Characterization and treatment of colored textile wastewaters from the typical Tunisian hat *Chechia* dyeing using newly isolated *Aspergillus niger*

Hajer Barouni [1,*], Haifa Chahed [2], Manel Ziadi [1], Kaouther Zaafouri [1], Mohamed Nejib Marzouki [2] and Moktar Hamdi [1]

[1] Laboratory of Ecology and Microbial Technology (LETMi). The National Institute of Applied Science and Technology INSAT. Carthage University. 2 Boulevard de la Terre, BP 676, 1080 Tunis, Tunisia.

[2] Laboratory of Protein Engineering and Bioactive Molecules (LIP-MB).The National Institute of Applied Sciences and Technology INSAT. Carthage University. 2 Boulevard de la Terre, BP 676, 1080 Tunis, Tunisia.

Abstract: This study aimed to characterize and investigate, for the first time, the treatment of real colored wastewaters from the artisanal dyeing of the typical Tunisian hat *Chechia,* using a newly isolated fungal strain. This textile effluent was a mixture called Mix of colored wastewaters from the three main types of *Chechia*. The major pollutant of the Mix was the toxic Azo dye Amaranth Acid or Acid Red 27. The fungal strain that made the cleanup was discovered in a *Chechia* dyeing wastewater's container and identified by ITS rDNA gene sequencing. This isolated *Aspergillus niger* showed interesting performances on the demonstration of *Chechia* wastewater's biodegradation in batch cultures. In order to understand the effect of agitation, Mix dilution and inoculum size on decolourisation and pollution removal, a full factorial experimental design 2^3 was set up. At the optimal conditions which were 20% inoculum size, 25% *Chechia* Mix dilution and an agitation of 100 rpm, *Aspergillus niger* was able to remove color as high as 70.18±2.84% at an initial dye concentration of 1346.6±0.01 mg/L, and to reduce COD to 74.17±14.52% at an initial COD of 4157±422 mg/L. FT-IR spectra analysis confirmed the decolourisation by biodegradation and transformation of the dyes. The treatment by the isolated *Aspergillus niger* could be successfully applied as a sustainable method to solve one of handicraft dyeing plants environmental management issues.

Keywords: *Chechia* artisanal textile wastewater; Red Azo dyes; decolourisation; *Aspergillus niger;* full factorial design.

Introduction

The wastewater generated from textile and tanning industries contains the highest amount of undesirable dyes and pigments. Field investigations on the Tunisian traditional red wool hat called *Chechia* artisanal dyeing plant revealed that the major chemical dye used for all types of *Chechia* was an anionic azoic dye, the Acid Red 27. This dye deserved a serious attention because of its sanitary risks. Suspected carcinogen, a risk of allergy was also reported particularly on salicylates sensitive subjects. With an increasing production of more than 81000 kg/year of *Chechias*, Tunisia is facing a huge environmental issue caused by more than 21.6 10^6 L/year of red wastewaters from a unique artisanal dyeing plant (personal data). The depollution of this colored effluent could contribute to achieving the global environmental management of this focal handicraft dyeing plant. This is a requirement of the Tunisian Eco Label for *Chechia* that is an artisanal product with a high patrimonial value. A particularly common aspect of textile effluents is the high COD. In order to overcome this problem, varied methods were explored to treat textile wastewater. Coagulation flocculation, membrane filtration, and chemical processes have all been widely used in the remediation of textile wastewaters [1]. Several advanced oxidation processes such as photocatalysis, Fenton and photo-Fenton were proposed [2]. However, most of the literature studies on destructive treatment methods for the cleanup of textile wastewaters reported decolourisation efficiencies, but not COD removal or mineralization. In case of toxic and biodegradable textile wastewaters, biological treatment is an alternative to these physical methods; it has a special interest and is in a constant development which contributes to the realization of high reactions yields with a sustainable treatment [3]. Biological treatment is a common process widely applied in the treatment of wastewater polluted with

dyes. It offers significant advantages in terms of cost and level of toxicity of the final products.

Bacteria are frequently solicited for the bioremediation of textile discharges because of their rapid growth and adaptability even in extreme conditions of salinity, pH and temperature, in addition to the expression of a dye specific enzyme system [4]. Many species have been sought for the bleach and the mineralization of various dyes [5]. However, most of the dyes from all chemically distinct groups are also prone to fungal oxidation [6]. Their large structural differences constitute a great challenge for the varying catalyzing properties of the fungal isolates. Biodegradation of azo and phalocyanin dyes was analyzed by using the common polypore mushroom *Trametes versicolor* and the white rot fungus *Bjerkandea adusta* [6]. Literature reported the degradation of azo, anthraquinone, heterocyclic and polymeric dyes by *Phanerochete chrysosporium* being the most studied fungi facing the degradation of xenobiotics such as azo dyes and synthetic one [7]. The Members of Zygomycetes such as the *mucoraceous* and *arbuscular mycorhizal* fungi, but also aquatic and anaerobic fungi can be applied for bioremediation. In the cleanup of industrial effluents from undesirable chemicals, the yeasts such *Candida tropicalis*, *Saccharomyces cerevisiae, Saccharomyces carlbergensis* and *Candida utilis* are other significant candidates. Furthermore, consortia of Fungi and Bacteria are used in composing a waste disposal practice [7]. Depending on the fungus species, several mechanisms could be involved in decolourisation: dyes can be removed by fungi via enzymatic mineralization using Lignin peroxidase (LiP), Manganese peroxidase (MnP), and Laccases (Lacc)[4], by bioadsorption [8] and also by biodegradation [7]. Oxidation of synthetic dyes by White Rot Fungi implies the expression of non-specific extracellular ligninolytic enzymes. The capability of laccase to act on chromophore compounds such as azo, triarylmethane, anthraquinonic and indigoid dyes leads to the suggestion that they can be applied in industrial decolourisation processes [9]. The enzyme laccase produced by *Pyricularia oryzae* oxidizes Phenolic azo dyes. Reactive dyes typically contain azo based chromophores attached to different types of reactive groups that interact with the active sites such as chitin, chitosan acidic polysaccharides, lipids, amino acid and other cellular components on the surface of the micro-organisms [10]. Several degradation mechanisms for non-phenolic and phenolic azo dyes are proposed in literature. Azo dyes can be degraded through a highly non-specific free radical mechanism, forming phenolic type compounds, without direct cleavage of the azo bond and avoiding the formation of toxic aromatic amines, which might be friendly for the environment [11]. In biodegradation, maximum decolourisation rate is generally reached during the exponential growth phase of the micro-organism. Also, additional

substrates such as glucose could enhance the decolourisation [12]. Literature also reported that various physicochemical conditions such as the type and concentration of dyes, additional carbon, nitrogen source, temperature and pH largely influence the fungal biodegradation. Other fungal species like *Aspergillus niger* are able to decolourise and /or absorb various dyes [7]. Jaidev and Narasimha (2010) showed that Rose Bengal dye at 100 ppm was effectively decolourised by *A. niger* within a 24 hour incubation period [13]. *Aspergillus niger*, isolated from the soil around textile industries, could tolerate high concentrations of dyes (up to 1000 mg/L) and displayed a high decolourising activity against various reactive dyes including azo dyes as reported by SA Omar [14].

This research could help respond to the increasing sustainability requirements of the artisanal processes as well as to the reduction of the ecological footprint of products. The objectives of the present study are threefold: *i)* reporting for the first time the characterization of the *Chechia* colored effluent, *ii)* demonstrating its biodegradation in batch cultures using an isolated *Aspergillus niger* and *iii)* determining the optimal conditions of depollution and decolourisation from its major pollutants, the acid azo dyes. Coupling handicraft with environment should increase the international influence of *Chechia*, placing this Tunisian symbol in the "green" rank of the cleanly processed products.

Materials and Methods

Effluent sampling
The colored textile wastewaters used in this study came from an artisanal dyeing plant of C*hechia* located in the old City of Tunis (capital of Tunisia). Samples have been collected from the *Chechia* dyeing marmites when the dyeing process was complete and before the colored wastewaters have been discharged into the public sewage system. Access to dye marmites was direct. Three types of colored wastewaters derived from three used types of *Chechia*: bright red wastewaters from the *Chechia Tounsi* dye, dark red wastewaters from the *Chechia Ben Ghazi* dye and black wastewaters derived from the black *Chechia Trabelsi* dye. A mixture of the three colored effluents at equal proportions called Mix was used as substrate for all the experiments.

