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Biodecolorization of indigo blue by *Trichoderma harzianum* native strains from Tehuacán, Puebla, Mexico

Miguel Alejandro Islas-Barrientos[1], Omar Romero-Arenas[2], María Eunice De Anda-Reyes[3], Nemesio Villa-Ruano[4], Conrado Parraguirre-Lezama[2A], Carlos Alberto Contreras-Paredes[5], Sandra Grisell Mora-Ravelo[6] and Amparo Mauricio-Gutiérrez[2B]*

Benemérita Universidad Autónoma de Puebla, [1]Posgrado en Manejo Sostenible de Agroecosistemas, [2]Centro de Agroecología, Instituto de Ciencias, Edificio VAL 1, [3]Instituto de Física, Edificio VAL 2, [4]SECIHTI, [5]Jardín Botánico, Km. 1.7 Carretera a San Baltazar Tetela, San Pedro Zacachimalpa 72960, Puebla, México, [6]Universidad Autónoma de Tamaulipas, Facultad de Ingeniería y Ciencias, Ciudad Victoria 87149, Tamaulipas, México. E-mail: *amg2510@hotmail.com

[1]ORCID: 0009-0000-9388-6901, [2]ORCID: 0000-0003-0076-3609, [2A]ORCID: 0000-0002-8510-5877, [2B]ORCID: 0000-0002-7525-9522, [3]ORCID: 0000-0003-0435-3009, [4]ORCID: 0000-0002-0787-374X, [5]ORCID: 0009-0001-8559-4957, [6]ORCID: 0000-0003-0015-4811

Abstract

In Tehuacán, Puebla, Mexico, textile wastewater contains the indigo blue dye. This research evaluated the biodecolorization capacity of indigo blue through native strains of *Trichoderma harzianum* isolated from agricultural soils impacted by the textile industry. Therefore, physical and chemical parameters were analyzed in samples of textile wastewater and agricultural soil contaminated with indigo blue. The wastewater samples showed parameters exceeding the Mexican Official Standard, such as an indigo blue concentration of 0.364 g/L, Electrical Conductivity (0.020 dS/cm), Total Suspended Solids (326.667 mg/L), Biochemical Oxygen Demand (437 mg/L), Chemical Oxygen Demand (706 mg/L), Total Kjeldahl Nitrogen (78.042 mg/L), Total Phosphorus (10.89 mg/L) and sulfides (163.758 mg/L). The contaminated agricultural soil, on the other hand, showed an indigo blue concentration of 0.083 g/L. Two fungal strains Buap Tehazo 1 and Tehazo 2 were isolated from this soil and identified as *T. harzianum* based on ITS sequences. These strains showed tolerance to 0.2 g/L of indigo blue on mineral media. Furthermore, they demonstrated the ability to biodecolorize 122.6 mg/L and 148.6 mg/L of indigo blue, respectively, transforming it into isatin, according to UV-VIS and FTIR spectroscopy analyses. These results indicate that *T. harzianum* strains biodecolorize indigo blue through an oxidative process, obtaining isatin as a reaction product.
Keywords: isatin, infrared spectroscopy, *in vitro*, bioaugmentation, agricultural soils, tolerance.

Biodecolorización de azul índigo por cepas nativas de *Trichoderma harzianum* aisladas de Tehuacán, Puebla, Mexico

Resumen

En Tehuacán, Puebla, México, las aguas residuales de origen textil contienen el colorante azul índigo. En la presente investigación se evaluó la capacidad de biodecoloración de este colorante en cepas nativas de *Trichoderma harzianum* aisladas de suelos agrícolas afectados por la industria textil. Para ello, se analizaron parámetros físicos y químicos de muestras de aguas residuales textiles y suelo agrícola contaminado con azul índigo. En las aguas residuales los parámetros estuvieron fuera de la norma oficial mexicana con una concentración de azul índigo de 0.364 g/L, Conductividad Eléctrica (0.020 dS/cm), Sólidos Suspendidos Totales (326.667 mg/L), Demanda Bioquímica de Oxígeno (437 mg/L), Demanda Química de Oxígeno (706 mg/L), Nitrógeno Total Kjeldahl (78.042 mg/L), Fósforo Total (10.89 mg/L) y Sulfuros (163.758 mg/L). En el suelo agrícola, además de medir la concentración de azul índigo que fue de 0.083 g/L se tomaron muestras, y fueron identificados dos cepas de hongos como *T. harzianum*, Buap Tehazo 1 y Tehazo 2 con base en secuencias ITS. Las cepas mostraron una tolerancia a 0.2 g/L de azul índigo en medio mineral y la capacidad de biodecolorar 122.6 mg/L y 148.6 mg/L de azul índigo, respectivamente, transformándolo en isatina, acorde al análisis de espectroscopía UV-Vis y FTIR. Estos resultados indican que las cepas de *T. harzianum* pueden biodecolorar el azul índigo con un proceso oxidativo y generar isatina producto de la reacción.
Palabras clave: isatina, espectroscopía infrarroja, *in vitro*, bioaumentación, suelos agrícolas, tolerancia.

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Introduction

The textile industry has established itself as one of the fastest-growing economic sectors globally and ranks second worldwide in terms of negative environmental impact (Bailey, Basu & Sharma, 2022; Gallego-Ramírez, Chica & Rubio-Clemente, 2022). Textile wastewater is alkaline and contains high concentrations of synthetic dyes and other chemicals that are resistant to biodegradation (Núñez-Moreno, Moreno-Albuja, Moscoso-Moreno & Velasteguí-López, 2023). In Mexico, textile-derived wastewater is reused for irrigating agricultural crops, such as in the agricultural area of San Diego Chalma, Tehuacán, Puebla, Mexico. The reuse of untreated textile wastewater alters soil fertility and damages the environment (Khan, Ahmed, Dhoble & Madhav, 2023). Additionally, these pollutants can bioaccumulate throughout the food chain, posing a risk to human health, flora, and fauna (Slama *et al.*, 2021; Gallego-Ramírez *et al.*, 2022).

