

Comparison of *Heterobasidion Insulare* with white rot fungi in Degradation wastewater of Pesticide plant

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Abstract. A *Heterobasidion Insulare* ability to degrade in different culture conditions was investigated and compared to that of *P. chrysosporium*. The results indicated that *H. Insulare* isolate is more efficient than *P. chrysoasporium* in decolorizing in the presence of added nutrients. *H. Insulare* is able to remove more than 50% of the color and phenols from Pesticide plant with in 6 days of incubation, whereas *P. chrysosporium* needs more than 12 days to reach similar results in the same conditions. Many factors affecting the treatment of diluted waste water of Pesticide plant (30%) by *H. Insulare* were studied, including the effects of added nutrients, initial pH, and temperature and inoculated biomass. Once the optimization of 30% waste water of Pesticide plant biodegradation process had been set up, higher waste water of Pesticide plant concentrations (50%) were tested. The results show that the fungus is capable of reducing all parameters analyzed at least 60%, after only 9 days of growth.

Introduction

Large amounts of pesticide is produced and used in China. Pesticide industry generates approximately 150 million tons of wastewater annually. Pesticide wastewater contains high concentrations of pollutants, the biological oxygen demand (BOD) and the chemical oxygen demand (COD) of this waste may be as high as 100 g/l and 200 g/l, respectively; moreover, the concentration of phenolic and polyphenolic compounds can exceed 10 g/l. Most of the color of waste water of pesticide plant is due to the aromatic compounds that are present and which have phototoxic and antibacterial effects [1]. Many difficulties have been encountered during aerobic and anaerobic bacterial treatment processes of this waste; therefore the elimination of phenols from waste water of Pesticide plant is an important objective in order to reduce its toxicity and to permit the occurrence of microbial fermentation. Recently, potential applications of white-rot fungi and their enzymes are gaining increasing importance in the detoxification of industrial waste waters and of a vast range of xenophobic environmental pollutants [2]. A strain of *H. Insulare* has been reported to efficiently decolorize Pesticide wastewater only when cultures are flushed with pure oxy-gen and supplemented with verity alcohol as inducer of the ligninolytic system. Furthermore, it is reported that the *P. chrysosporium* mycelium is able to remove phenols and to detoxify 10% diluted waste water of Pesticide plant in the absence of any external added nutrient. Nevertheless, it has also been reported that the addition of simple or complex nutrient is essential in order to obtain an efficient decolonization. The present work was aimed at studying the ability of a white-rot fungus of *H. Insulare*, isolated from *H. Insulare* strain was isolated from fallen wood of *Korean pine* in forest of Xiaoxing'anling and classified as *H. Insulare*, to modify the polluting properties of diluted Pesticide wastewater in comparison with that of *P. chrysosporium* [3]. Optimal conditions for the utilization of *P. chrysosporium* in the treatment of Pesticide wastewater were also explored.

Materials and methods

Pesticide wastewater was collected from a pesticide factory, Jiamusi City, northeast China, centrifuged at 5,000 rpm for 10 min to eliminate solids and Insoluble matters, and stored at -20°C. Strain and culture conditions Two different fungal strains were used in the pesticide wastewater

treatment experiments: a strain isolated using the agar plate technique and classified by PDA. Fungi were maintained through periodic transfer at 4°C on potato-glucose (2.4%) agar plates in the presence of 0.5% yeast extract. Incubations in liquid medium of both fungi were carried out at 28°C in the dark by pre inoculating 300ml of potato–dextrose broth (2.4%) containing 0.5% yeast extract in 1 l shaken flasks with *P. ostreatus* or *H.Insulare* mycelia. After 5 days culture, samples of 25 ml of culture were transferred into 1 l flasks containing 250 ml of different media [4]. Cultures were incubated at 28°C under continuous agitation (120 rpm).