Isolation and identification of the fungal strain
Isolation and characterization of the fungus
The fungal strain used in the present work was isolated from an abandoned effluent sample; as it already seemed to visually present interesting dye removal abilities. The isolation of the strain was carried out by successive subcultures on a specific medium composed of: 250 mL of diluted Mix (25%) supplemented with 7.5 g Agar. The incubation was carried out at 30°C during 5 days. For phenotypic and molecular characterization, the strain was grown

in the classical media Potatoes Dextrose Agar (PDA) and Potatoes Dextrose Broth (PDB) (REACTIFS RAL) and incubated at 30℃ for 3 days. Microscopic observations were done at 10, 40 and 100 magnifications using an optical microscope (LIIOOA) equipped with a camera.

Total genomic DNA extraction

The total genomic DNA of the fungus was prepared from mycelium. After 3 days of preculture on PDB medium, the fungal mycelium was ground in liquid nitrogen until obtaining a viscous liquid suspension. Three hundred milligrams of the ground material were weighed in an Eppendorf sterile tube to which were added 400 µL of genomic DNA extraction buffer. The sample was gently homogenized and added with 250 µL of 3M ammonium acetate (pH 4.8). After 30 min of incubation on ice, the sample was centrifuged for 15 min at 13 000 rpm at 4℃. A carefully recovered aqueous phase was added to 1V of phenol saturated in extraction buffer (pH 8). The whole was thoroughly mixed and centrifuged for 5 min at 10 000 rpm. The upper aqueous phase was recovered for re-extraction with 1V of chloroform. After homogenization and centrifugation for 5 min at 10 000 rpm, 2V of absolute ethanol and 1/10 V of 3M sodium acetate (pH 4.8) were added to the aqueous phase. The whole was incubated for 2 hours at -80℃, then centrifuged for 15 min at 12 000 rpm at 4℃. The pellet was then washed with 70% ethanol, centrifuged 2 min at 10 000 rpm, dried and finally re-suspended in 30 µL extraction buffer. The genomic DNA obtained was analyzed by electrophoresis agarose gel 0.8% in the presence of the 1 Kb Ladder molecular weight markers. The genomic DNA is then stored at -20℃.

Polymerase Chain Reaction (PCR) amplification, DNA sequencing and phylogenetic analysis

The fungal genomic DNA was subjected to amplification by PCR using specific primers ITS1:5'-TCCTCCGCTTATTGATATGC-3'and ITS4:5'-GAAGTAAAAGTCGTAACAAGG-3'. PCR was performed using a 50 µL reaction mixture containing the following (per reaction) : 100 ng of genomic DNA ; 1X DreamTaq reaction buffer (Thermo) ; 10 mM of dNTP mix ; 20 µM of each primer ; 1.25 U DreamTaq DNA polymerase (Thermo). Amplifications were performed in a BIORAD Thermocycler and the different PCR steps were as follows: an initial denaturation step at 94℃ for 5 min, 30 cycles of: denaturation at 94℃ for 1 min, annealing at 49℃ for 1min, and extension at 72℃ for 1 min 30 s, followed by a 10 min final extension step at 72℃. Five microliters of the mixture were finally analyzed by electrophoresis on 1% agarose gel and visualized by Ethidium Bromide. The nucleotide sequence of the ITS region of the cleaner fungus genomic DNA was amplified by PCR. The amplified PCR products were then sequenced, using the BigDye® Terminator v3.1 Cycle Sequencing kit. The obtained sequence was then analyzed using the Blast alignment program. Results were represented in a phylogenic tree. The Neighbor-Joining method [15] was used. The evolutionary distances were computed using the Jukes-Cantor method and were in the units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences. Evolutionary analyses were conducted in MEGA6 [16].

Analytical methods

Determination of pH, conductivity, TS, TVS, TSS, COD, BOD₅ and Color

The determination of pH, conductivity, Total Solids (TS), Total Volatile Solids (TVS), Total Suspend solid (TSS) and Chemical Oxygen Demand (COD) of the effluent were carried out according to standard methods [17]. pH values were measured using the pH meter (METTLER TOLEDO Education Line). The conductivity was measured using a conductivity meter (TOLEDO type MC 226). TS were determined by evaporation of a volume V (10 ml) of the test sample in a crucible by incubation in a drying oven (JOUAN S') at 105 °C for 24 hours. It was then calcined in the muffle furnace (PROTHERM FURNACES) model at 550 °C for 2 hours to determinate the TVS. Samples were centrifuged 3000 rpm / min for 20 min; the COD was measured using a PALINTEST 5000 photometer on the supernatant after the end of the centrifugation; the pellet was placed in a crucible and in an oven at 105 °C for 24 hours to determine TSS. The determination of the Biological Oxygen Demand (BOD₅) was made by the electrochemical method according NF 1899 (1998). The biological oxygen demand was the amount of oxygen in mg of oxygen that is consumed by the biodegradable material in one liter of sample under the conditions of the test, after five days incubation at 20 °C in the dark. For Color determination, the sample was centrifuged 20 min at 3000 rpm and the optical density (OD) of the supernatant was measured at a Λ_{max} of 525 nm with an UV-Visible (SECOMAN XS2 model) calibrated with distilled water. The decolourisation yield was expressed in percentage of color removal (%).

Determination of Nitrogen, Phosphorus, Nitrates, Chlorides, Fluorides, Potassium and Heavy metals

Ammonia and organic Nitrogen concentration expressed in mg N/L were determined following the method after mineralization in selenium according to NF 25663/ ISO 5663 (1994). This method permitted to determinate nitrogen in the negative trivalent state. The sample was mineralized to 350 °C in an acid medium in the presence of Selenium as catalyst and Potassium sulfate (elevation of boiling point). Phosphorus was determined following the spectrometric dosing method using Aluminium molybdate, according to ISO 6878 (2004). The sample was mineralized using persulfate at 120℃,

transforming many organophosphates into orthophosphates. The concentration of orthophosphate in the sample was determined after absorbance measurement at 880 nm. Determination of Nitrates, Chlorides and Fluorides in contaminated waters were made by chromatography in liquid phase ion according to NF ISO 10 304 (1995). Separating anions by liquid chromatography column involved the use of a low-capacity ion exchange as stationary phase and an aqueous solution of a monoacid and low diacid (solution of sodium hydrogencarbonate and sodium carbonate) as mobile phase. The ion detection was made by conductimetry. Potassium and Heavy metals : Aluminium (Al), Chromium (Cr), Manganese (Mn), Cadmium (Cd), Lead (Pb), Iron (Fe), Zinc (Zn), Cobalt (Co), Copper (Cu) and Nickel (Ni) were dosed using atomic emission spectrometry with inductively coupled plasma, according to NF ISO 11885 (1998). .The concentration of the elements was expressed in mg/L from their calibration curves taking into account the dilution factor.

Fourier Transform Infrared Spectroscopy (FT-IR) analysis

Fourier Transform Infrared Spectroscopy (FT-IR) analysis was performed to determine the distribution of functional groups of *Chechia* Mix before and after treatment in order to illustrate the biotreatment effect. Samples were prepared by lyophilizing the *Chechia* Mix obtaining a powder and then analyzed by FT-IR in the spectral range 4000 cm^{-1}-500 cm^{-1} using JASCO, FT/IR-4100.

Chechia Mix biodegradation

The biodegradation of *Chechia* Mix using the fungus was preliminarily carried out in non stirred batch system, in triplicate. Erlenmeyer flasks containing 100 mL of sterilized diluted *Chechia* Mix (25%) were inoculated with biomass at an inoculum size of 10% and placed in static conditions at 30 °C during 28 days in an incubator (ENO55 model). At the end of the treatment, samples were withdrawn from the flasks; centrifuged 20 min at 3000 rpm and the supernatant was used for OD at 525nm measurement and COD $_{soluble}$ determination. For biomass estimation expressed in TVS, 10 mL of the samples were dried at 105 °C for 24 hours, weighted

and then calcined at 550 °C for 2 hours. Color and pollution removals respectively expressed in OD525 and COD yields were studied during the experiment as well as the fungal biomass growth expressed in TVS yield.

