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Study on the influence of high salts content on fungal treatment of saline wastewaters

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ABSTRACT

We investigate in this study an assay of aerobic treatment of wastewaters from the sea-food-processing industry with selected strains of white rot fungi. Because the effluent was highly charged with salts, the effect of high salts concentrations on growth and enzyme production for *Trametes trogii* and *Phanerochaete chrysosporium* was firstly studied. Results showed that the two selected strains tolerated high concentrations of NaCl up to 20 g l^{-1} for both mycelia growth and enzymes production. The production of laccase by *T. trogii* was inhibited by 50% for 30 g l^{-1} of added NaCl (512 mM). The enzyme production inhibition was less important for MnP and LiP produced by *P. chrysosporium*. On the other hand, the industrial wastewaters, not highly charged with organic matter (COD = 3 g l^{-1}), contained 25 g l^{-1} of salts and 0.3 g l^{-1} of ammonia. But, the wastewaters were toxic according to microtoxicity and phytotoxicity tests since the percentage of bioluminescence inhibition of *Vibrio fischeri* (% BI) was 60% and the germination index of *Lepidium sativum* not exceeded 40%. The two strains grew on 80% of the effluent and the enzymes production was less than the enzymes production in the case of the control. This effluent concentration partially inhibited the COD removal efficiency but the strains were able to decrease the amount of organic matter contained up to 90%. Furthermore, the toxicity exhibited for the raw effluent was removed and the treated wastewaters by the two strains remained non toxic.

Keywords: *Phanerochaete chrysosporium*; *Trametes trogii*; Salts; Sea-food-processing; Wastewaters treatment

1. Introduction

Sea-food-processing industries generate large amounts of highly polluted wastewaters [1,2]. These wastewaters, rich in both organic matter and total dissolved solids (TDS), are difficult to treat using conventional biological wastewater treatment processes [1]. Until recently, little attention has been paid on the treatment of such effluents and only a rough removal of solids and fats has been carried out [3]. Although, the characteristics of effluents largely depend on the raw material and the type of the process employed, usually, they have high content of chemical oxygen demand (COD) and proteins and a very high salinity. The presence of high sodium and/or chloride concentration was considered as inhibitory for the anaerobic wastewater treatment [4]. Consequently, the use of halophilic bacteria is required [5]. The interest in treating that kind of wastewaters is growing at a fast rate [6].

Therefore, the use of salt tolerant microorganisms in biological wastewaters treatment systems could be a solution for COD removal from saline wastewaters.

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Most of the studies focused on biological saline wastewaters treatment are based on the use of aerobic halophilic microorganisms and technologies [7]. The COD removal efficiencies in the biological treatment of saline wastewaters are usually low because of inhibitory effects of salt on microbial flora. Salt (NaCl) concentrations more than 1% cause plasmolysis of cells resulting in reduced biological activity. A number of studies have reported on adverse effects of salts on microorganisms [8].

Many industrial wastewaters were found to be highly charged with salts content [9]. Treatment of saline wastewaters has been not easy. With high saline content, the wastewater invariably has a rather high electric conductivity because of the presence of anions and cations in the aqueous solution [10]. Biological treatment of such effluents is usually of great importance and minimizes considerably the amount of organic pollutants contained in wastewaters. On the other hand, biological treatment using fungi [11], mainly, the white rot group among them [12] is reported as successful. These fungi have developed a non-specific oxidative system to degrade organic substrates including extracellular oxidoreductases such as laccases, peroxidases, tyrosinases, low molecular weight metabolites and activated oxygen species [13].

Their enzymes are produced in optimal conditions and media [14]. The physiological requirements of white rot fungi are variable. Hence, considerable research has been carried out on the influence of many factors such as agitation, pH, nitrogen level, and other culture conditions for the production of ligninolytic enzymes [15] but very few studies focused on the inhibitory effects of salts and demonstrated the variability of inhibitory concentrations with species [13,16]. Fungal strains differ in their sensitivity towards salts, heavy metals and organics and in the protection mechanisms involved [17]. However, many studies reported salts tolerance of some fungal species [17–21].

The aim of this study is to investigate the effect of high salts content on the treatment of saline industrial wastewaters by selected strains of basidiomycetes.