It is estimated that 50% of dyes used in the dyeing process end up in wastewater, with concentrations ranging from 100 to 500 mg/L (Zaruma-Arias, Proal-Nájera, Chaires-Hernández & Salas-Ayala, 2018). Among these dyes , indigo blue stands out as one of the most persistent and widely used in dyeing denim and other textile products, and even at low concentrations, it is toxic (Castillo-Suárez, Sierra-Sánchez, Linares-Hernández, Martínez-Miranda & Teutli-Sequeira, 2023). Indigo blue in water bodies reduces light absorption, thereby affecting photosynthesis and biodiversity, which in turn impacts water quality (Francisco, Moreira & de Albuquerque, 2023).

The removal of textile dyes using conventional (physical and chemical) methods is costly and inefficient due to the high recalcitrance of these compounds (Slama *et al*., 2021). In contrast, biological processes, especially those employing dye-degrading microorganisms such as fungi, offer a sustainable and effective alternative. This is attributed to their enzymatic capacity to break down complex and recalcitrant compounds into less toxic substances (Bernal *et al.*, 2021). The genus *Trichoderma* is cosmopolitan and has been reported to biodegrade textile dyes thanks to its enzymatic machinery (including ligninases, laccases, peroxidases, among others) and its adaptability to contaminated environments (He *et al.*, 2018).

Trichoderma is used in mycoremediation processes, and its implementation could provide an effective and low-cost solution in the San Diego Chalma area, Tehuacán, Puebla, Mexico. Therefore, the objective of this study was to evaluate the indigo blue biodecolorization capacity of native strains of *T. harzianum* isolated from agricultural soils impacted by the textile industry in the study area.

Materials and Methods

Sampling

Sampling was carried out in San Diego Chalma, Tehuacán, Puebla, Mexico, located at coordinates 18.43145° N and 97.35834° W during the year 2023. The region has a warm semi-arid climate, with average annual temperatures ranging from 18°C to 22°C and annual rainfall between 200 and 1 800 mm. The predominant vegetation is xerophytic and is characterized by crops such as corn, beans, squash, and chili, among others. Samples of textile wastewater and well water used for agricultural irrigation were analyzed in accordance with NOM-230-SSA1-2002 (DOF, 2005). Additionally, composite samples of soil contaminated with indigo blue and uncontaminated soil (not affected by the textile industry) were collected according to NOM-021-SEMARNAT-2000 (DOF, 2002). Water and soil samples were stored at -4°C in containers for later analysis.

Determination of Physical, Chemical, and Biological Parameters of Water and Soil

Physical, chemical, and biological parameters in water were determined according to NOM-230-SSA1-2002: pH, electrical conductivity (EC), (dS/cm), fecal coliforms (MPN/100 mL), total suspended solids (TSS), biochemical oxygen demand (BOD), chemical oxygen demand (COD), N-nitrates, N-nitrites, total Kjeldahl nitrogen (TKN), total phosphorus (TP), sulfates, sulfides, and levels of As, Cu, and Hg (mg/L), (DOF, 2005). In soil samples, the following parameters were analyzed according to NOM-021-SEMARNAT-2000: pH, EC (dS/cm), texture (%), organic matter (OM), total nitrogen (N), (%), available phosphorus (mg/kg), cation exchange capacity (CEC, cmol(+)/kg), macronutrients (Na, Ca, Mg), (cmol(+)/kg), micronutrients (Cu, Fe, Mn, Zn), (mg/kg), and potentially toxic elements such as Pb, Cd, Cr, and Ni (mg/kg), (DOF, 2002).

Quantification of Indigo Blue in Water and Soil

The quantification of indigo blue in water and soil samples was carried out using UV-Vis spectrophotometry at a wavelength of 649 nm. A 50 mL sample of previously centrifuged water (10 000 rpm for 5 min) was diluted 1:4 with triple-distilled water and read at 649 nm using UV-Vis (Buscio, Crespi & Gutiérrez-Bouzán, 2014).

For soil, 50 g of previously sieved dry soil (mesh no. 16) were used. Aqueous extraction was performed using 1 g of soil with 13.5 mL of triple-distilled water and maintained under constant agitation at 250 rpm for 30 min. The extract was filtered through Whatman no. 1 filter paper, and the supernatant was analyzed for indigo blue using the previously described UV-Vis method.

Isolation of Indigo Blue-Tolerant Fungi

For the isolation of tolerant fungi, 1 g of soil was inoculated into Erlenmeyer flasks containing 50 mL of liquid nutrient

medium with the following composition (g/L): 7 g NH_4NO_3, 1 g K_2HPO_4, 1 g KH_2HPO_4 0.2 g malt, 1 mL micronutrients, 15 µL erythromycin (in 96% ethanol), and 0.2 g indigo blue, at pH 5.6. Micronutrient solution (g/L): 4 g $MgSO_4 \cdot 7H_2O$, 0.2 g $FeSO_4 \cdot 7H_2O$, and 0.2 g $CaCl_2 \cdot 2H_2O$. Samples were incubated at 28°C under constant agitation at 140 rpm for 7 days (Guzmán-Cedeño *et al.*, 2014). After this period, 1 mL was transferred to a new flask under the same conditions, and this subculturing was repeated twice. From the final flask, isolation was performed by inoculating 100 µL onto Petri dishes with malt-enriched mineral medium until axenic strains were obtained. The tests were performed in triplicate.

The composition of the malt-enriched mineral medium was (g/L): 20 g malt extract, 0.5 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g NH_4NO_3, 1 mL micronutrients, 1 mL erythromycin (96% ethanol), and 20 g agar, at pH 5.6.

Microscopic Identification

Morphological identification of fungi was based on colonial morphology, macroscopic growth, and microscopic appearance using the microculture technique described by Agu and Chidozie (2021), to identify reproductive structures. Fungal genera were identified by comparing microscopic characteristics using the identification guide by Barnett and Hunter (2006).

Molecular Identification and Phylogenetic Analysis

Molecular identification was carried out through amplification of the ITS regions between the small subunit (18S rRNA) and large subunit (28S rRNA), including the 5.8S rRNA, using universal primer pairs ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3'). The product was amplified as described by White et al. (1990) and sequenced at the Colegio de Postgraduados, Texcoco, State of Mexico, Mexico.