Optimization of Chechia Mix biotreatment

The optimization of the biodegradation of *Chechia* Mix using the fungal strain was carried out through full factorial experimental design 2^k as described by Goupy [18]. The full factorial design was commonly used to study the effects of (k) factors selected at the same time, each factor having a low level (-1) and a high level (+1). 2^3 experiments were done. Three independent quantitative variables were selected to reach the optimal conditions for fungal biotreatment of *Chechia* Mix: agitation (rpm) F1, Mix effluent dilution (%) F2 and inoculum size (%) F3. The full factorial experimental design was set up using 250 mL Erlenmeyer flasks containing 100 mL of *Chechia* Mix effluent previously autoclaved: eight Erlenmeyer flasks (E1 to E8) were inoculated with different percentages of biomass and 8 Erlenmeyer flasks were not inoculated and used as control. Inoculation was done with a preculture from Petri dishes scrapped in *Chechia* Mix. Erlenmeyer flasks were then incubated at 30°C for 35 days, either in a Shaker (LABTECH model) stirred at 100 rpm or in an incubator (ENO55 model) according to the full factorial experimental design theoretical matrix described in Table 2 matrix. At the end of the experiment, samples were withdrawn from each flask for COD determination. For color determination, samples were centrifuged and OD 525 measurement was done on the supernatant. The experiments were carried out in duplicate. The studied responses of the experimental designs were the color removal Y_1 expressed in OD 525 yield (R_{OD} %) and the pollution removal Y_2 expressed in COD yield (R_{COD} %). In order to evaluate the effect of the three operating factors F1, F2 and F3 on the two responses Y_1 and Y_2, the regression coefficients C_c for color removal and C_p for pollution removal were determined as follow:

$$Cc = \frac{1}{4} * \left[\sum_{i=1}^{4} Aj * Xi \right] \qquad Cp = \frac{1}{4} * \left[\sum_{i=1}^{4} Aj * Yi \right]$$

Where:
Aj: means either high (+) or low (-) level in experimental assay i;
Xi: the color removal $R_{OD\ 525}$ (%);
Yi: the pollution removal R_{COD} (%);
The optimal conditions showing the best yields of color and pollution removals were adopted for the subsequent experiments as FT-IR analysis of post treated *Chechia* Mix.

Statistical analysis and mathematic models

All the measurements were made in triplicate. The statistical analysis of the responses obtained after running the experimental design and the calculation of the coefficients were done with NemrodW® software version 9901 (published by Didier Mathieu, Jean Nony, Roger Phan-Tan-Lw)

$$Y = b0 + \sum_{i=1}^{k} bi\, Xi$$

Results and Discussion

Effluent characterization

The characterization of the *Chechia* Mix was conducted for the first time in literature through physical, chemical, biological and spectral analysis. Twenty six parameters have been evaluated and compared to Tunisian Standards NT106.002 defining

and Minitab® version15 software. Checking the relationship between the three operating factors and the two responses, a first-order polynomial model studying the main effects of factors was set up. The coded mathematical model for the full factorial experimental design 2^3 was expressed in this equation:

Where:
b_0: squared effect term;
bi: regression coefficients;
Y: predicted response value

the limits for discharge into public sewage and other receiving environments (Table 1). This real textile wastewater seemed different from the reported domestic wastewaters generally dark brown with bland odors. Olfactive and visual observations of *Chechia* Mix revealed no pronounced odor and a dark red color indicating the presence of specific pollutants that were dyes in strong concentrations.

Table 1. Characteristics of *Chechia* Mix, a real textile effluent from an artisanal dyeing plant

Parameters	Units	*Chechia* Mix average values	Tunisian Standard (NT106.002) limits for effluent discharge on: Public sewage - Earth - Public channel		
pH at T =14.8°C	-	**4.78**±0.18	6.5<pH<8.6		
Electric conductivity at T=14.8°C	μS/cm	**7745**±704.5	no data		
Salinity	g/L	**4.25**±1.34			
COD	mgO₂/L	**4157**±422	1000	90	90
BOD₅	mgO₂/L	**1920**±171	400	30	30
Nitrogen NTK	mg/L	**9**±2.22	100	30	1
Organic Nitrogen	mg/L	1.85±0.20			
Ammonia Nitrogen	mg/L	7.14±2.01			
Phosphorus	mg/L	**2.05**±0.26	10	0.1	0.05
Potassium	mg/L	**119.85**±11.32	50	1000	50
Nitrates NO₃	mg/L	**2425**±213.17	90	90	50
Chlorides	mg/L	**225**±35.35	700	No exigence	600
Fluorides	mg/L	**2500**±267.76	3	5	3
Heavy metals					
Lead Pb	mg/L	**< 0.005**	1	0.5	0.1
Cadmium Cd	mg/L	**<0.0005**	0.1	0.05	0.05
Iron Fe	mg/L	**0.722**±0.44	5	1	1
Copper Cu	mg/L	**1.986**±0.79	1	1.5	0.5
Zinc Zn	mg/L	**0.733**±0.31	5	10	5
Manganese Mn	mg/L	**0.185**±0.15	1	1	0.5
Cobalt Co	mg/L	**< 0.005**	0.5	0.5	0.1
Aluminium Al	mg/L	**0.137**±0.03	10	5	5
Chromium Cr	mg/L	**<0.008**	0.5	0.5	0.01
Nickel Ni	mg/L	**< 0.005**	2	2	0.2