2. Material and methods

2.1. Chemical solutions preparation and effluent sampling

NaCl solution was added at 10, 20, 30 and 40 g l^{-1} respectively from a sterilized stock solution prepared at 200 g l^{-1}. The effluent was generated from a seafood-processing industry in Sfax region, Tunisia. If not immediately analysed, samples were stored at 4 °C until use.

2.2. Culture conditions and activities determinations

2.2.1. Stains and culture conditions

The strain *Phanerochaete chrysosporium* HD used in this study was an isolate from strain BKM-F-1767 (ATCC 24725). The strain was cultivated on a basal optimized medium as described by Ellouze et al. [22]. *T. trogii* (CTM 10156) was isolated in our laboratory from a Tunisian biotope by Dhouib et al. [23]. The strain was cultivated on an optimized producing laccase medium described by Ellouze et al. [18]. Strains were maintained at 4 °C on malt extract Agar medium. The choice of the two strains was based on the originality and the wide range use of each basidiomycete. Firstly, *P. chrysosporium* secreted Manganese Peroxidase and Lignine Peroxidase, which are described as enzymes well involved in the treatment of various effluents and many problematic compounds. Secondly, *T. trogii* produces a large amount of laccases (reaching 200,000 U l^{-1}) which are well involved in biodegradation and biodetoxification of many industrial effluents.

2.2.2. Growth measurements

Growth was measured in terms of dry weight of washed mycelium after filtration and drying an overnight at 105 °C on glass-fiber filters (GF/D Whatman Inc.). Yields were expressed as grams of dry weight mycelia per litre of culture.

2.2.3. Enzymatic activities

Laccase activity was determined according to [24] and using ABTS (2; 2_-azinobis (3-ethylbenzthiazoline-6 sulphonic acid)) as substrate. The oxidation of ABTS by laccase leads to a green product and increases the absorbance measured at 420 nm.

MnP activity was determined using the vanillylacetone oxidation assay [25]. We detected the oxidation of vanillylacetone (substrate) which increases the absorbance measured at 334 nm.

LiP was assayed according to [26] using veratryl alcohol oxidation increasing the absorbance measured at 310 nm.

Enzymatic activities were determined using the coefficient of extinction expressed in units per litre of laccase, MnP and LiP, respectively.

2.3. Toxicity determination

Microtoxicity was carried out with *Vibrio fischeri* (luminescent bacteria LCK 480) using LUMIStox 300 measuring instrument, according to ISO, 1998 [27]. The

inhibition of the bioluminescence of *V. fischeri* using the LUMIStox test kit was determined as reported by Dhouib et al. [28]. Phytotoxicity was estimated by the determination of the germination index of *Lepidium sativum* seeds according to Zucconi et al. [29].

2.4. Physico-chemical determinations of the effluent

Electric conductivity (EC) and pH were measured using a conductivity-meter (Consort C 831) and a pH-meter (Metrohm), respectively. Biological oxygen demand (BOD_5) was determined after 5 days by the manometric method with a respirometer [BSBController Model 620 T (WTW)] according to APHA [30]. COD was estimated as described by Knechtel [31]. Samples were centrifuged at 4,000 rpm for 20 min. A total reflux digestion was achieved by reaction with H_2SO_4 and potassium dichromate at 150 °C for 2 h. COD was then determined by dosage with Mohr salt (N/40) and with ferroine as indicator. The total Kjeldahl nitrogen content (TKN) and N-NH_4^+ were analysed and quantified as described in Kjeldahl-N method [32]. Dry matter (DM), total suspended solids (MLSS), volatile matter (VM) and mixed liquor volatile suspended solids (MLVSS) were determined according to APHA [33]. Heavy metals and metals determination was carried out with flame atomic absorption of samples previously digested with an acid mixture of HCl and HNO_3.

3. Results and discussion

In a previous study, the treatment of non-diluted complex wastewaters (landfill leachates) by selected white rot fungi was unachieved because the effluent exhibited high toxicity. Studies were focused on the main causes of the treatment inhibition. The objective of this study was to demonstrate if the presence of high sodium chloride concentration would affect the fungal treatment efficiency of wastewaters from the sea-food-processing industry.