The ITS nucleotide sequences of each strain were compared using the NCBI database (National Center for Biotechnology Information; www.ncbi.nlm.nih.gov) through the Basic Local Alignment Search Tool for Nucleotide Sequences (BLASTN). Sequence alignment was performed using MEGA VII software (Molecular Evolutionary Genetics Analysis), (RRID: SCR_000025). Phylogenetic analysis was conducted using the Akaike Information Criterion model (model= TrNef) of jModelTest. Bayesian inference (BI) was performed four Monte Carlo Markov Chains (MCMC) in Mr. Bayes software v.3.2.7 (RRID:SCR_012067). A total of 752 trees were generated, and 745 were used to calculate posterior probabilities with 500,000 generations and a standard deviation of 0.000456. Figtree v1.4.4 software (RRID:SCR_008515) was used as a graphic viewer of the resulting tree (https://github.com/rambaut/figtree/). The nucleotide substitution model was ML+rapid bootstrap with the raxmlGUI 2.0 software v.2.0.8. The phylogenetic tree includes Bayesian inference support values (PP: posterior probability) and maximum likelihood bootstrap values (BS). Sequences of the three isolated fungal strains were deposited in the NCBI GenBank database with accession numbers PV707074, PV70707075, and PV707076.

Tolerance Assays of Fungal Strains

Tolerance assays for the isolated fungal strains were performed using a modified technique from Al-Jawhari (2015).

A 10 mm mycelial disc from each strain was inoculated into Petri dishes with PDA culture medium supplemented with indigo blue at different concentrations: 0 (control), 0.05, 0.10, 0.15, and 0.20 g/L. Cellophane was placed in advance to collect the mycelium. Plates were inoculated in triplicate and incubated at 28°C for 7 days. Mycelial growth was measured every 24 h. At the end of the experiment, dry biomass and spore viability were determined. The latter involved collecting spores with sterile water and inoculating 100 spores onto PDA medium at 28°C for 7 days. Germinated spores were counted every 24 h. Spore viability percentage was calculated using the equation:

$$\%PRGI = \frac{\text{Control radial growth mm-Treatment radial growth mm}}{\text{Control radial growth mm}} \text{ x } 100.$$

The percentage of radial growth inhibition (PRGI) was also determined using the equation: , according to the method of Martínez-Salgado, Andrade-Hoyos, Romero-Arenas, Villa-Ruano, Landeta-Cortés & Rivera-Tapia (2021).

Indigo Blue Biodecolorization Assays

Indigo blue biodecolorization was conducted in triplicate using the most tolerant strains BUAP TehAzo1 and BUAP TehAzo2. Three 10 mm diameter discs were inoculated into Erlenmeyer flasks containing 50 mL of liquid mineral medium with the following composition (g/L): 0.5 g NH_4NO_3, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 8 g malt, 1 mL micronutrients, and 0.2 g indigo blue, at pH 5.6. Flasks were incubated under constant agitation at 100 rpm at 28°C for 14 days; uninoculated flasks were used as controls. Indigo blue biodecolorization was measured every 24 h by UV-Vis at 649 nm, culture medium without indigo blue was used as a blank and a calibration curve of 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.15, and 0.20 g/L ($R^2 = 0.9804$). Isatin was quantified at the end of the experiment by UV-Vis in a sweep the range of 325 to 400 nm using a calibration curve (0.05 to 0.20 g/L of isatine, $R^2 = 0.9934$ at 325 nm). The degradation product isatin was confirmed by Fourier Transform Infrared (FTIR) spectroscopy (Tello-Burgos, López-Montes, Valero, Nieves & Blanc, 2022). Samples were previously filtered (Whatman No. 1) and dried at 50°C for 7 days. They were then analyzed using ATR-FTIR spectroscopy (Model Cary 360, Agilent Technologies) in the spectral range of 5 000 to 650 cm^{-1}, with a resolution of 4 cm^{-1} and 32 scans per sample. Indigo blue biodecolorization was calculated as: %Biodecolorization = [(Control - Treatment) / Control] x 100, where: Control=

concentration of indigo blue in medium without inoculating; Treatment= concentration of indigo blue in inoculated medium.

Statistical Analysis

The generated data were analyzed using the Statgraphics Centurion version 16.1 statistical software for Windows. Analysis of variance (ANOVA) and Tukey's multiple mean comparison tests were performed at a significance level of $p = 0.05$.

Results

The physical and chemical parameters analyzed in the textile-derived wastewater from San Diego Chalma, Tehuacán, Puebla, showed higher concentrations compared to well water (Table I). Indigo blue dye was quantified at a concentration of 0.364 g/L. Electrical conductivity (EC) increased by 11.1%, indicating a higher concentration of dissolved salts. Total suspended solids (TSS) increased 19 times more than the well water, revealing a higher load of suspended particles. Similarly, total Kjeldahl nitrogen (TKN) increased by 15%, the biochemical oxygen demand (BOD) was 437 mg/L, the chemical oxygen demand (COD) value was 706 mg/L, total phosphorus (TP) was quantifiable at 10.89 mg/L, and sulfide concentration was 163.758 mg/L. The quantification of potentially toxic elements (As, Cu, Hg) ranged from 0.001 to 0.100 mg/L.

On the other hand, the agricultural soils in the study area presented a sandy loam texture, organic matter (OM) ranging from 8.51–8.61%, with N values between 0.68–0.70%, P from 0.0044–0.0047%, and K at 0.041%. Soil analysis indicated a difference between control agricultural soil (irrigated with well water) and contaminated agricultural soil exposed to indigo blue due to irrigation with textile wastewater (Table II). The quantification of indigo blue in the contaminated soil was 0.083 g/L, and it was not detected in the control soil. Additionally, the pH of the impacted soil (8.18) increased by 3% compared to the control soil (7.93). As well as EC increased by 12.5% (0.18 dS/cm), and the cation exchange capacity (CEC) increased by 40% (17.5 cmol/kg), indicating that textile wastewater is causing alkalinity in the soil due to its high EC of 0.020 dS/cm. Regarding micronutrients, Fe levels were higher in the control soil than in the contaminated soil. The presence of potentially toxic elements (Pb, Cd, Cr, Ni) showed low concentrations ranging from 0.28 to 6.90 mg/kg. These results evidenced the environmental impact caused by textile industry pollution in water bodies and agricultural soils (Tables I and II).

Due to the presence of indigo blue in wastewater and its reuse for agricultural irrigation in the study area, three fungal strains were isolated: BUAP TehAzo 1, BUAP TehAzo 2, and BUAP TehAzo 5. Each strain showed a population of 10^2 CFU/g of soil on culture media supplemented with indigo blue. The macroscopic morphology of the strains showed uniform growth with a greenish color and rough mycelium. Microscopically, typical structures such as conidiophores, phialides, and conidia were observed, like those described for the genus *Trichoderma*

Table I. Water analysis for agricultural irrigation in San Diego Chalma, Tehuacán, Puebla.