Chechia Mix did not comply with the Tunisian Standard in most of pollution indicators like COD, BOD, Nitrates and Color (Table 1). This pollution was mainly due to dyes which were present at a concentration of 1346.6 ± 0.01 mg/L. The major pollutant is the "3051 Acid Amaranth 166%" (BORUTA COLORS) or Acid Red 27 which molecular formula is $C_{20}H_{11}N_2Na_3O_{10}S_3$. Listed as a food additive (E123) this dye is banned from use in several developed countries. *Chechia* Mix presented an acidic pH of 4.78 ± 0.18 and its high electric conductivity correlated to its salinity of 4.25 ± 1.34 g/L explained the major inorganic nature of the effluent in which TVS represented 25% of the TS. The TSS, Nitrogen and Phosphorus contents of the effluent were complying with the Tunisian Standard limits for effluent discharge in public sewage (Table 1). *Chechia* effluents could contain high levels of organic compounds which can be dangerous to environmental life, as depletion of dissolved oxygen could have an adverse effect on the marine ecological system and the whole ecosystem[1]. *Chechia* Mix COD and BOD_5 were respectively 4157±422 mg O_2/L and 1920±171 mg O_2/L. Thus, the *Chechia* Mix was qualified as highly polluted compared to olive mill wastewater characteristics with COD of 95 g O_2/L, total solids of 84.2 g/L and total suspended solids of 14.5 g/L [19] or also with regard to other real textile wastewaters [20,21] with COD of 1000 mg O_2/L and 2870.6 mg O_2/L, BOD_5 of 30 mg O_2/L and 1295.3 mg O_2/L, TS of 5.810 g/L and 10.8 g/L, and also TSS of 0.115 g/L and 0.08 g/L. Chlorides and all the heavy metals except Copper were the only parameters that complied with the Tunisian Standard NT106.002 limits. Traces elements like Iron, Zinc and Copper detected in the wastewater could be related to the metallurgic composition of the artisanal dyeing bath which is an alloy of these metals. *Chechia* dyeing is an artisanal operation subjected to frequent changes as a result of its artisanal side. This is the main reason for the variable volume and the wide variety of chemical pollutants present in this wastewater. With a COD/BOD_5 ratio of 2.16, *Chechia* effluent was not considered easily biodegradable but amenable to biological treatment with selected micro-organisms.
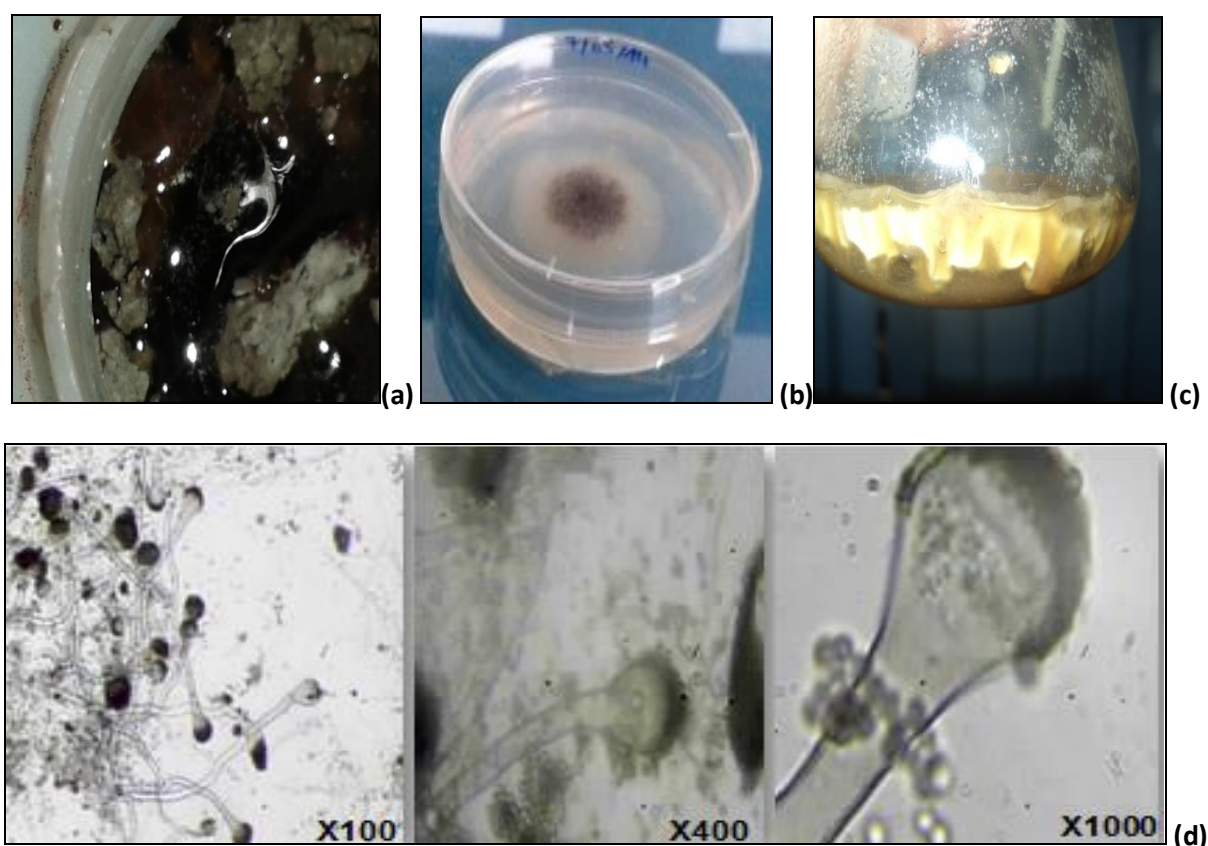

Figure 1. Morphological characteristics of the C*hechia* Mix cleaner fungal strain: (a) spontaneous growth on C*hechia* Mix, (b) cultivated in PDA, (c) cultivated in PDB and (d) through optical microscopic observations at 10,40 and 100 magnifications, after 3 days at T=30℃

Identification of the fungal strain and phylogenetic analysis

The isolated fungus has grown spontaneously on *Chechia* wastewater and has presented visually an interesting potential on dye removal (Figure 1.a). The strain cultured at 30 ℃ in PDA for 3 days showed colonies with the following phenotypic characteristics: circular, curved, hairy, white at the

circumference and black going to the middle with a colony diameter of 25-45 mm (Figure 1.b). When incubated in PDB, the fungus had a better white mycelium growth (Figure 1.c). The optical microscopy permitted the description of the appearance and the shape of mycelium and spores at different magnifications (Figure 1.d).

According colony morphology, conidial size and ornamentation, the strain can be assigned to *Aspergillus* section *Nigri* [22]. Figure 1.d showed that the mycelium has branched filaments with vesicles 45-80 μm wide, nearly globose. Phialides are flask-shaped terminated by spores or conidia which are very small in size 3.5-5 μm, not chinulate, very volatile and giving its color to the colonies. There were no sclerotia observed in the culture. The studied fungal strain exhibited the growth and the morphological characteristics of *Aspergillus niger*. Black aspergilli were isolated from various sources but the main one remains soil [23]. However, Samson et al. underlined that morphological structures can be helpful but the growth pattern was not sufficient to distinguish species including the species related to *A. niger* in particular [22]. Indeed, Black aspergilli are difficult to be recognized based only on their phenotypic characters. Frisvad et al. exposed some interesting methods that can be used in the

diagnostics of the known and accepted species [24]. To identify the fungus cleaning *Chechia* Mix, the genomic fungal DNA (Figure 2.a) was amplified by PCR (Figure 2.b) which was realized using the universal primers ITS1 and ITS4. Those primers encompass the nuclear gene for the 5.8S rRNA and the flanking transcribed spacers ITS1 and ITS4 approximately 600pb. The ITS nucleotide sequence of the amplified DNA was determined and analyzed with the NCBI database using the BLAST program. The nucleotide sequence showed 100% homology with ITS of *Aspergillus niger* strain (Figure 2.c). The sequences that showed the highest similarity with the query sequence were found to belong to Ascomycetus fungi. The multiple alignment and phylogenetic tree (Figure 2.c) showed that the strain is *Aspergillus niger*. But ITS can only be used for the uni- and biseriate species classification while only four groups of *A. niger* related taxa can be identified [22]. Samson et al. highlighted that almost all species in *Aspergillus section Nigri* can be distinguished from each other using calmodulin and β-tubulin sequence data [22]. More analytic investigations could be carried out to specify the identification of the cleaning fungus following Abarca et al. [23].
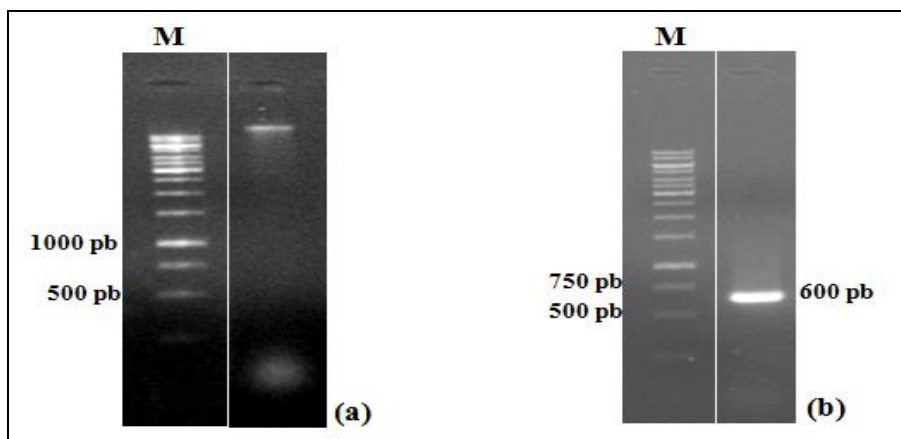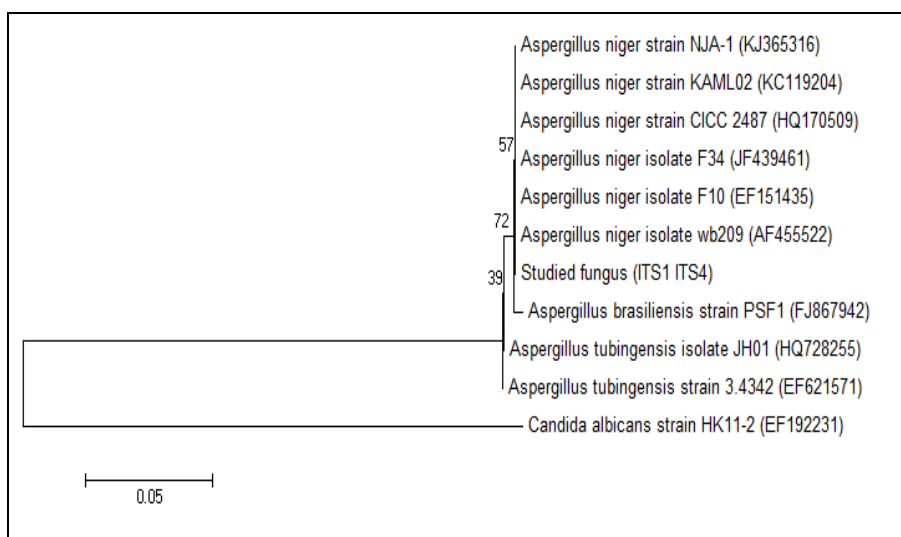

