is relatively inexpensive, the running costs are low, and the end products of complete mineralization are not toxic [5].

Thus, biodegradation is a promising approach for the remediation of synthetic dye wastewater because of its cost-effectiveness, efficiency, and environmentally

Journal of Chemical Health Risks

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JCHR (2023) 13(6), 2906-2913 | ISSN:2251-6727

friendly nature [7-9]. As the best alternative, much interest is now focused on biodegradation of dyes [10,11]. This study deals with the biological decolorization and biodegradation of a textile azo dye Acid Yellow 25 by *Marinobacter gudaonensis AYAF-130.*

II. Materials and Methods

a. Collection of samples

Soil samples from salterns (Saltpan), areas near waste disposal sites of the textile industry, sewage, sludge from effluent treatment plants (ETP), marine water, and compost were collected as the source of microorganisms; these soil samples were kept in a container and refrigerated till use.

b. Acclimatization and isolation of microorganisms

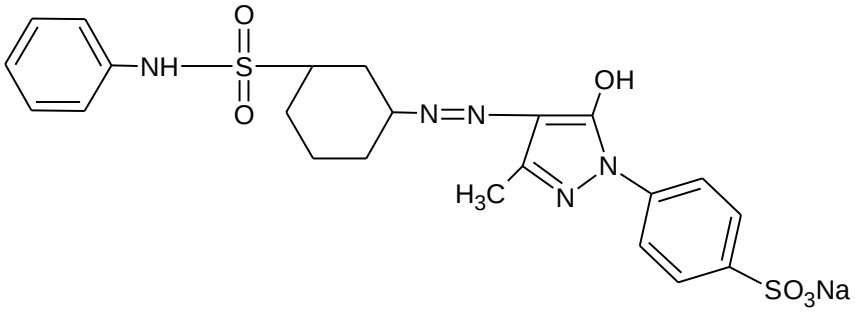
For the isolation of microorganisms capable of decolorizing the dye soil was used as a source of microorganisms. The soil samples were subjected to enrichment using nutrient broth amended with increasing concentrations of NaCl (5%-20.0%) and of dye (10µg ml^{-1} to 1000µg ml^{-1}) with an incubation time of 24 hours at 37℃. Repeated transfers were carried out to isolate stable dye decolorizing strain and the isolated strain was stored at 5℃ for further use. The high decolorizing bacteria were screened by performing a decolorization assay with the dye using UV- VIS spectrophotometer (Systronics-106 model) at its respective λ max 392nm and designated isolate AYAF-130 and used for further studies.

c. Determination of biodegradation activity

The isolate AYAF-130 was inoculated in a nutrient medium containing the dye acid yellow 25 (figure 1) at a concentration of 1000µg ml^{-1} and incubated at 37℃. An aliquot of 5 ml was removed after different time intervals. The aliquot was centrifuged at 10,000 rpm for 20 minutes to remove the cell mass. The supernatant was then used to investigate the decolorization of the dye by observing the change in the absorbance at the maximum absorption wavelength (λ max) 392nm on a spectrophotometer.

Dye Name - Acid Yellow 25. (CI No. 18835) λmax 392nm

Structure



Properties

Molecular Formula	: $C_{22}H_{24}N_5NaO_6S_2$
Formula Weight	: 541.575629
Composition	: C (48.79%) H(4.47%) N(12.93%) Na(4.24%) O(17.73%) S(11.84%)

Figure 1: Properties of the dye

i. Percent decolorization in nutrient broth, half (½) strength nutrient broth

Isolate AYAF-130 was used to inoculate in 20 ml nutrient broth (peptone:1.0g, NaCl:0.5g, beef extract:0.3g, distilled water: 100 ml) containing 6.5% NaCl and 1000µg/ml concentration of dye. These tubes were then incubated at ambient temperature for 24 hours and observed for decolorization of the dye. In addition, half-strength nutrient broth (peptone: 0.5g, NaCl: 0.25g, beef extract: 0.15g, distilled water100 ml) was also used to test for the ability of isolate AYAF-130 to decolorize the dye Acid Yellow 25 with the same NaCl and dye concentration.