Enzyme assays

Lignin peroxidase (LiP) activity was determined using vertebral alcohol as substrate. The assay mixture contained 2.0 mM vertebral alcohol and 0.4 mM H₂O₂ in 50 mM sodium titrate buffer, pH 2.5. Oxidation of vertebral alcohol was followed by measuring the absorbance increase at 310 nm. Manganese peroxidase (MnP) activity was determined using MnSO₄ as substrate. The assay mixture contained 0.5 mM MnSO₄, and 0.5 mM H₂O₂ in 50 mM sodium malamute buffer, pH 4.5. Oxidation of Mn₂⁺ to Mn₃⁺ was followed by measuring the absorbance increase at 270 nm due to the formation of Mn₃⁺-malonate. Enzyme activity was expressed in IU. Laccase activity was determined using ABTS as substrate. The assay mixture contained 2 mM ABTS in 0.1 M sodium citrate buffer, pH 3.0. Oxidation of ABTS was followed by measuring the absorbance increase at 420 nm. Enzyme activity was expressed in IU.

Determination of phenol content

Phenol concentration was determined by a colorimetric assay as described based on the oxidative coupling of phenol and 4-amino-antipyrine [5].

Chemical analyses

Analyses of pH, suspended matter, chlorine (Cl⁻), potassium (K⁺), ammoniac nitrogen (NH₄⁺), COD were determined according to APHA standards methods [6].

Results

The composition of pesticide wastewater chemical characteristics of the crude waste used in this study is shown in Table1. The presence of the reported concentrations of polluting substances, particularly phenols (12.0g/l) and other organic compounds (COD, 50g/l), noticeably reduces the ability of most micro organisms to grow on this waste. Treatment of pesticide wastewater by *Pl.ostreatus* and *H.Insulare* In order to study the effects of fungal treatment on pesticide wastewater, two different white-rot fungi were tested in batch cultures of diluted pesticide wastewater (20%), supplemented with potato dextrose (0.24%), yeast extract (0.05%) and maltose (1%). Both fungi are able to decolorise 20% pesticide wastewater in the presence of added nutrients; *Pl. ostreatus* and *H.Insulare* cause about 65% and 95% decrease of absorbance at 395 nm, respectively, after 15 days of growth (Fig.1). It is worth noting that *Pl. ostreatus* is able to reduce more than 50% of the initial color after 6 days of incubation. Phenol content and COD decrease were also compared (Fig.2). The maximum reduction of phenol content and COD is 62% and 52% for *Pl. ostreatus*, whilst it is 82% and 77% for *H.Insulare* after 15 days of treatment. The time course of absorbance decrease is similar to that of phenol content and COD reduction for both fungi, suggesting the existence of a correlation between these parameters and the colored components present in pesticide-water.

Table 1. Composition of wastewater of Pesticide plant

pH	4.2	SM (g/l)	4.00
Colour (A ₃₉₅)	16.0	Cl ⁻ (g/l)	11.9
COD (g/l)	50.0	K ⁺ (g/l)	2.5
Phenol (g/l)	12.0	NH ₄ ⁺ (g/l)	0.15

Note: COD chemical oxygen demand, SM suspended matter.

The results obtained indicate that *H. Insulare* is able to decolorize pesticide wastewater and to degrade its phenolic component more efficiently than *Pl. ostreatus* of different parameters for pesticide wastewater treatment by *H. Insulare*. On the basis of the above results, *H. Insulare* was chosen to set up better conditions for pesticide wastewater biodegradation. For this purpose several parameters were analyzed, monitoring the events occurring after 6 days of fungal treatment. Different carbon sources were utilized to supplement 20% diluted pesticide wastewater in order to investigate their effect on colors, phenols and COD reduction during fungal growth. The best results were obtained using glycerol as an additional substrate. No significant difference was found between cultures grown in the presence or absence of added nutrients; therefore, the following experiments were performed with cultures in the absence of nutrients, thus taking into account this economic aspect for potential biotechnological applications. The mycelia of *H. Insulare* were grown in 20% pesticide wastewater at different temperatures (20, 28 and 37°C) starting from mycelia originally grown at 28°C (Table 2).