Figure 2. Electrophoretic analysis of genomic DNA on (a) Agarose gel 0.8% and (b) Polymerase Chain Reaction products on Agarose gel 1% M: Molecular Weight Marker. (c) Phylogenetic analysis based on ITS sequences of some *Aspergilli* strains, using the neighbor-joining method on MEGA6.

Biological Treatment
Chechia Mix biodegradation

Despite its specific pollutant, *Chechia* Mix could be successfully treated using a biological process with selected micro-organisms. That was prognosticated after *Chechia* Mix characterization with a COD/BOD_5 ratio of 2.16 and then demonstrated through the preliminary batch culture results depicted in Figure 3.
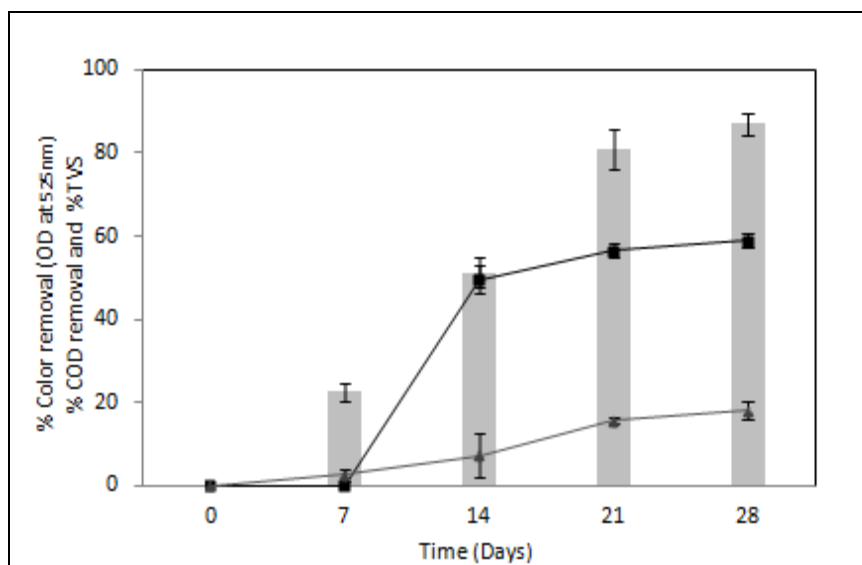

Figure 3. Variation of decolourisation (━■━) $R_{OD\ at\ 525nm}$, pollution removal (�) R_{COD} and biomass growth (━▲━) R_{TVS} yields during diluted *Chechia* Mix biotreatment by isolated *Aspergillus niger* inoculated at 10 %, under static conditions

Indeed, diluted *Chechia* Mix (25%) treatment using the isolated *Aspergillus niger* (inoculum size 10%) in static conditions presented increasing pollution and color removal. An interesting decolourisation yield of 59.06±1.51% and a large depollution performance illustrated by a COD removal yield of 87.05±2.68% was registered at the end of the treatment. In parallel, fungal biomass growth expressed in TVS yield increased progressively and achieved 18.16± 1.94% at the end of the experience. Unlike in agrifood fermentation processes applications, a reduced biomass growth could be appreciated on the environmental field. Also, *Aspergillus niger* growth seemed to be strongly associated with enzymatic activities production as an increase of pH from 4.78±0.18 to 8.73±0.12 was noticed between the beginning and the end of treatments. Indeed, Fu and Viraraghavan explained that biomass cells produce enzymes such as laccase, manganese peroxidase and lignin peroxidase to mineralize the dyes [25].

Factors Effects

Several authors have studied the degradation of textile wastewaters through the statistical design of experiments to optimize the treatment [10]. Even though, most of these studies were based on synthetic industrial effluents, the goal of the present work was to determine optimal conditions for the biodegradation of a real artisanal wastewater, the *Chechia* Mix. According to literature, several factors such as pH, agitation, effluent dilution, inoculum size and substrate concentration affected the fading rate while salts and other nutrients had little effect on color removal. After the preliminary batch results demonstrating the biodegradation efficiency of *Aspergillus niger*, the *Chechia* Mix biotreatment conditions were optimized through full factorial experimental design repeated twice. Three factors were explored and chosen as independent quantitative variables, namely: inoculum size, Mix dilution and agitation. The monitoring of eight different batch systems generating two responses, decolourisation and pollution removal, was described in Table 2. In order to determine whether the observed differences between responses were significantly different, statistical analyses were carried by means of NemrodW® software. Differences between factors effects (P) were compared with 0.05. The NemrodW® software analysis of the data indicated that all factors effects were statistically significant ($P < 0.05$).

Table 2. Biodegradation of *Chechia* Mix with *Aspergillus niger* by full factorial experimental design 2^3- Responses Matrix of eight experiments (E1 to E8) with High (+1) and Low (-1) coded levels Factors (F1 to F3) and Regression coefficients (b_0, b_1 b_2 and b_3) calculated for color removal (Y1) and pollution removal (Y2)

	F1 Agitation	F2 Mix dilution(%)	F3 Inoculum size(%)	Y1 Color		Y2 Pollution removal(%)	
Low level -1	0	3,3	10				
High level +1	100	25	20				
E1	-1	-1	-1	59.66	±3.63	68.63	±32.83
E2	+1	-1	-1	24.03	±1.66	95.53	±2.33
E3	-1	+1	-1	58.70	±1.06	80.45	±9.37
E4	+1	+1	-1	55.46	±0.08	83.14	±7.96
E5	-1	-1	+1	23.89	±0.70	90.62	±9.54
E6	+1	-1	+1	38.39	±4.44	84.99	±17.92
E7	-1	+1	+1	73.82	±0.10	41.24	±1.08
E8	+1	+1	+1	70.18	±2.84	74.17	±14.52
b_0				50.518		77.348	
b_1		F1 Agitation		-3.501		7.111	
b_2		F2 Mix dilution		14.024		-7.597	
b_3		F3 Inoculum size		1.054		-4.591	

The statistical p values are calculated from analyses between different cultures ($p < 0.05$).

According to the Table 2, the E7 system had remarkable performances with 73.82±0.10% of decolourisation and 40.5±1.08 % of COD reduction. Also, the E2 system achieved 95.53 ±2.33% of pollution removal while it registered only 24.03±1.66% of color removal. The best performances were registered for the E8 system with a decolourisation of 70.18±2.84% and a COD removal of 74.17±14.52%. There was no decolourisation in any of the uninoculated controls confirming the absence of intrinsic autoxidation phenomenon on the *Chechia* Mix. Thus, the best conditions for the *Chechia* Mix biotreatment corresponded to the initial conditions of the batch E8: 20% of inoculum size, 25% of Mix dilution and an agitation of 100 rpm. Ali et al.[12] achieved more than 95% of decolourisation efficiency within 48 h for azo reactive dyes using *Aspergillus foetidus,* but digging into the mechanisms involved, no significant biotransformation in dye was noticed and the entire color removal was found to be due to bioadsorption .Similarly, divalent dyes like Acid Orange II and Acid Red 114 showed a great affinity to the fungal biomass cell wall of *Myrothecium verrucaria* and A*spergillus niger* [12]. The fungal decolourisation mechanism involves the adsorption and/or enzymatic degradation [7]. Robinson et al. indicated that the textile effluent is not the adequate environment for the growth and preservation of fungal biomass, but fungi have a high capacity for accumulation of heavy metals and dyes in addition to ligninolytic enzymes secretion that has a strong oxidizing power[4]. Unlike bacteria like *Bacillus subtilis*, *Bacillus cereus*, *Pseudomonas.sp*, *Klebsiella pneumonia*, *Acinetobacter liquefaciens* and *Aeromonas*

hydrophila where the degradation of the dyes is intracellular, degradation by fungus is extracellular[26]. As related by Shyamala Gowri et al., the decolourisation could be a secondary metabolic activity linked to the fungus ligninolytic degradation activity [27].