Parameters		Well water	Waste water
pH		7.8	7.2
EC (dS/cm)		0.018	0.020
Fecal coliforms (MPN/100 mL)		15	93
Indigo Blue (g/L)		N.D.	0.364
TSS	(mg/L)	17	326.667
BOD		3.2	437
COD		34	706
N-Nitrates		13.082	0.680
N-Nitrites		0.012	0.010
TKN		0.500	78.042
TP		0.25	10.89
Sulfates		169.450	118.679
Sulfides		0.100	163.758
As		0.017	0.0134
Cu		0.100	0.100
Hg		0.001	0.001

N.D.: No detected; MPN: Most probable number.

Table II. Physical and chemical characterization of agricultural soils in San Diego Chalma, Tehuacán, Puebla.

Parameters	Control soil	Contaminated Soil
pH	7.93	8.18
EC (dS/cm)	0.16	0.18
Texture (%) (sand: silt: clay)	53.0: 34.0: 13.0	54.0: 36.0: 10.0
OM (%)	8.51	8.61
N (%)	0.68	0.70
Available phosphorus (%)	0.0044	0.0047
K (%)	0.041	0.041
Indigo Blue (g/L)	N.D.	0.083
CEC (cmol(+)/kg)	12.5	17.5
Secondary macronutrients (cmol(+)/kg)		
Na	3.80	3.80
Ca	39.50	40.53
Mg	5.41	6.41
Micronutrients (mg/kg)		
Cu	2.52	3.80
Fe	1118.9	40.53
Mn	26.58	6.41
Zn	9.08	1.04
Potentially toxic elements (mg/kg)		
Pb	6.90	3.40
Cd	0.28	0.38
Cr	1.99	2.3
Ni	5.84	1.32

N.D.: Not detected.

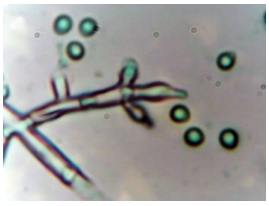
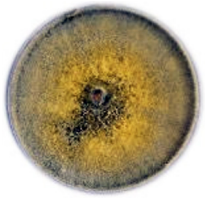
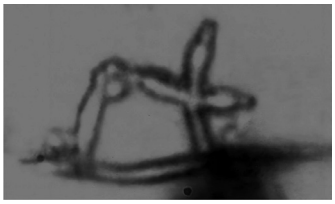
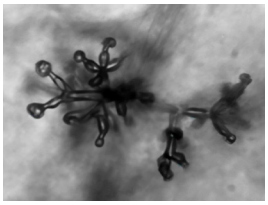
(Table III). Therefore, molecular identification through amplification and sequencing of the ITS regions confirmed the phylogenetic affiliation with the species *T. harzianum* (Figure 1).

The tolerance to indigo blue evaluated in the three *T. harzianum* strains at low concentrations (0.05 and 0.10 g/L) showed normal green and uniform mycelial growth. However, at higher concentrations (0.15 and 0.20 g/L), a reduction in mycelial growth was observed; the strains no longer showed uniform growth, and the green coloration decreased (Table IV).

At an indigo blue concentration of 0.2 g/L, strains BUAP TehAzo 1 and BUAP TehAzo 2 produced the highest spore counts (3.16 and 3.26 ± 0.001 x 10^6 spores/ml) with significant viability rates of 67.67% ± 1.528 and 66.0% ± 2.0, respectively, compared to strain BUAP TehAzo 5 ($p \leq 0.05$). Notably, BUAP TehAzo 1 maintained consistent spore production across different indigo blue concentrations, while BUAP TehAzo 2 tended to decrease as the indigo concentration increased. In contrast, strain BUAP TehAzo5 showed a significant reduction ($p \leq 0.05$) in spore production at 0.690 ± 0.010 x 10^6 spores/ml with a viability of 21.33 ± 3.215% at 0.2 g/L indigo blue. Regarding the radial growth inhibition percentage (PRGI), strain BUAP TehAzo 2 had the lowest value (20.84 ± 0.510%) at the highest indigo concentration (0.2 g/L), indicating high tolerance, whereas BUAP TehAzo 1 and BUAP TehAzo 5 showed approximately twice the inhibition (38.08 ± 0.415% and 40.99 ± 0.948%, respectively) at the same concentration. Regarding dry mycelial weight, all three strains showed progressive reductions with increasing indigo blue concentrations, more noticeably in BUAP TehAzo 5 (Table IV).

Based on the tolerance results, the *T. harzianum* strains BUAP TehAzo 1 and BUAP TehAzo 2 were selected for biodecolorization experiments due to their high capacity to grow in media containing indigo blue. BUAP TehAzo 1 showed 36.32% biodecolorization at 24 h with a residual indigo concentration of 0.1247 g/L. At 336 h, it presented

Table III. Morphological identification of fungi isolated from the study area.

Isolate	Morphology		CFU g^{-1}
	Microscopic	Macroscopic	($x10^2$)
BUAP TehAzo1			1.45±1.6
BUAP TehAzo2			0.10±0.6
BUAP TehAzo5			0.01±0.01

The values represent the mean ± standard deviation. The fungi were observed under a compound microscope (10X eyepieces, 40X objective).