3.1. Effect of NaCl on P. chrysosporium and T. trogii growth and enzyme secretion

The effect of the sodium chloride concentrations on fungal growth and on laccase, MnP and LiP activities was studied. Experiments were carried out, as described in Materials and Methods section, at 10 g l^{-1}, 20 g l^{-1}, 30 g l^{-1} and 40 g l^{-1} of NaCl added to the synthetic media since salts concentrations founded in wastewaters from sea-food-processing industries are high and can reach 40 g l^{-1}.

Table 1
Biomass quantification of *P. chrysosporium* and *T. trogii* as function of added NaCl (g l^{-1}) to the synthetic media

[NaCl] (g l^{-1})	*P. chrysosporium*	*T. trogii*
0	4.68 ± 0.5	15.50 ± 1.50
10	4.21 ± 0.4	12.25 ± 1.30
20	4.46 ± 0.4	12.50 ± 1.20
30	4.04 ± 0.4	9.20 ± 1.00
40	4.29 ± 0.4	7.99 ± 0.80

The synthetic medium optimized for *P. chrysosporium* growth did not contain sodium chloride but an amount of 1 g of sodium was present in the medium when associated to the tartrate at 10 mM. The pH and the enzymes production were determined during the process. Toxicity was also evaluated and the biomass was quantified at the end of the culture. During the culturing time, the pH decreased from 5.5 to 4.4 for all sodium chloride containing cultures as well as for the control. The process of acidification, sign of a normal growth of the strain, seemed to be achieved independently of the presence of sodium chloride. The differences in values were not significant and could be associated to the experiment errors as shown in Table 1. The strain of *P. chrysosporium* showed tolerance to salts up to 20 g l^{-1}. Therefore, higher concentrations of sodium chloride (30 and 40 g l^{-1}) affected the enzyme production. The production of MnP was important for 10 and 20 g l^{-1} containing cultures for the two concentrations cited (Fig. 1a). The maximum of production reached 712 U l^{-1} for the control and 643 U l^{-1} for 20 g l^{-1}, at the 9th day. For 30 and 40 g l^{-1}, the production of MnP decreased by 50% and 80%, respectively. Then, a concentration of 30 g l^{-1} exerted an inhibitory effect on the MnP production. For LiP, the production was more important for the control (144 U l^{-1}) than for the 20 g l^{-1} containing culture (116 U l^{-1}) at the 8th day. The production of LiP was decreased by 50% and 67% for 30 and 40 g l^{-1}, respectively (Fig. 1b). This inhibitory behaviour was not similar to the biomass production where the reduction was not significant between the control and culture containing NaCl at concentrations up to 30 g l^{-1} as mentioned in Table 1.

For the strain *T. trogii*, the monitoring of pH evaluation showed that the decrease of this parameter was similar for the control as well as for cultures containing 10 and 20 g l^{-1} of NaCl (data not shown). The production of *T.trogii* biomass depend on the NaCl concentration. However, 20 g l^{-1} seemed not to be an inhibitory concentration since the biomass obtained for 20 g l^{-1} was little less than the control (15.5 g l^{-1} for the control and 12.5 g l^{-1} for 20 g l^{-1}). A concentration of 40 g l^{-1} of

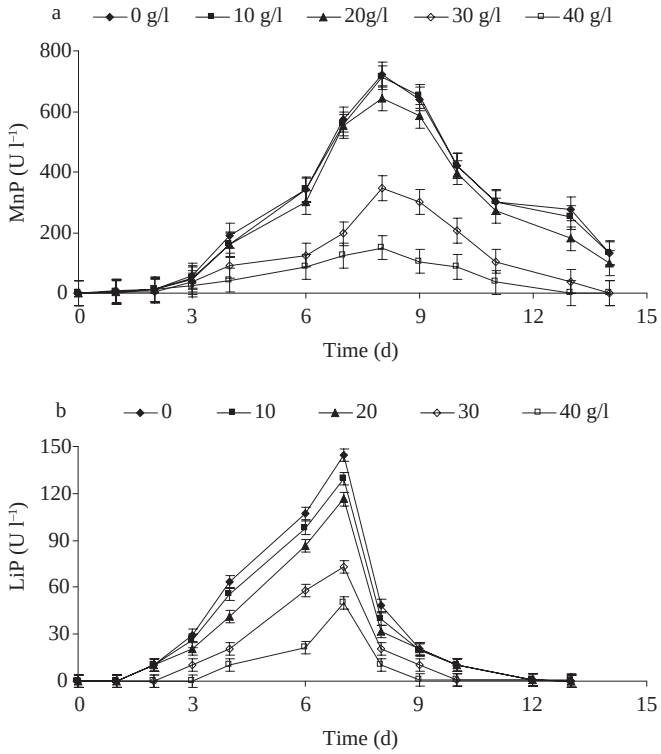

Fig. 1. MnP (a) and LiP (b) activities of *P. chrysosporium* cultures containing NaCl (◆) : 0 g l^{-1}, (■) : 10 g l^{-1}, (▲) : 20 g l^{-1}, (◇) : 30 g l^{-1}, (□) : 40 g l^{-1}