Statistical analysis and selection of the optimal conditions

NemrodW® software provided considerable useful information and reaffirmed the utility of statistical analysis for experiments conduct in order to be decisive when it came to the selection of the optimal conditions for *Chechia* Mix biotreatment. In order to evaluate the effect of each factor on the studied responses, weighting coefficients were generated by NemrodW® software and summarized in Table 2. The regression coefficient b_1 was positive for Y2 and negative for Y1 which indicated that agitation had a positive effect on pollution removal at its high level while its effect is positive at its low level for color removal. On the contrary, the study of the regression coefficients b_2 and b_3 revealed that Mix dilution and inoculum size had positive effects at their high levels for color removal and at their low levels for pollution removal. To summarize, the best conditions for pollution removal were agitation at 100 rpm, mix effluent dilution at 3.3% and biomass inoculation at 10%, whereas the optimal operating conditions for color removal were Mix dilution at 25% and 20% as inoculum size without agitation. As the major and specific pollutant of this wastewater was an azo dye, the most important biotreatment target of this study remained the color removal. Thus, selected optimal conditions for *Chechia* Mix

biotreatment were defined as follows: agitation at 100 rpm, 25% of mix dilution and 20% of inoculum size. Due to the weight coefficients presented in Table 2, mathematical models were also established. The experiences can be described through the following mathematical models in which the factors F1, F2 and F3 were respectively agitation, Mix dilution and the inoculum size; and the responses Y1 and Y2 were respectively the color and pollution removals yields:

Y1 = R$_{OD525}$ = 50.518 - 3.501Agitation +14.024 Mix dilution +1.054Inoculum Size

Y2 = R$_{COD}$ = 77.348 +7.111Agitation - 7.597Mix dilution - 4.591Inoculum Size

(a)

(b)

(c)
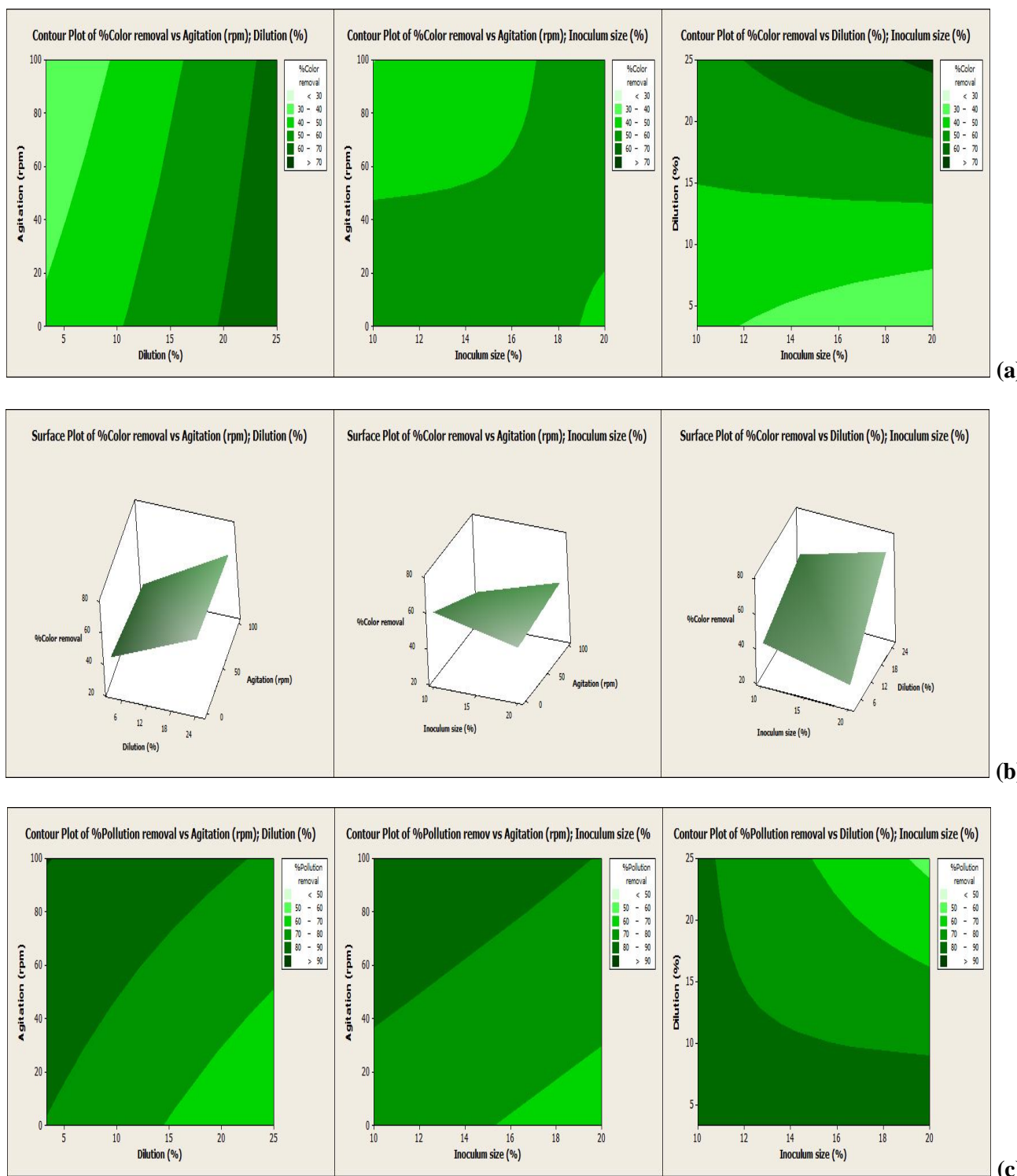
Figure 4. Contour and surface plots (a and b) showing interactions between agitation, Mix dilution and inoculum size on the color removal response. Contour plots (c) presenting interactions between agitation, Mix dilution and inoculum size on the pollution removal response

To investigate the interaction between different factors and visualize how the studied factors affected color and pollution removal after 35 days of experiment, a graphical representation of contour plots was generated using Minitab (Figure 4). The shapes of the contour plots indicated the nature and extent of the interactions. Contour plots of color removal showed in Figure 4.a underlined that color removal was in a range of 40-50% when inoculum size was around 20% combined to agitation at a range of 0-20 rpm. Color removal efficiency increased at a range of 60-70% if agitation at 50-100 rpm was combined to inoculum size between 16-20%. Moreover, color removal exceeded 70% when Mix dilution of 20-25% is combined to agitation of 0-100 rpm or when inoculum size ranged from 15-20 % was combined to Mix dilution 24-25%. Surface Plots of color removal illustrated in Figure 4.b confirmed that high decolourisation rates were strongly correlated with the high level of inoculum size and Mix dilution. The increase of Mix dilution improved decolourisation while high level of agitation could give a positive effect. Agitation enhanced oxygen availability but Ikehata et al. explained also that LiP and MnP production by *P. chrysosporium* could be suppressed because of agitation on submerged culture[28]. On another side, the decrease in COD on *Chechia* Mix led to an increase in the pollution removal rate. Contour plots of pollution removal in Figure 4.c indicated that the pollution removal rate was between 70 and 80% when Mix dilution was around 25%, inoculum size 15-20% and agitation ranged 50-100 rpm. Hence, to maximize pollution removal, the variables must be kept at the lowest tested levels for Mix dilution and inoculation size, but kept at the highest ones for agitation. Regarding the effects of the different factors, Mix dilution and then inoculum size were the largest influencing factors on the color removal, while concerning agitation it was more important to achieve the best pollution removal rate rather than to attend the best decolourisation performance. To summarize, Mix dilution of 25%, inoculum size of 20% and agitation at 100 rpm seemed to be the optimal conditions for *Chechia* Mix biotreatment by *Aspergillus niger*. With no special high energy consumer operating conditions, biotreatment by *Aspergillus niger* seemed to be a viable eco-friendly and low cost process way to remove colors from *Chechia* wastewaters.