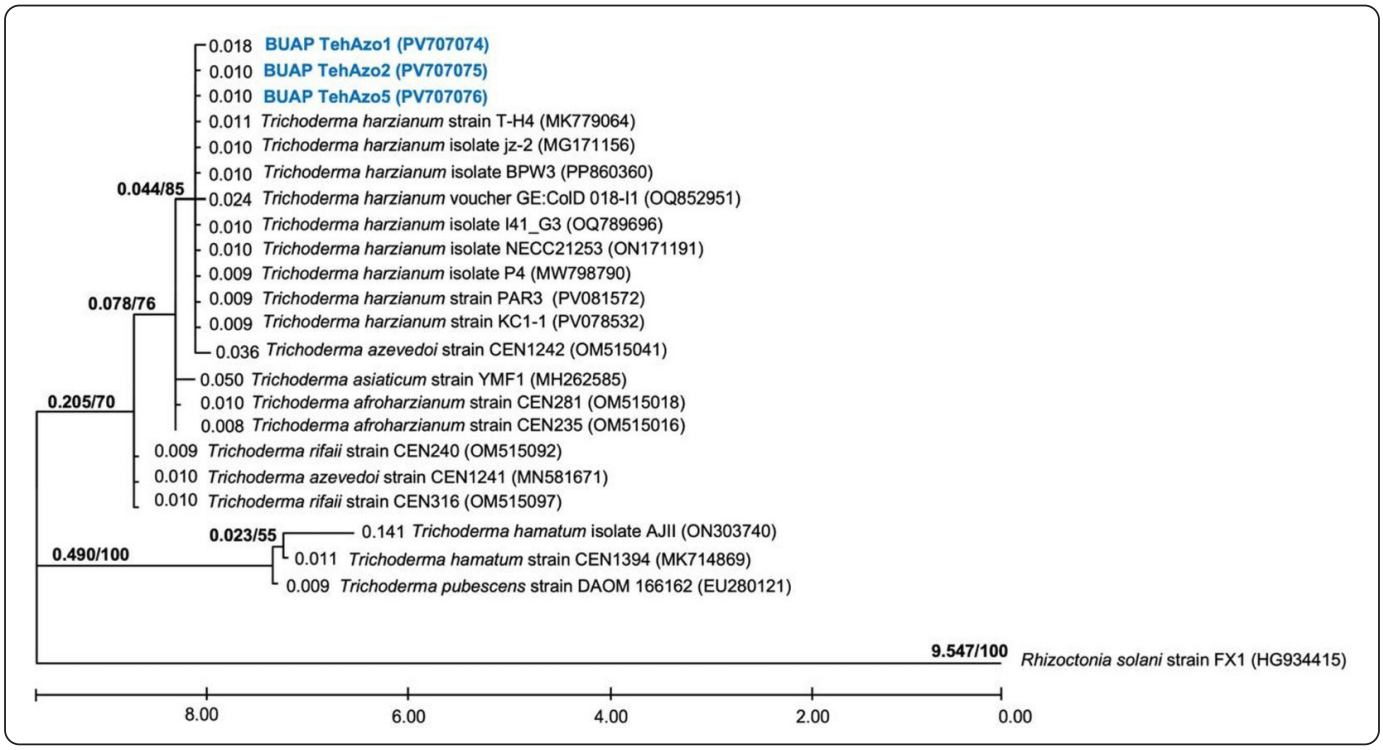


Figure 1. Phylogenetic tree based on the sequences of the ITS regions. The numbers on the nodes represent Bayesian inference (posterior probability) and maximum likelihood (bootstrap) values. GenBank accession numbers of the ITS sequences of each reference species are shown in parentheses. The bar indicates to nucleotide substitution per site.

Table IV. Evaluation of tolerance to indigo blue by native fungal strains.

Fungal strain	Indigo blue concentration (g/L)	No. Spores (x10^6 mL^{-1})	Spore viability (%)	PRGI (%)	Mycelium weight (mg)
BUAP TehAzo1	0	4.55 ± 0.050 ^{b}	100 ± 0.001 ^{a}	NA	376 ± 0.461 ^{a}
	0.05	3.21 ± 0.005 $^{f g}$	72.00 ± 2.646 ^{b}	2.65 ± 0.455 ^{a}	363 ± 0.577 ^{a}
	0.10	3.18 ± 0.005 $^{f g}$	69.33 ± 2.517 ^{b}	11.18 ± 1.290 ^{c}	296 ± 0.577 $^{a b c}$
	0.15	3.17 ± 0.015 ^{g}	68.67 ± 1.528 ^{b}	24.54 ± 2.180 ^{f}	250 ± 0.346 $^{b c d}$
	0.20	3.16 ± 0.010 ^{g}	67.67 ± 1.528 ^{b}	38.08 ± 0.415 ^{g}	230 ± 0.001 $^{b c d}$
BUAP TehAzo2	0	4.77 ± 0.075 ^{a}	100 ± 0.001 ^{a}	NA	390 ± 0.173 ^{a}
	0.05	3.41 ± 0.010 ^{c}	71.33 ± 0.577 ^{b}	4.20 ± 0.498 $^{a b}$	310 ± 0.100 $^{a b}$
	0.10	3.36 ± 0.010 $^{c d}$	70.33 ± 0.577 ^{b}	5.84 ± 0.240 ^{b}	236 ± 0.230 $^{b c d}$
	0.15	3.30 ± 0.001 $^{d e}$	68.33 ± 2.082 ^{b}	11.00 ± 0.620 ^{c}	213 ± 0.152 $^{c d}$
	0.20	3.26 ± 0.020 $^{e f}$	66.00 ± 2.000 ^{b}	20.84 ± 0.510 ^{e}	203 ± 0.152 $^{c d}$
BUAP TehAzo5	0	2.31 ± 0.010 ^{h}	100 ± 0.001 ^{a}	NA	323 ± 0.404 $^{a b}$
	0.05	1.41 ± 0.015 ^{i}	44.67 ± 3.055 ^{c}	5.90 ± 0.098 ^{b}	263 ± 0.251 $^{b c d}$
	0.10	1.21 ± 0.010 ^{j}	37.33 ± 2.082 ^{d}	18.07 ± 0.396 ^{d}	240 ± 0.346 $^{b c d}$
	0.15	0.885 ± 0.015 ^{k}	27.67 ± 3.215 ^{e}	35.90 ± 0.865 ^{g}	206 ± 0.208 $^{c d}$
	0.20	0.690 ± 0.010 ^{l}	21.33 ± 3.215 ^{f}	40.99 ± 0.948 ^{h}	183 ± 0.057 ^{d}

The values represent the mean ± standard deviation. PRGI: percentage of radial growth inhibition. Means with different letters in the column indicate significant differences with Tukey test (p<0.05). NA: Not applicable.

61.28% biodecolorization with 0.0629 g/L of residual indigo. Isatin was detected as an oxidative product at a wavelength of 342 nm with an absorbance of 0.275. The control showed 19% chemical biodecolorization of indigo blue at the end of the experiment (336 h), (Figures 2a and 2b).