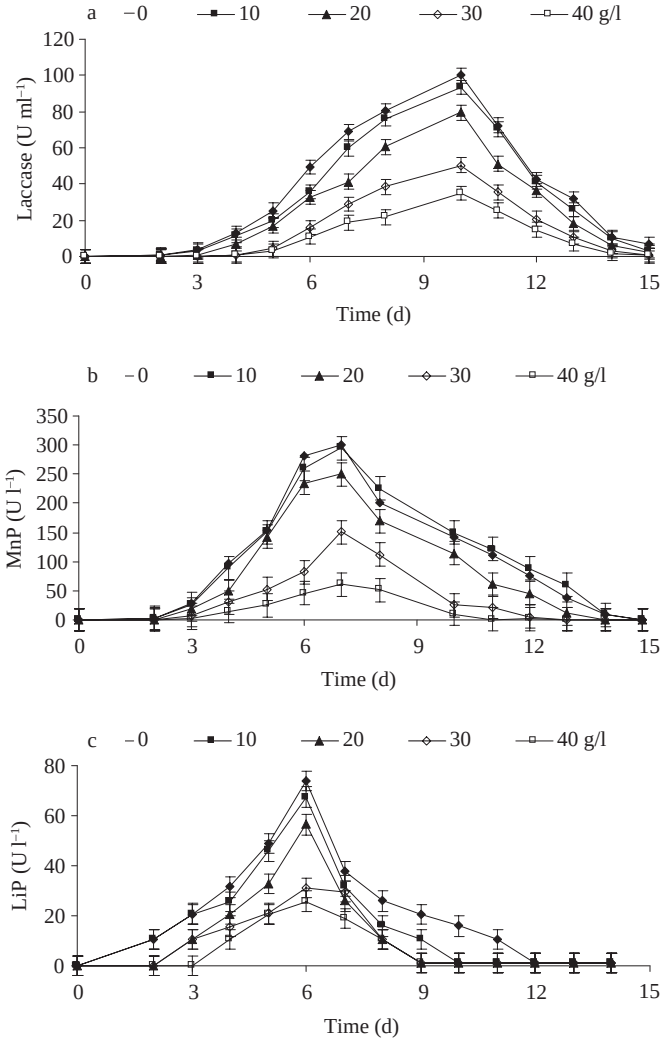

Fig. 2. Laccases (a), MnP (b) and LiP (c) activities of *T. trogii* cultures containing NaCl (◆) : 0 g l^{-1}, (■) : 10 g l^{-1}, (▲) : 20 g l^{-1}, (◇) : 30 g l^{-1}, (□) : 40 g l^{-1}.

NaCl caused 50% of biomass production inhibition (Table 1).

It has been reported that tolerance to salts in term of fungal growth differs with fungal species. Hence, according to Thangavelu, biomass production of *Aspergillus foetidus* was found to be promoted at 10 ppm of salt but was reduced at high salinity level (500 ppm) [19]. These results suggest that low concentrations of salts appear to promote the growth of the fungi, whereas higher concentrations affected intracellular growth activities. In contrast, Leitão et al. reported that *Penicillium chrysogenum* was halotolerant and was able to grow in a nutrient-rich medium with 5.8% NaCl (1 M) [34]. This strain was able to mineralize phenol and to reduce phenol toxicity in medium containing 1 M of NaCl. In any case, for some species, these cellular responses were long-term reactions, which would be consistent with the need for the organism to produce osmo-protective compounds for cell wall integrity and maintenance of vacuolar morphology. Salt stress can imbalance the osmotic potential in fungi cells generating a water deficit and the influx of sodium may lead to metabolic toxicity. It has been observed that salinity stress affects the growth but not the energy metabolism of the organism [19].