Journal of Chemical Health Risks

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JCHR (2023) 13(6), 2906-2913 | ISSN:2251-6727

ii. Percent Decolorization in the presence of different Co-substrates

The isolate was supplemented with different carbon sources and nitrogen sources *viz.* 1% glucose, 1% yeast extract, and 1% starch. The effect of these sources on the decolorization was then observed.

iii. Percent Decolorization in Cell-Free Extract

The cells of isolate AYAF-130, grown in nutrient broth containing 6.5% NaCl were separated by centrifugation using a cooling centrifuge (BIO-LABS 165-R) at 8000 rpm for 20 minutes at 4℃. The pellet was then suspended in 50mM phosphate buffer pH 7.2. The cell suspension in the buffer was properly cooled and lysed by using an ultra-sonicator (Sonic-Vibra Cell System-130) the output was kept at 50amp with 6 strokes of 25s each, time interval kept was 2 minutes at 4℃. This homogenate was centrifuged at 10,000 rpm for 10 minutes to separate the cell debris from the intracellular enzymes. The supernatant was used as a crude intracellular enzyme source. The supernatant containing the crude enzyme was then added with 1000µg/ml concentration of dye solution and observed for dye decolorization. The percent decolorization studies were monitored by using a spectrophotometer.

The percent decolorization of the dye by the isolate was determined by the following formula,

$$\text{Decolorizatin (\%)} = \frac{A_0 - A_t}{A_0} \times 100$$

Where,

A_0 = Absorbance of the blank (dye solution).

A_t = Absorbance of the treated dye solution.

c. Percent COD reduction Studies

The percent COD reduction value of the dye decolorized in nutrient broth by isolate AYAF-130 was calculated by COD analysis using $K_2Cr_2O_7$ as a strong oxidizing agent under reflux conditions.

d. GC-MS Analysis

Degradation of the dye Acid Yellow 25 by the isolate AYAF-130 was confirmed by GC-MS analysis. The samples for GCMS were prepared by extracting the degraded products in Di-Chloro Methane (DCM). The decolorized broth was centrifuged at 10,000 rpm for 20 minutes. The supernatant was decanted and collected in a separating funnel. An equal amount of DCM was added to the separating funnel containing the supernatant. The funnel was shaken vigorously for 20 minutes to extract the products in DCM. The separating funnel was kept undisturbed for the separation of the solvent phase and the liquid phase. The separated solvent was then taken out from the funnel. The products that were extracted in the solvent were concentrated in the vial by evaporation of the solvent at room temperature. This was then analyzed by Gas chromatography and Mass spectroscopy (GC-MS). The GC-MS analysis of metabolites was carried out using a Shimadzu 2010 MS Engine, equipped with an integrated gas chromatograph with an HP1 column (60 m long, 0.25 mm id, non-polar). Helium was used as carrier gas at a flow rate of 1 ml minutes^{-1}. The injector temperature was maintained at 280℃ with oven conditions as 80℃ kept constant for 2 minutes and increased up to 200℃ with 10℃minutes^{-1} raised to 280℃ with 20℃minutes^{-1} rate.

e. Identification of the isolate PCR Amplification and sequencing of 16S rRNA gene

The 16S rRNA was determined in the National Center for Cell Sciences, University of Pune Campus, Pune. Genomic DNA isolation of isolate was carried out using Qiagen DNA isolation kit as per the manufacturer's instruction. Its presence was checked by running in agarose gel (0.8%) stained with ethidium bromide.

PCR amplification of 16S rDNA from all the strains was performed using 16S rDNA specific universal oligonucleotide primers 16F27N (5'-AGA GTT TGA TCM TGG CTC AG-3') and 16R1488 (5'-CGG TTA CCT TGTTAC GAC TTC ACC-3') hybridizing respectively at positions 8-27 and 1488-1511 relative to *E. coli* 16S rDNA numbering (12). The PCR reactions were carried out in a PE 9700 thermal cycler (Perkin Elmer, USA). The amplification conditions were: initial denaturation at 94℃ for two minutes, followed by 35 cycles of denaturation at 94℃ for one minute, annealing at 55℃ for one minute, and extension at 72℃ for one minute, and final extension at 72℃ for 10 minutes [13]. PCR was carried out in a 25µl reaction mixture consisting of 10 x *Taq polymerase* buffer Bangalore Genei, Bangalore, India), 2mM dNTPs, 10 pM primers, 1-unit *Taq polymerase* (Bangalore Genei, Bangalore, India), and 10ng DNA. The PCR amplification products were checked on 1% (weight/volume) agarose gels. The PCR product was purified by PEG-NaCl precipitation [15]. Briefly, the PCR product was mixed with 0.6 volumes of PEG-NaCl solution (20% PEG 6000, 2.5 M NaCl) and incubated for 10 min at 37℃. The precipitate was collected by centrifugation at 12,000 rpm for 10 minutes. The pellet was washed twice with 70% ethanol