Strain BUAP TehAzo 2 showed 37.81% biodecolorization at 24 h, with a residual indigo concentration of 0.1234 g/L. It presented increasing biodecolorization capacity between 24 and 168 h, and at the end of the experiment (336 h), it achieved 74.29% biodecolorization with a residual indigo

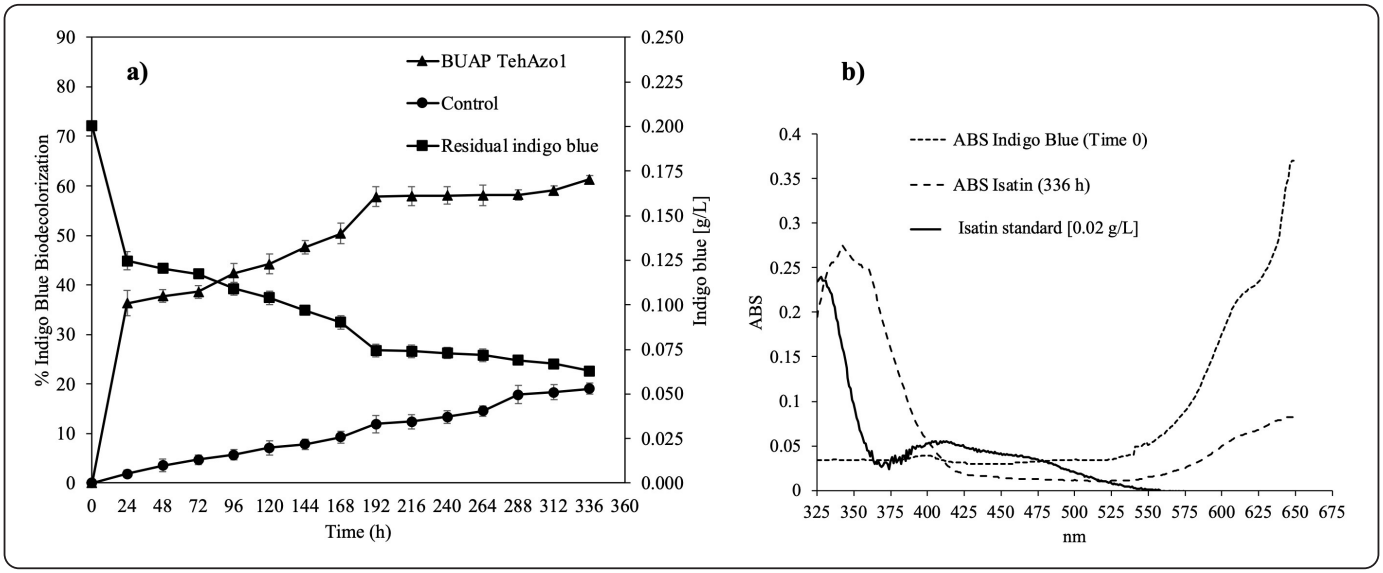


Figure 2. a) Indigo blue biodecolorization percentage by *T. harzianum* strain BUAP TehAzo 1 at 28 °C. b) Absorption spectrum of indigo blue biodecolorization.

concentration of 0.0426 g/L (Figure 3a). Isatin was also detected in this strain as an oxidation product of indigo blue at a wavelength of 336 nm with an absorbance of 0.315 (Figure 3b).

In both strains, isatin was detected within a wavelength range of 336 to 342 nm (Figures 2b and 3b). This indicated that most of the dye was transformed into one of the oxidative products of indigo blue, such as isatin, as evidenced by the emergence of these peaks and the disappearance of the indigo peak at 649 nm. There was no significant difference in biodecolorization capacity between the two strains according to a mean comparison analysis (Tukey test, $p = 0.05$). Both strains showed effective biodecolorization at 336 h.

FTIR analysis confirmed the oxidative biodecolorization of indigo blue by the *T. harzianum* strains BUAP TehAzo 1 and BUAP TehAzo 2 (Figure 4). The spectra obtained from the strains were compared with reference standard of isatin. The isatin standard showed bands at 3300, 1615, 1350, and 1092 cm^{-1}, as well as bands in the region between 943 and 658 cm^{-1}. The band observed at 3300 cm^{-1} would represent the -NH group. The band at 2300 cm^{-1} was attributed to CO_2 molecule as part of the metabolism microbial. A band at 1615 cm^{-1} indicating a carbon-carbon simple bond (C-C) and the band at 1350 cm^{-1} was assigned to the ketonic group (C=O) in the aromatic ring part of the dye´s chromophore region. It´s noteworthy that the band at 1092 cm^{-1} indicate the stretching vibration of single bonds involving C-N. The spectra of *T. harzianum* strains BUAP TehAzo 1 and BUAP TehAzo 2 showed characteristic bands at 3300, 1615, 1350, and 1092 cm^{-1}, like the isatin standard spectrum. The similarity between the spectra indicates that both strains could transform indigo blue to isatin, confirming that *T. harzianum* BUAP TehAzo 1 and BUAP TehAzo 2 carried out dye oxidation.

Discussion

In the locality of San Diego Chalma, Tehuacán, Puebla, Mexico, economically important crops such as maize, beans, and squash are produced (SADER, 2025). Soil analyses conducted in the agricultural lands of this region show low fertility in terms of total nitrogen (N_t) for crop development, while organic matter (OM), phosphorus (P), potassium (K), and micronutrients are present at medium fertility levels, according to NOM-021-RECNAT-2000 (DOF, 2002). Additionally, the soils exhibited a loamy sand texture; this condition can facilitate the retention of nutrients and water, which supports crop growth (Ding, Zhang, Yang, Xin, Zhu & Huang, 2021). On the other hand, the concentration of potentially toxic elements (Pb, Cd, Cr, Ni) was low compared to the current Mexican regulations (DOF, 2002), suggesting no toxicological risk for present organisms. However, the pH of the contaminated soil (pH = 8.18) showed an increase of 0.25 compared to the control soil (pH = 7.93). This slight shift in alkalinity suggests that the use of textile wastewater in local agriculture may be altering the soil's chemical conditions, causing an increase in electrical conductivity (EC) and cation exchange capacity (CEC), and thereby promoting the dispersion of indigo blue dye (0.083 g/L), (Tables I and II).