On the other hand, *T. trogii* showed tolerance to NaCl at a concentration reaching 20 g l^{-1}, for its enzymes production. The same laccase activity in the control was obtained in cultures containing 10 g l^{-1} of NaCl, without delay in the production timing. The inhibition of this enzyme not exceeded 20% for medium containing 20 g l^{-1}. For 30 g l^{-1} containing cultures, the production of laccase was important but the inhibition reached 50% comparing to the control. This concentration of NaCl seems to be an inhibitor concentration of laccase secretion (Fig. 2a). This finding was not in agreement with the literature since Abadulla et al. have reported that the *Trametes hirsuta* laccase retained 50% of its activity at 50 mM NaCl (2.9 g l^{-1}) for static culture and at 85 mM for immobilized enzyme which tolerated a higher NaCl

concentration [13]. Other authors have reported a wide IC_{50} range, between 0.4 and 600 mM for Cl_2, for fungal laccases [21] (Yaropolov et al.). It has been suggested that the magnitude of inhibition of laccases by halides depends on the accessibility of the copper atoms and can thus vary between different laccases and inhibitors [35]. Among various halides, F_2 was the strongest inhibitor for the *T. hirsuta* laccase, followed by Cl_2 and Br_2. The same trend has been previously observed for a number of fungal laccases [35]. Especially, Trovaslet demonstrated that LAC-1 activity was slowly decreased in presence of salts (NaCl or Na_2SO_4) and that this enzyme was more stable in presence of Na_2SO_4 and it is noteworthy that its stability was enhanced rather than inhibited by chloride [16]. Currently, only a few comparative data are available concerning the activity and/or the stability of laccases at different ionic strengths. The effects of ionic force on LAC-1 activity seem to be dependent on the nature of the added salt (chloride, sulphate). But, the addition of chloride ions usually inhibits the catalytic activity of the enzyme, while recently NaCl has also been reported as an activator of *Bacillus halodurans* laccase activity as reported by Ruijssenaars and Hartmans [36]. On the other way, according to Valentín et al., salinity characteristic of sea water (3.2%) had minimal effects on ligninolytic activity of *Irpex lacteus* and *Lentinus tigrinus*, while in *Bjerkandera adusta*, activity was inhibited by salinity levels of 32 g l^{-1} [36]. Trovaslet demonstrated that laccase activity of *Pycnoporus sanguineus* retained more than 50% of its activity in presence of salts concentrations up to 1 M (58 g l^{-1} of NaCl) [16]. Moreover, its stability was enhanced rather than inhibited by chloride.

At the same time, MnP production was not influenced by the presence of sodium chloride up to 20 g l^{-1} since the production inhibition didn't exceed 20% for this concentration of NaCl. The inhibition reached 50 and 80% for 30 and 40 g l^{-1} (Fig. 2b). In the same way, the production of LiP by *T. trogii* was not affected by the presence of this salt at 20 g l^{-1} in the medium. 68 U l^{-1} and 56 U l^{-1} were detected in 10 and 20 g l^{-1} containing cultures respectively, while the production was about 74 U l^{-1} for the control as shown in Fig. 2c. On the other hand, 30 g l^{-1} caused more than 50% of LiP production inhibition.

Moreover, the added concentrations didn't cause any toxicity to *V. fischeri* for both used strains (data not shown). This can be explained by the tolerance of this marine strain to salts. That is, even it exhibited any toxicity to *P. chrysosporium* or to *T. trogii*, it wouldn't be manifested in toxicity evaluated by *V. fischeri*.

Table 2
Characterization of the wastewaters from the sea-food-processing industry and Tunisian Standards for discharge in public environment

	Sea-food industrial wastewaters	Tunisian standards of discharge
pH	6.3 ± 0.2	6.5–6.8
EC (mS cm^{-1})	29.0 ± 3.0	–
Salts (g l^{-1})	25.0 ± 2.4	–
COD (g l^{-1})	3.10 ± 0.3	1
BOD_5 (g l^{-1})	1.90 ± 2.0	0.4
BOD_5/COD	0.7 ± 0.08	–
SS (g l^{-1})	2.7 ± 0.20	0.4
DM (g l^{-1})	3.55 ± 3.5	–
VM (g l^{-1})	1.96 ± 2.2	0.4
VSS (g l^{-1})	1.78 ± 0.18	–
TNK (mg l^{-1})	987 ± 95.0	100
NH_4^+ (mg l^{-1})	310 ± 30	100
SO_4^{2-} (g l^{-1})	2.65 ± 0,28	0.4–0.6
Ca^{2+} (mg l^{-1})	15.0 ± 1.5	500
Mg^{2+} (mg l^{-1})	32.0 ± 3.2	300
Na^+ (mg l^{-1})	59.0 ± 5.4	1,000
K^+ (mg l^{-1})	9.5 ± 0.7	50
BI (%)	60 ± 10	–
GI (%)	40 ± 5.0	80