Similar results have been found by Kim and Shoda [29] on molasses decolourisation and Asses et al. [19] on olive mill wastewater decolourisation by *Geotrichum candidum*. When studying the interaction between pH and agitation speed for the decolourisation and enzyme synthesis, Asses et al. showed that the utmost of decolourisation was obtained with low tested levels [19]. According to Manai et al [21], the filamentous fungus *Chaetomium globosum* seemed to have a great capacity for dye removal via the biodegradation mechanism. Thus, it should be necessary to investigate, deeply, the relation between decolourisation and extracellular enzyme activities from the isolated *Aspergillus niger*. The biological action of the isolated *Aspergillus niger* on *Chechia* dyes degradation and/or transformation could be further evaluated by FT-IR spectra analysis of the post biotreated *Chechia* effluent at optimal conditions.

FT-IR analysis of Chechia effluents

Infrared spectroscopy is largely employed for qualitative materials characterization. Indeed, the signals obtained from FT-IR are caused by substance-specific vibration modes of the molecules and can be attributed to functional group [21]. Infra red spectra of C*hechia* Mix confirmed the theoretical major presence of the Acid Red 27 dye as Infra red spectrum of the *Chechia* Mix before treatment (Figure 5.a) complies completely with the pure dye one (Figure 5.b).
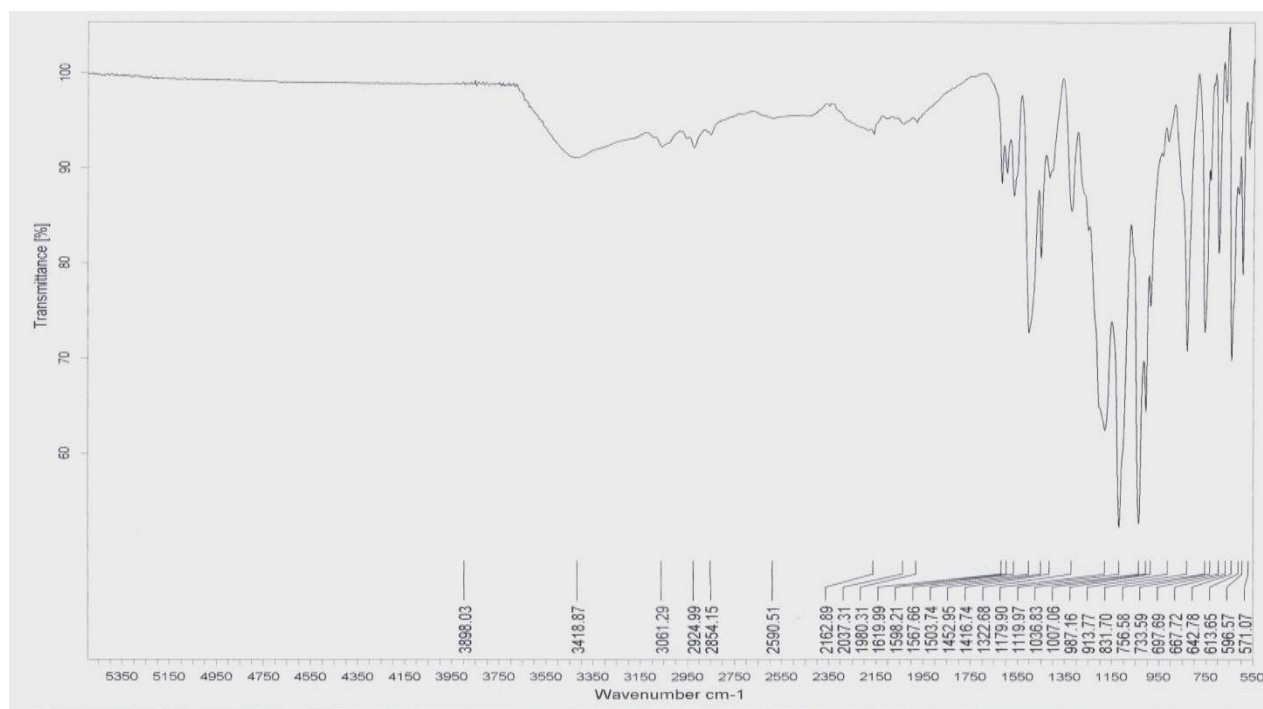

(a)
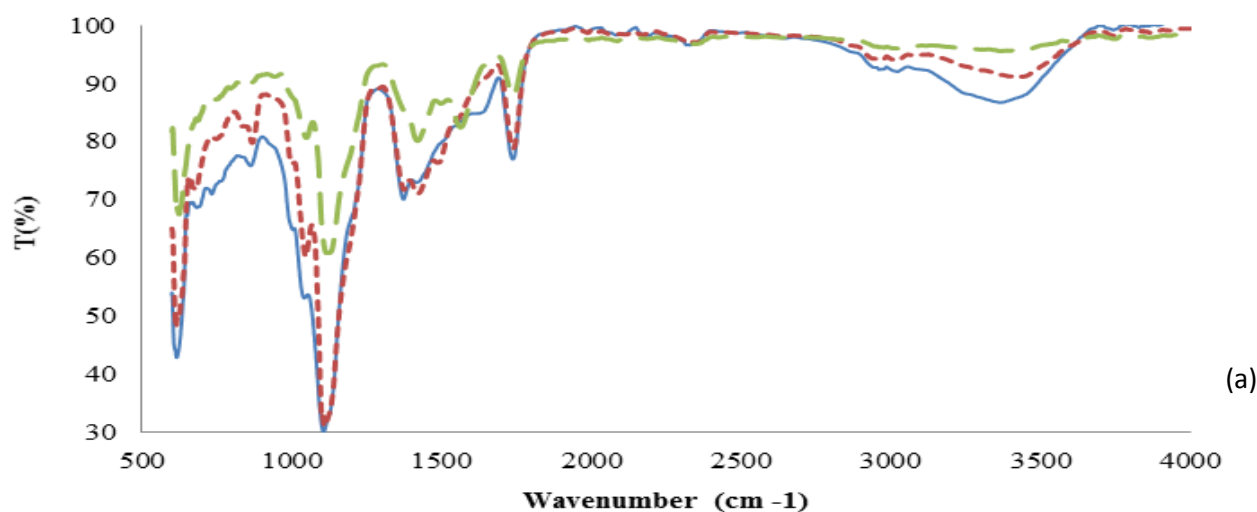
Figure 5. FT-IR spectra of *Chechia* effluents before and after biotreatment (a) under optimized conditions: 20% Inoculum size, T = 30 °C and Agitation = 100 rpm (━━ ━━ untreated Mix, ━ ━ ━ diluted Mix (25%) before treatment, ━━ diluted Mix (25%) after treatment). (b) Infra-red spectra of the major Dye Acid Red 27 $C_{20}H_{11}N_2Na_3O_{10}S_3$