Table 2. Colour, phenol removal and chemical oxygen demand (COD) and Colour by *H. Insulare* and *P. chrysosporium*

Carbon source	Colour removal	Phenol removal (%)	COD removal	Incubation temperature	Phenol removal (%)	Colour removal (%)	COD removal (%)
20% PW	49	50	46	20	20	22	25
20% PW-PDYm	65	64	59	28	49	47	45
20% PW-PDY	58	60	54	37	28	30	29
20% PW-mal	60	63	57	21	23	19	24
20% PW-glu	63	67	61	22	25	23	23
20% PW-gly	67	70	64	19	21	25	27

Note: PW:means Pesticide wastewater

The highest decrease in colors, phenol content and COD was observed at 28°C. The reduction of these parameters, determined at 20 and 37°C, were similar and noticeably lower than that observed at 28°C. Therefore 28°C was chosen as the incubation temperature for the following experiments.

The effect of the initial pH on the fungus' ability to degrade pesticide wastewater was explored in the pH range 3.0-7.0. The results show that cultures starting at pH values ranging from 4.0 to 5.0 lead to the most efficient removal of phenol, colors and COD (Fig.2). Different amounts of *H. Insulare* mycelia were also used as inoculums in treatment experiments of 20% pesticide wastewater. The results (data not shown) demonstrate that greater inoculate give faster decolonization up to 4.0 g/l (inoculums dry weight/liter of medium) whilst no significant differences were observed after doubling this amount.

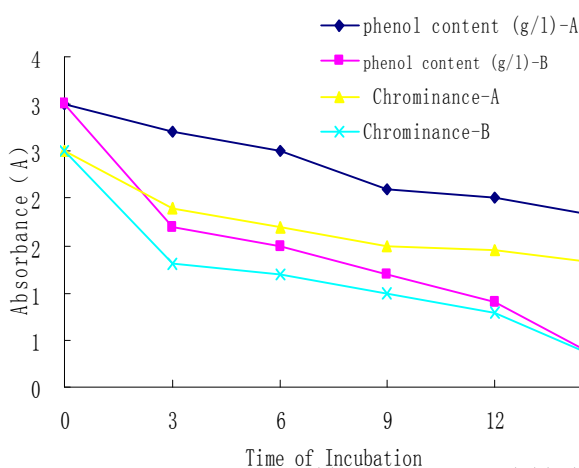


Fig 1. course of colour (A), phenol content (g/l) (B)

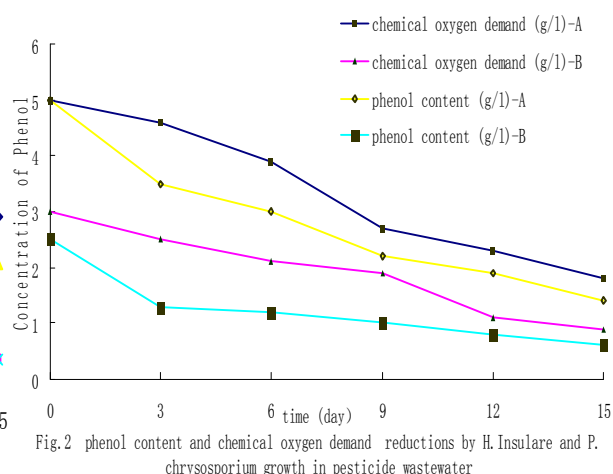


Fig.2 phenol content and chemical oxygen demand reductions by *H. Insulare* and *P. chrysosporium* growth in pesticide wastewater

H. Insulare treatment of pesticide wastewater

The effect of fungal treatment on higher concentrated pesticide wastewater was investigated using the optimal conditions previously set up [7]. The results obtained when pesticide wastewater concentration was increased from 20% to 50% are shown in Fig. 2. After 6 days of incubation, colors and phenol content reduction was about 50% in both culture conditions.