The textile wastewater from San Diego Chalma, Tehuacán, Puebla, Mexico contained indigo blue dye concentrations (364 mg/L) toxic to aquatic life. According to studies by Francisco et al. (2023), a concentration of 100 mg/L of indigo blue causes ecotoxicological effects such as reduced reproduction rates and gonadal histological abnormalities in *Danio reiro*. Furthermore, previous studies have indicated that textile dyes

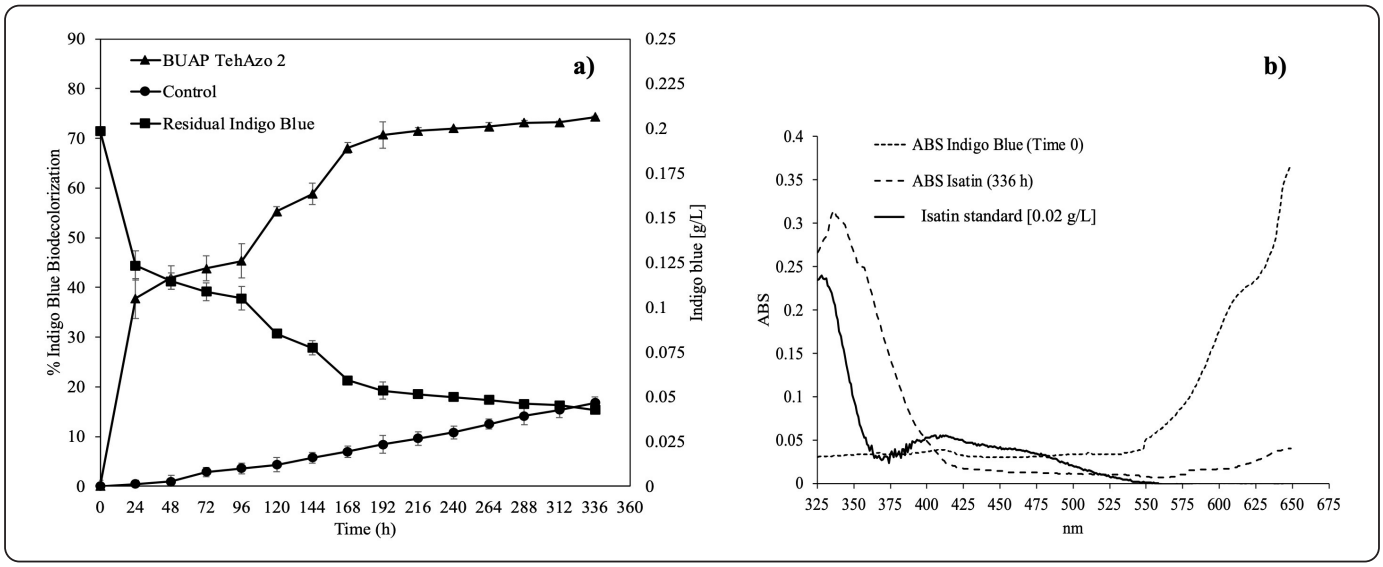


Figure 3. a) Indigo blue biodecolorization percentage by *T. harzianum* strain BUAP TehAzo 2 at 28 °C. b) Absorption spectrum of indigo blue biodecolorization.

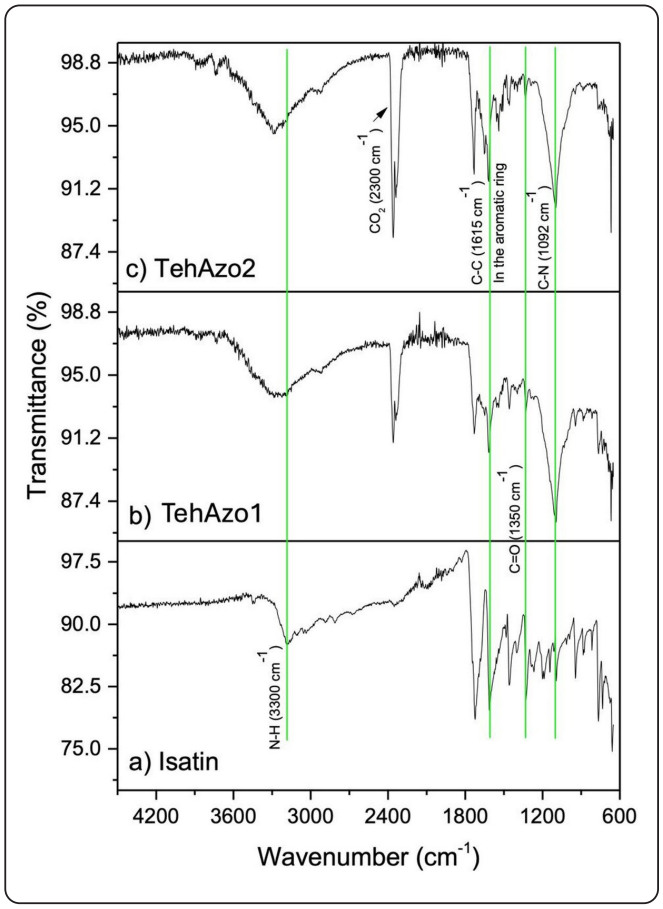


Figure 4. FTIR spectra of *T. harzianum* strains compared with isatin standard. a) isatin standard, b) BUAP TehAzo 1 strain and c) BUAP TehAzo 2 strain.

can bioaccumulate in plants posing risks to human and animal health (Singh, Gupta & Das, 2021; Slama *et al.*, 2021; Gallego-Ramírez *et al.*, 2022). The wastewater from the study area is a source of indigo blue, organic matter (high levels of BOD, COD, TSS), and sulfides (due to the use of sulfur soaps in textile dyeing processes), (Verma, Sankhla, Rathod, Sonone, Parihar & Singh, 2021), (Table I), all of which negatively impact water quality and alter the fertility parameters of agricultural soils.