3.2. Treatment of saline wastewaters by T. trogii and P. chrysosporium: influence of excessive salinity on the fungal treatment process

3.2.1. Characterisation of wastewaters from a sea-food-processing industry

The physical-chemical characterisation of many samples of wastewaters from a sea-food-processing industry is mentioned in Table 2. These wastewaters were quietly charged with organic matter (COD = 3.12 g l^{-1}). This organic matter is biodegradable since the BOD_5/COD ratio is equal to 0.66. In the other hand, the effluent was highly charged with salts, with a concentration reaching 25 g l^{-1}. But, it contained low concentration of ammonia which not exceeding 310 mg l^{-1}. Moreover, the effluent showed high level of toxicity for microorganisms and plants since the % BI of V. *fischeri* reached 60% and the GI of *L. sativum* didn't exceed 40%. The concentrations of the monitored parameters are under Tunisian standards for discharge. The treatment of such wastewaters is imperative before their discharge in the environment. Because salts could inhibit the performance of wastewaters treatment with conventional microorganisms, the fungal treatment of such wastewaters using the two halotolerant basidiomycetes may be a solution for biodegradation and detoxification.

Table 3
Biomass quantification (g l^{-1}) of *P. chrysosporium* and *T. trogii* cultivated on different concentrations of effluent

[Effluent] (%)	*P. chrysosporium*	*T. trogii*
0	5.68 ± 0.6	15.50 ± 1.5
30	4.96 ± 0.5	14.50 ± 1.5
50	4.54 ± 0.4	10.9 ± 1.0
80	4.39 ± 0.4	8.99 ± 0.8

3.2.1. Fungal treatment of wastewaters from a sea-food-processing industry

In order to treat these wastewaters, we choose the biological treatment with the two strains of basidiomycetes (T and Pc) were chosen for their their tolerance to high salt concentrations, to treat biologically the saline wastewater. The application of this treatment assay was successful when the effluent was quietly diluted (80% of wastewaters). The presence of salts and ammonia would not affect the biodegradation process. In fact, we obtained normal growth of the two strains on the effluent since the production of biomass reached 4.4 and 9 g l^{-1} for *P. chrysosporium* and *T. trogii*, respectively (Table 3).

Fig. 3 represents the production of enzymes by the two basidiomycetes during the treatment of saline

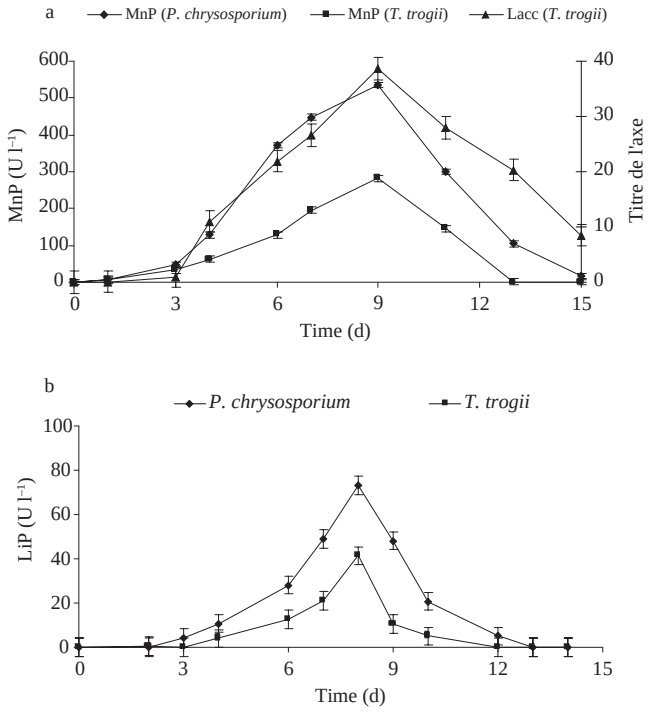

Fig. 3. Laccases, MnP (a) and LiP (b) activities of *P. chrysosporium* and *T. trogii* cultured on 80% of effluent.