Journal of Chemical Health Risks

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JCHR (2023) 13(6), 2906-2913 | ISSN:2251-6727

and dried under vacuum, which was then resuspended in glass distilled water at a concentration of >0.1 pmol/ ml. Purified products were sequenced on both strands on an AB 3730 DNA analyzer using the Big Dye terminator kit (Applied Biosystems, Inc. Foster City, CA). Internal primers used were 16S-704F-5'GTAGCGGTGAAATGCGTAGA3'; 16S-907R-5' CCGTCAATTCMTTTGAGTTT3'; 16S-355F-5' GGCGGACGGGTGAGTAAT3. The sequence was deposited to the European Bioinformatics Institute (EBI).

The sequence was analyzed at the Ribosomal Database Project (RDP-II) (http://rdp.cme.msu.edu/) for closed homology. The sequences downloaded from the RDP II database were aligned by using CLUSTAL X2 multiple sequence alignment tools. The Phylogenetic tree was constructed by the neighbor-joining method using Kimura-2-parameter distances in MEGA 4.0 [16].

f. Phylogenetic analysis and sequence alignment

Initially, the 16S rRNA gene sequence was analyzed at the NCBI server (http://www.ncbi.nlm.nih.gov) using BLAST (blastn) tool and corresponding sequences of homologous species were downloaded and used for phylogenetic analysis. The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) was shown next to the branches (Felsenstein 1985). The phylogenetic tree was linearized assuming equal evolutionary rates in all lineages. The clock calibration to convert the distance to time was 0.01 (time/node height). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [15] and were in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA4 [16].

III. Results

a. Screening and Identification of the isolate

One isolate was selected and designated as AYAF-130 showing the zone of decolorization on nutrient agar containing 6.5% NaCl and 1000µg/ml dye. The isolate was a Gram-negative, highly motile rod. Colonies on nutrient agar containing 6.5% NaCl and dye were transparent and circular in shape. Based on biochemical tests and 16S rRNA analysis, the isolate was identified as *Marinobacter gudaonensis AYAF-130.* The phylogenetic tree was constructed using MEGA 4.0 (figure 2).

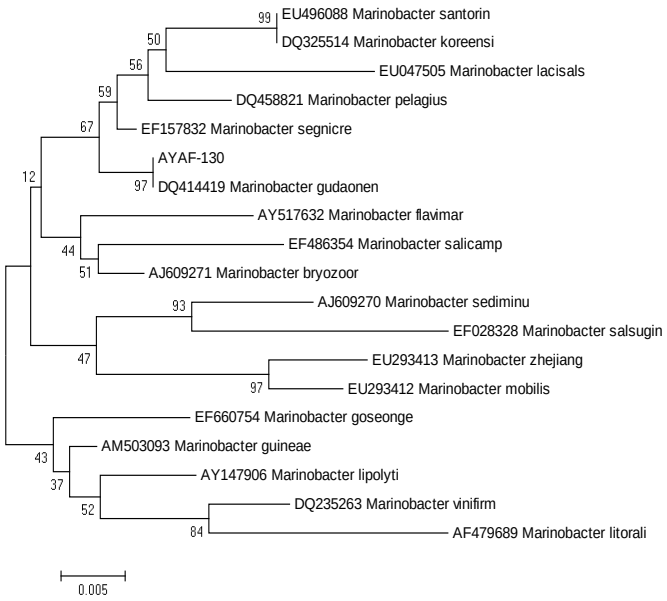

Figure 2: Neighbor-joining tree showing the estimated phylogenetic relationships of the isolate AYAF-130 and other closely-related strains of the genus *Marinobacteria*. Bootstrap values out of 1000 are given at the nodes.

b. Decolorization studies

Journal of Chemical Health Risks

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JCHR (2023) 13(6), 2906-2913 | ISSN:2251-6727

The decolorization capacity of a microorganism can be tested by examining its potential to degrade various dyes [31].

i. Decolorization studies in nutrient broth, half (½) strength nutrient broth, and in the presence of co-substrate

The decolorization was conducted with the Acid Yellow 25, supplemented with nutrient broth having 6.5% NaCl and dye at 37℃. So as depicted in Table 2 and Table 3, *Marinobacter gudaonensis AYAF-130* showed the decolorization of the dye Acid Yellow 25 to a greater extent in the presence of 1% Co-substrates was slightly higher than that in its absence. Also, the decolorization of the dye in half (½) strength nutrient broth was slightly less.