The right section (<1500 cm^{-1}) of an infrared spectrum refers to its "fingerprint", the large number of bands and their nature revealed several structural pieces of information. All the bands in this right section of both spectra (Figure 5.a and b) were found in the same positions and relative intensities confirming that Acid Red 27 dye was the major dye of the *Chechia* Mix. The left section (> 1500 cm^{-1}) included most of the characteristic bands of functional groups. The CH absorptions peaks around 3000 cm^{-1} illustrated the organic nature of *Chechia* Mix effluent. Infrared analysis based on Coates (2000) revealed bands υ C $=$ O (1700 cm^{-1}), ABC by conjugation to 1600 cm^{-1}, υ C $=$ N (1650 cm^{-1}), υ NH (3300 cm^{-1}) and υ CH (3000-3100 cm^{-1}). FT-IR spectra of treated and untreated *Chechia* Mix shown in Figure 5.a. permitted further qualitative investigations on the effect of the best treatment conditions batch bioreactor E8. The FT-IR spectra of untreated dyes showed the specific peaks in the fingerprint region (1500-500 cm^{-1}) for the mono-substitued and para-disubstituted benzene rings[30]. The peak around 1640 cm^{-1} stands for the C=C stretching of the benzene ring. The observed peaks in the region (3200-3500 cm^{-1}) are assigned to the O-H stretching. The dye effluent presented a characteristic peak in the range 1350-1000 cm^{-1} related to the S=O stretch band with a first significant absorbance peak at 1100 cm^{-1} and a second for the C-S stretching vibrations due to sulphur compound bonded to the activated carbons. Comparison of FTIR spectra of *Chechia* effluent dye after decolourisation clearly indicated the

biodegradation of the initial dye compound by *Aspergillus niger*. It can be seen that the bands located within the range 1610 cm^{-1}-1630 cm^{-1} and at 1500 cm^{-1} were due to azo linkages -N=N- on aromatic structures and -N=N- stretching on Acid Red 27. The bands at 1710 cm^{-1} are assigned to the C=O stretching of Acid Red. The characteristic band at 1640 cm^{-1} of C=C of the benzene ring persisted even after the increase of the exposure time with the fungus. However, the bands assigned to aromatic skeletal vibrations have been reduced after biological treatment confirming the degradation of the dyes. The analysis of the spectral characteristics of treated effluent revealed a peak at 1261 cm^{-1} for -C-N- stretch with supporting peak at 1098 cm^{-1} which might be attributed to the formation of primary and secondary amines [30]. The fact that no new peaks appeared between 3300 cm^{-1} and 3500 cm^{-1} attributed to azo bonds and OH groups in α position relative to the azo linkage and in the region between 1340 cm^{-1} and 1250 cm^{-1} (-NH$_2$) after the treatment suggested that the azo linkage could be converted into NH$_3$ or N$_2$ [31]. Moreover, the presence of new peaks at 850 cm^{-1} and 950 cm^{-1} and the peak at 1140 cm^{-1} that could belong to acetate, formats, propionates, benzoates, etc. suggested that the products were subjected to irreversible chemical changes, probably due to concomitant biodegradation and autoxidation reactions of the products formed during the reductive dye degradation [31]. In addition, the persistence of the sulphur groups of sulphonated dyes even after treatment reflects their general stability. These

results suggested the ability of *Aspergillus niger* in the biodegradation of xenobiotics and recalcitrant compounds such as *Chechia* effluent dyes, characterized by a non-specific action on aromatic groups.

Conclusion

    Chechia Mix effluent was first time characterized as highly polluted especially because of its specific dark red color linked to its major pollutant the Acid Red 27 dye. An interesting *Chechia* dye removing *Aspergillus niger*, isolated from the wastewater ecosystem, having a potential application bioremediation of artisanal and industrial dye-rich textile effluents, was cultured and characterized. A full factorial experimental design 2^3 confirmed the *Chechia* Mix biotreatment efficiency using the selected fungal strain and revealed its optimal conditions: agitation at 100 rpm, Mix dilution of 25% and inoculum size of 20%. The optimized biotreatment assays reached 70.18±2.84% in terms of color removal, and 74.17±14.52% of COD reduction. Decolourisation by biodegradation of the major dye Acid Red 27 was first time bioremediation of *Chechia* wastewater as an eco-friendly and low cost method to solve a national environmental issue.

Acknowledgements

References

1- A. B. dos Santos, F. J. Cervantes, J. B. Van Lier, Bioresour Technol., **2007**, 98, 2369-2385.

2- A. Alinsafi, F. Evenou, E. Adbulkarim, M. Pons, O. Benhammou, A. Yaacoubi, Dye Pig., **2007**, 4, 439-445.

3- B. Lodha, S. Chaudhari, J. Hazard. Mater., **2007**, 148, 459-66.

4- M. Solís, A. Solís, H. I. Pérez, N. Manjarrez, M. Flores, Process Biochem., **2012**, 47,1723-1748.

5- V. K.. Gupta, Suhas, J. Env. Manag., **2009**, 90, 2313-2342.

6- S. Erum, S. Ahmed, African J. Biotechnol., **2011**, 10, 3399-3411.

7- M. Sudha, A. Saranya, G. Selvakumar, N. Sivakumar, Int. J. Curr. Microbiol. App. Sci., **2014**, 3, 670-690.

8- Y. Fu, T. Viraraghavan, Water Qual. Res. J. Canada, **2000**, 35, 95-111.

9- P. Blanquez, N. Casas, X. Font., M. Gabarrell, M. Sarrá, G. Caminal, Water Res., **2004**, 38, 2166-2172.

10- F. Ay, Cocay, E. Catalkaya, F. Kargi, J. Hazard. Mater., **2009**, 162, 230-236.

11- G. M. B. Soares, M. T. Pessoa Amorim, R. Hrdina, M. Costa-Ferreira, Process Biochem., **2002**, 37, 581-587.

12- N. Ali, Ikramullah, G. Lutfullah, A. Hameed, S. Ahmed, World. J. Microbiol., **2008**, 24, 1099-1105.

13- C. L. R. Jaidev, G. Narasimha, Asian J. Micro., Biotech. Env. Sci., **2010**, 12, 353-357.

14- S. A. Omar, J. Env. Sci. Tech., **2016**, 9, 149-156.

15- N. Saitou, M. Nei, Mol. Biol. Evol., **1987**, 4, 406-425.

16- K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, Mol. Biol. Evol., **2013**,30, 2725-2729.

17- J. Wang, K. Li, C. Yang, Y. Wang, J. Jia, Electrochem. Commun., **2012**, 18, 51-54.

18- J. Goupy, Anal. Chim. Acta., **2005**, 544,184-190.

19- N. Asses, L. Ayed, H. Bouallagui, I. Ben Rejeb, M. Gargouri, M. Hamdi, Bior. Technol., **2009**,100, 2182-2188.

20- R. Khlifi, L. Belbahri, S. Woodward, M. Ellouz, A. Dhouib, S. Sayadi, T. Mechichi, J. Hazard. Mater., **2011**,175, 802-808.

21- I. Manai, B. Miladi, A. El Mselmi, I. Smaali, A. Ben Hassen, M. Hamdi, H. Bouallagui, J. Environ. Manage., **2016**,170, 8-14.

22- R. A. Samson, P. Noonim, M. Meijer, J. Houbraken, JC. Frisvad, J. Varga, Stud. Mycol., **2007**, 59, 129-145.

23- M. L. Abarca, F. Accensi, J. Cano, F. J. Cabañes, Antonie Van Leeuwenhoek, **2004**, 86, 33-49.

24- J. C. Frisvad, T. O. Larsen, R. Vries, M. Meijer, J. Houbraken, F. J. Cabañes, K. Ehrlich, R. A. Samson, Stud. Mycol., **2007**, 59, 31-37.

25- Y. Fu, T. Viraraghavan, Bioresour. Technol., **2001**,793, 251-262.

26- E. Khelifi, H. Bouallagui, Y. Touhami, J. Godon, M. Hamdi, Desalin.Water Treat., **2009**, 2, 310-316.

27- R. Shyamala Gowri, R. Vijayaraghavan, P. Meenambigai, Int. J. Curr. Microbiol. App. Sci., 2014, 3, 421-436.

28- K. Ikehata, I. D. Buchanan, D. W. Smith, J. Environ. Eng. Sci., **2004**, 3, 1-19.

29- J. Kim, M. Shoda, Appl. Environ. Microbiol., **1999**. 65, 1029-1035.

30- J. P. Coates, P. H. Shelley, *Encyclopedia of Analytical Chemistry*, Ed. R.A. Meyers. J. Wiley & Sons, Ltd., Chichester, UK, **2000**, 8217-8240.

31- E. Franciscon, A. Zille, F. Dias Guimaro, Inter. Biodet. Biodeg. , **2009**, 63,280-288.