In the agricultural soil contaminated with indigo blue, three strains were isolated and identified: BUAP TehAzo 1, BUAP TehAzo 2, and BUAP TehAzo 5. These strains showed morphological and microscopic characteristics consistent with the genus *Trichoderma* (Table III), and their ITS region sequence analyses showed 100% similarity for BUAP TehAzo 1 and BUAP TehAzo 2 with *T. harzianum*; BUAP TehAzo 5 showed 99.69% similarity with this species (Figure 1). *T. harzianum* has been reported in various systems contaminated with textile dyes (Hadibarata, Syafiuddin, Al-Dhabaan, Elshikh & Rubiyatno, 2018; Kulkarni, Kumar, Kulkarni & Satpute, 2024). The presence of these strains in soils contaminated with indigo blue suggests they are adapted to pollution, likely due to their ability to secrete oxidative enzymes such as veratryl alcohol oxidase, aryl-alcohol oxidase, manganese peroxidase, and laccases, which enable them to tolerate and degrade complex compounds like synthetic dyes (Salem, Mohamed, Shaban, Mahmoud, Eid & El-Zawawy, 2024). Therefore, the removal of dyes is through processes of fungal lignolytic enzymes such as laccases (Lac), manganese peroxidases (MnP) and lignin peroxidases (LiP) (Nagraj, Chaurasia, Bharati, Sharma, Kumar & Sivalingam, 2025). Different basidiomycetes (RCK-1, RCK-3, RCK-4 strains) isolated from sugarcane bagasse and degraded wood showed a significant positive correlation ($r>0.72$) in the activity of ligninolytic enzymes such as Lac (>1.7 U/mL) and MnP (<0.3 U/mL) with the ability to biodecolorize indigo carmine under *in vitro* cultures (Diwaniyan, Kharb, Raghukumar & Kuhad, 2010). As well, *Stenotrophomonas* sp. CFB-09 showed a correlation of LiP activities mainly, followed by MnP and Lac with the ability to decolorize 75.0-86.5% of indigo carmine at 100 mg/L in 120 h (Olajuyigbe, Afere, Adetuyi & Fatokun, 2022). On the other hand, MnP from white rot fungi such as *Irpex lacteus* CD2 has been purified and characterized, the purified CD2-MnP presented a molecular mass of 42 kDa and was a heme protein with iron protoporphyrin IX as compound II, with the ability to decolorize up to 91.5% of indigo carmine (50 mg/L) within 5 h (Qin, Zhang, Zhang & Yang, 2014).

As observed in our study, the *T. harzianum* strains exhibited normal mycelial growth with uniform green coloration at low indigo blue concentrations (0.05 and 0.10 g/L), (Table IV), but at higher concentrations (0.20 g/L), the BUAP TehAzo 2 strain was the most tolerant, based on its growth and development in culture medium. This tolerance may be attributed to its metabolic plasticity in expressing degradative enzymes and its adaptive capacity to environments contaminated with indigo blue (Mendarte-Alquisira, Alarcón & Ferrera-Cerrato, 2024). The degradation of dyes is intrinsically linked to the genes responsible for lignin metabolism, recent transcriptomics studies of *Trametes gibbosa* show that, in the presence of alizarin red dye *lac, mnp,* and *lip* genes are upregulate which encoding these enzymes (Lac, MnP, LiP); furthermore, transcriptome analysis identified that the Glutathione metabolism pathway (ko00480) is involved in detoxify through Glutathione S-transferase (GST) and Cytochrome P450 (CYP450) (Zhang & Feng, 2021).

Due to their high tolerance, strains BUAP TehAzo 1 and BUAP TehAzo 2 were selected for *in vitro* indigo blue biodecolorization assays. These strains achieved biodecolorization efficiencies of 61.28% and 74.29% after 336 hours (14 days) at 0.20 g/L initial concentration. (Figures 2a and 3a). In contrast, strains of *T. atroviride* obtained results greater than 90 % indigo carmine dye decolorization at initial concentration of 0.0088 g/L for 8 days (Lisboa *et al.*, 2017). Although it has been reported that *T. asperellum* cell-free supernatant biodecolorizes three different

dye families (reactive, azo and anthraquinone) at 0.05 g/L initial concentration with or without HBT (1-hydroxybenzotriazole) it showed 60-80% biodecolorization of Remazol Brilliant Blue R (RBBR), Direct Red 75 (DR75) and Turquoise Blue (TB); 9-90% biodecolorization of Reactive Black 5 (RB5) and 75-80% biodecolorization of Acid Orange 51 (AO51) over 48 h (Ben-Ali *et al.*, 2020). Other genera such as *Aspergillus* strain H1T showed biodecolorization of 100% indigo blue at an initial concentration of 50 mg/L at 100 rpm for 48 h at 28 ºC (Valdez-Vazquez, Robledo-Rizo, Muñoz-Páez, Pérez-Rangel & de la Luz Ruiz-Aguilar, 2020) and *Penicillum cf. oxalicum* strains LAMAI 2400 and LAMAI 2402 biodecolorized 81-98% of 0.4 g/L sulphur indigo blue for 7 days at 140 rpm and 28 ºC (Kita, Giovanella, Yoshinaga, Pellizzer & Sette, 2022). Moreover, the strains demonstrated a significant capacity to transform the dye into the oxidative product isatin, as indicated by the emergence of peaks at 336 and 342 nm and the decrease in the indigo blue peak at 649 nm in UV-Vis spectroscopy (Figures 2b and 3b). FTIR analysis revealed characteristic bands at 3300, 1615, 1350, and 1092 cm^{-1}, like the reference standard spectrum for isatin (Figure 4). The presence of isatin suggests that the *T. harzianum* strains oxidized the dye via oxidative enzymes, possibly peroxidases and laccases, as reported in *Trichoderma* spp. (Salem *et al.,* 2024). This oxidation process observed in both strains were like the biodecolorization processes reported by Campos, Kandelbauer, Robra, Cavaco-Paulo & Gübitz (2001) and Rodrigues *et al.* (2024), where indigo blue was transformed into isatin through catalytic enzymes from ligninolytic and filamentous fungi. The biodecolorization of indigo blue into isatin is of great environmental relevance, as indigoid dyes are highly recalcitrant, lipophilic, and capable of bioaccumulating in organisms, posing environmental and toxicological risks (Hemashenpagam & Selvajeyanthi 2023). The ability of *T. harzianum* to significantly reduce indigo blue concentrations *in vitro* suggests its potential for scale-up studies in the remediation of soils and water impacted by the textile industry.

Conclusions

In agroecosystems contaminated by the textile industry, such as the San Diego Chalma area in Tehuacán, Puebla, Mexico, microorganisms like *T. harzianum* have been identified. These strains showed the ability to tolerate and biodecolorization indigo blue through oxidative transformation into isatin under *in vitro* conditions. Bioremediation processes such as bioaugmentation with *Trichoderma* could be a promising biotechnology for restoring agroecosystems contaminated with indigo blue and could help mitigate the environmental risks associated with textile dyes.

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