Table 2: Percent Decolorization in Nutrient Broth, Half (½) Strength Nutrient Broth, Cell Free Extract by *Marinobacter gudaonensis AYAF-130* in 24 hrs at 392nm λ max.

	% Decolorization in		
Culture Code	**1% Glucose**	**1% Yeast Extract**	**1% starch**
AYAF-130	100	99	99. 5

Table 3: Percent Decolorization in presence of Co-substrates *Viz*. 1%Glucose, 1%Yeast Extract and 1% Starch by *MarinobactergudaonensisAYAF-130*in 24 hours at 392nm λ max.

Culture Code	**% Decolorization in Nutrient Broth**	**% Decolorization in Half Strength Nutrient Broth**	**% Decolorization in cell-free extract**	**COD Reduction**
AYAF-130	98	90.03	80.13	65

ii. Percent decolorization in cell-free extract

Microbes can acclimatize themselves to toxic wastes and new resistant strains develop naturally, which can transform various toxic chemicals to less harmful forms. The action of the cell-free extract of the *Marinobacter gudaonensis AYAF-130 to* decolorize the dye Acid Yellow 25 was observed to be more than that in the nutrient medium (Table 3).

Percent COD reduction

To evaluate the level of biodegradation of Acid Yellow 25 by *Marinobacter gudaonensis AYAF-130*, we have determined the percentage of mineralization (represented by COD removal) by measuring the initial and final organic content. 65% of COD was removed which is significant removal of COD was observed after a period of 24 hours.

Confirmation of biodegradation of dye

The GCMS analysis report showed that the dye Acid Yellow 25 was degraded by *Marinobacter gudaonensis AYAF-130* and not decolorized (Figure 3). The molecular weights of the degraded products are 65, 91, 92, 158, 168, 207, 242, 247, 66, 94, 113, 207, 237, 239, 281.

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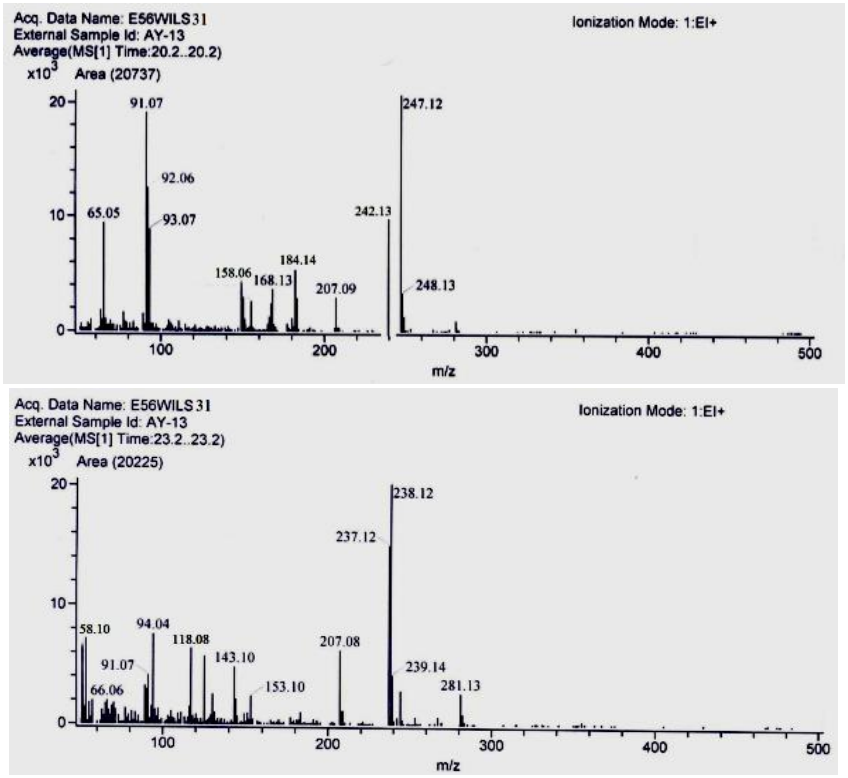