As far as COD is concerned, its time course was close to linearity until after 15 days of fungal growth in 50% pesticide wastewater culture, whilst in 20% pesticide wastewater medium COD was almost unchanged between the 9th and the 15th days. It is worth to note that all the parameters tested reached similar values either for 20% or 50% pesticide wastewater after 15 days of treatment, although the initial values were very different. Furthermore, the time courses of ligninolytic enzyme production by *H. Insulare* grown on pesticide wastewater were analyzed (Fig.2). LiP, MnP and laccase activity production were monitored during *H. Insulare* incubation in 20% and 50% pesticide wastewater. LiP activity showed a maximum at about the 6th day and then decreased. On the other hand, MnP and laccase activities were almost undetectable till the 6th day and then increased from the 7th day onwards. No significant differences in enzyme production could be observed between the two conditions analyzed [8].

In order to investigate the performance of *H. Insulare* when larger volumes of pesticide wastewater are used, a 101 vessel filled with 8 l of 20% pesticide wastewater was inoculated with 32 g (dry weight) of mycelium. The vessel was kept at room temperature under agitation and air was inflated for about 5 h/day. Absorbance at 395 nm, phenol content and COD were followed during 15 days of treatment.

The decrease in all the parameters was slower than in the case of incubation performed in shaken flasks at 28°C in the dark. After 6 days of treatment, a decrease of only 15% A_{395} , 13% phenol content and 32% COD was observed; whilst a reduction of 93% A_{395} , 90% phenol content and 74% COD was obtained in 15 days.

Non-sterilized pesticide wastewater (20%) was also treated with *Ph. chrysosporium* in the optimal treatment conditions previously described. After 15 days, the reductions in phenol content and COD were 55% and 69%, respectively; in spite of this, no color reduction as well as no variation in the visible absorbance spectra was detected.

Discussion

Some authors have reported that nitrogen concentration and nature of the carbon source markedly influences pesticide wastewater decolonization by white-rot fungi [9]. Furthermore it has also been reported that *H. Insulare* HD is able to grow and decolorize diluted pesticide wastewater only in the presence of a complex medium containing nutrients, and vertebral alcohol as inducer of ligninolytic enzymes [10]. On the other hand, our results prove that the absence of added nutrients does not significantly reduce the ability of *H. Insulare* to decolorize pesticide wastewater. The optimal decolonization temperature for *H. Insulare* M₁ is 28°C; a temperature lower than that used for *H. Insulare* (37°C). Furthermore, the optimal pH for pesticide wastewater treatment is in the range 4.0-5.0.

Since the pH of diluted pesticide wastewater is between 4.0 and 5.0, the process does not require any pH alteration of the effluent. Degradation of 20% or 50% pesticide wastewater, expressed as colour, phenol and COD removal, is almost the same after 15 days of fungal growth. Hence, not only is this fungus able to grow in 50% pesticide wastewater as sole carbon source, but the degradation rate of the effluent increases in these cultural conditions [11]. This is not an obvious conclusion, taking into account that the concentration of pesticide wastewater negatively affects the decolonization process. It is worth noting that the achieved reduction of the parameters tested is enough to obtain a complete abatement of pesticide wastewater toxic effect on *B. cereus* bacteria. Moreover, scaling-up the fungal pesticide wastewater treatment resulted in a slower degradation recess, even if similar final results were obtained [12]. The observed difference in the rate of the processes can possibly be ascribed to a reduced aeration of the larger volume system respect to the shaken flask [13].

Since it has been reported that ligninolytic enzymes are involved in pesticide wastewater degrading process [14], ligninolytic enzyme production by *H. Insulare* was analyzed during pesticide wastewater treatment. Time courses of the production of three enzymatic activities (POX, MnP, LiP) are quite different; LiP activity reaches a maximum after 6 days of fungal growth and then decreases, whilst MnP and POX activities significantly increase from the 6th day onwards [15]. Because the

most notable reduction in all parameters analyzed occurs during the first 6 days, it can be hypothesized that LiP enzymes play a key role in the degradation process. The isolated *H. Insulare* strain is therefore able to grow using diluted pesticide wastewater, as sole carbon source, and to notably reduce colors, phenol content and COD, thus proving to be a good candidate for the effective treatment of this waste water.

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