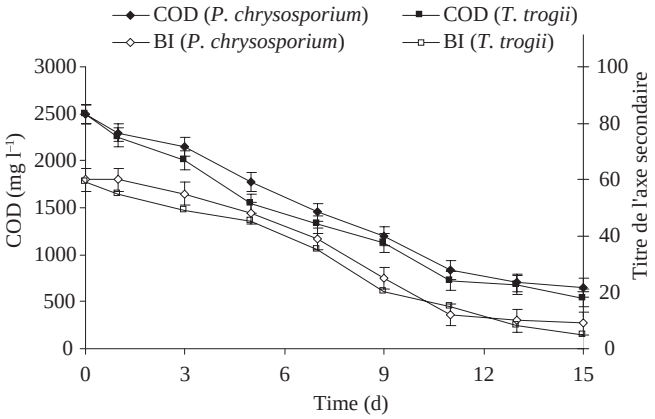

Fig. 4. COD and toxicity evaluation during the treatment process of the effluent.

industrial wastewaters. Results showed that *P. chrysosporium* produces LiP and MnP at its enzymes which are LiP and MnP at relatively high levels. In fact, the maximum productions reached are 535 U l^{-1} for MnP, and a LiP activity approximately equal to the control and 73,2 U l^{-1} for LiP. For the later enzyme, the amount produced was less than that found in the control case.

On the other hand, the strain *T. trogii* produced a large amount of laccase reaching 38,700 U l^{-1}. This concentration of produced enzyme was similar to the amount produced when the synthetic medium was added with 20 g l^{-1} of NaCl. We have also obtained important production of MnP and LiP (282 U l^{-1} and 41.5 U l^{-1}, respectively). But, the inhibition was clearer for de la the LiP secretion. This would be attributed to the high salt concentration which was responsible of the LiP production decrease. This was demonstrated when studying the effect of high concentrations of salts on enzymes production.

Thus, this important production of enzymes for the two strains was responsible of the decrease of the COD effluent which was important, too. This was observed according to the monitoring of COD effluent which was synchronised with the enzymes production. Results of this monitoring showed an important reduction of the COD, reaching 74% and 78% for *T. trogii* and *P. chrysosporium*, respectively, at the end of the treatment (Fig. 4). Consequently, a considerable decrease of the toxicity was obtained with the strain *V. fischeri*. In fact, after 7 days of treatment, the toxicity was reduced by 50%, for the two strains. A total detoxification was achieved after 9 days of the wastewaters treatment involving the two basidiomycetes when the percentage of BI was less than 20% as shown in Fig. 4.

As conclusion, the two strains of basidiomycetes were able to remove the organic matter as well as the toxicity at high levels and the percentages of these

reductions for the two strains were similar while they didn't produce the same enzymes. Consequently, we can suppose that the activity of these enzymes wasn't specified on the biodegradation process of organic pollutants present in this effluent.

4. Conclusion

The behaviour of two strains of white rot fungi *P. chrysosporium* and *T. trogii* to additional sodium chloride was carried out in this study in order to study the effect of high salts concentrations on fungal treatment of saline wastewaters.

The two strains *P. chrysosporium* and *T. trogii* tolerate an important concentration of added NaCl up to 20 g l^{-1}. The mycelium growth was not inhibited and the enzymes secretion was not affected. Above this concentration, the strains showed mycelia growth inhibition and retard enzymes secretion with an important inhibition of the production. Laccase, produced by *T. Trogii*, was quietly more affected by the presence of this salt.

As a consequence, the organic matter of the saline wastewaters was reduced by the two strains. The COD removal reached 75%. On the other hand, the two strains were able to reduce the toxicity of the effluent which reached 60% for the raw wastewaters. They allowed a total detoxification of the effluent after 9 days of treatment.

We can also conclude that the concentration of ammonia found in these wastewaters, which not exceeded 0.3 g l^{-1}, not affected the fungal growth neither the production of enzymes involved in the biodegradation and detoxification process.

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