Figure 3: GCMS analysis report of isolate AYAF-130

IV. Discussion

Guo *et al*., (2008) isolated *Halomonas* strains was isolated from coastal sediments contaminated by chemical wastewater [17]. They showed that under high salt concentrations, the isolated *Halomonas sp.* decolorized five azo dyes in 24 hours with a decolorization above 90%. These results were very much similar to our results in which *Marinobacter gudaonensis AYAF-130* could decolorize the textile azo dye Acid Yellow 25 up to 98% in nutrient medium in 24 hours. Previous studies have shown that the strains of *Halomonas sp.* isolated from textile industries effluents were able to decolorize seven azo dyes-Remazol Black B, Remazol Black N, Sulfonyl Scarlet BNLE, Sulfonyl Blue TLE, Maxilon Blue, and Entrazol Blue IBC. The strains were identified as *Halomonasaquamarina, Halomonas meridian, and Halomonas salina* [18]. Halotolerant bacteria have been reported to decolorize textile azo dyes [19]. The azo dyes are degraded by bacteria using their extracellular hydrolytic and oxidative enzymes [20,21]. The *Halomonas* elongated decolorized and degraded all three azo dyes (Reactive blue 81, Reactive Red 111, and Reactive yellow 44) efficiently [22].

Due to acclimatization, this isolate was resistant to 10,000 ppm of dye concentration. Such similar findings were reported by Khehra *et al*., (2005) [23]. Since the dyes are deficient in carbon sources, it seems necessary to supplement additional carbon or nitrogen sources to assist the biodegradation of dyes by the bacterial consortium (Senan and Abraham 2004) [31]. Jadhav *et al*., (2010) pointed out that the presence of various carbon and nitrogen sources in a medium might have a stimulatory or inhibitory effect on enzyme systems involved in the decolorization [24]. Saratale *et al*., (2006) showed that consortium GR could convert and degrade the complex substrates such as extract of rice husk and rice straw, producing some volatile organic acids or alcohols (such as acetic acid and ethanol), which act as electron donors and induces the reductive cleavage of azo bonds (Yoo *et al*., 2000) [25,26]. The role of *oxidoreductive enzymes* in the decolorization of sulfonated reactive azo dyes has been characterized in *Rhizobium radiobacter MTCC 8161* (on Reactive Red 141), and *Pseudomonas sp. SUK1* on Reactive Red 2 [27,28]. A major mechanism behind the biodegradation of different recalcitrant compounds in microbial systems is driven by the biotransformation enzymes

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JCHR (2023) 13(6), 2906-2913 | ISSN:2251-6727

[25,29]. Moreover, the COD removal efficiency is better than that reported earlier as a COD reduction of 55.55 and 52% was observed for Reactive Blue 172 by *Exiguobacterium sp. RD3* and Reactive Red 2 by *Pseudomonas sp. SUK1*, respectively [27,30].

V. Conclusion

The present work focuses on the use of halophilic microorganisms to remediate the environment contaminated by azo dyes, which are extensively used in the textile industries. The presence of high salt content in the textile dye effluents hinders the development of an efficient bio-treatment system to bioremediate the textile azo dyes. This imposes a great need to develop bacterial strains that could thrive under high salt conditions. Salterns or Solar salt crystallizer ponds contain hyper-saline waters and the soil present there serves as a rich source of marine microorganisms. The bacteria isolated from such saline soil will yield a good collection of halophilic and halotolerant bacterial strains. Bioremediation has proved to be a very effective method in countering textile dye pollution in an eco-friendly way. This approach creates a promising hope to remediate the environments polluted by textile azo dyes.

The results presented here indicated that the dye Acid Yellow 25 was biodegraded in 24 hours by the isolate AYAF-130 which was identified as *Marinobacter gudaonensis AYAF-130*. The degradation of the dye was enhanced when 1% glucose was used as a carbon source. Also, the intracellular enzymes showed a considerable amount of degradation of the dye. The isolate reduced the COD of the dye to a much greater extent proving it a good agent for bioremediation. So, the isolate might play a possible role in the bioremediation of the dye-contaminated soil.

VI. Acknowledgment

We would like to offer our sincere thanks to the Staff of the Indian Institute of Technology Powai, Mumbai for GCMS analysis and National Center for Cell Sciences, University of Pune Campus for the 16s rRNA sequencing of the isolate.

VII. Conflict of Interest

The authors declare that no conflict of interest.

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