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Comparative Study of Olive Oil Mill Wastewater Treatment Using Free and Immobilized *Coriolopsis polyzona* and *Pycnoporus coccineus*

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The efficiency of the two white-rot fungi *Pycnoporus coccineus* and *Coriolopsis polyzona* in the Olive Oil Mill Wastewater (OOMW) treatment was investigated. Both fungi were active in the decolourisation and COD removal of OOMW at 50 g/L COD, but only the first fungus remains effective on the crude effluent (COD=100 g/L). Moreover *P. coccineus* was less affected by oxygen supplementation and exhibited a high tolerance to agitation in comparison to *C. polyzona*. However, it required a nitrogen supplementation to obtain faster and higher COD removal. To overcome the negative effect of agitation on fungi growth and efficiency, immobilisation of *C. polyzona* and *P. coccineus* in polyurethane foam was applied. The immobilized system showed better COD decreases during three consecutive batches without remarkable loss of performances. The results obtained in this study suggested that immobilized *C. polyzona* and especially immobilized *P. coccineus* might be applicable to a large scale for the removal colour and COD of OOMW.

Keywords: olive oil mill wastewater, biodegradation, *Coriolopsis polyzona*, *Pycnoporus coccineus*, cell immobilization

Introduction

Olive Oil Mill Wastewater (OOMW) poses a critical problem for the olive oil industries, well implanted in Tunisia. The volume of this liquid waste (vegetation water) depending on the method used for the oil extraction, varies from (per 100 kg of olives) 40–60 L for pressing method to 80–100 L for three-phase centrifugation technique (Harwood and Aparicio, 2000). Total polyphenols (TP) and chemical oxygen demand (COD) as the major chemical characteristics of the vegetation water obtained by different processing methods are (g/L) 6.2 and 145 for the pressing method and 2.7 and 86 for the centrifugation method, respectively (Fadil *et al*., 2003). The characteristic dark brown colour of the effluents is due to polymerization of low molecular weight phenolics and is chemically related to lignin and tannin derivatives (Jaouani *et al*., 2003, 2005, 2006; Jarboui *et al*., 2008). These phenolic compounds are also the main determinants of antimicrobial and phytotoxic olive-mill wastes actions (Zouari and Ellouz, 1996; Mantzavinos and Kalogerakis, 2005).

Stringent environmental regulations impose increasing efforts towards the development of new technologies and improved methods for reduction of the biorecalcitrant organics in wastewaters, such as OOMW. The physico-chemical treatments (coagulation, precipitation or flocculation of OOMW organic compounds) are very expensive and/or do not completely solve the problem of the need to dispose the sludge or the by-products that derive from the process (Paredes *et al*., 2005). Several studies have reported the biological disposal of this wastewater by anaerobic digestion, being the main interest the production of energy (biogas) and the potential re-use of the effluent in irrigation (Marques, 2001; Roig *et al*., 2006). The major limitation of this type of treatment is the inhibition of metanogenic bacteria by the phenolic compounds and the organic acids present in the OOMW (D'Annibale *et al*., 1998), showing that a pre-treatment is necessary to remove undesirable compounds.

Several treatments focused on the degradation of phenolic compounds showed that fungi are more effective than bacteria in OOMW detoxification (Oliveri *et al*., 2006; Sampedro *et al*., 2007; Asses *et al*., 2009). These fungi appear quite effective achieving removal rates as 40–88% for COD, 60–100% for phenolics, and 45–80% for colour (Morillo *et al*., 2009). The reason for this lies in the structure of the aromatic compounds present in OOMWs that is analogous to that of many lignin monomers and only a few microorganisms, and particularly white-rot fungi (WRF), are able to completely oxidize phenols *via* their ligninolytic enzymes (Hattaka, 1994). Laccase (Lac, E.C. 1.10.3.2), lignin peroxidase (LiP, E.C. 1.11.1.14) and manganese peroxidase (MnP, E.C. 1.11.1.13) are among the major enzymes of WRF involved in lignin degradation (Sayadi and Ellouz, 1992; Jaouani *et al*., 2006) The structure of the aromatic compounds present in OOMW present obvious similarities to lignin components, and it is generally admitted that ligninolytic enzymes are involved in the fungal degradation of a wide spectrum of aromatic compounds responsible for the recalcitrant brownish color of OOMW (D'Annibale *et al*., 1998; Sayadi *et al*., 2000; Jaouani *et al*., 2003; Dias *et al*., 2004; Ergul *et al*., 2011).

Among different methodologies used to improve the useful life of these WRF for a particular treatment while the cost is reduced, the immobilization strategy was found promising. Several supports for immobilization of WRF cells have

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been tried such as polyurethane foam, porous ceramic, porous poly (styrenedivinylbenzene) and fibrous nylon sponge (Ahmadi *et al*., 2006a, 2006b). Indeed, immobilization of whole cells for the degradation of different compounds in wastewater has several advantages as providing high yield, good operational stability and separating cell mass from bulk liquid for possible reuse (Lan *et al*., 2009).

Although the efficiency of WRF in OOMW treatment is well established, more efforts are needed to standardize experimental approaches in order to compare fungi performances for full scale application. C*oriolopsis polyzona* and *Pycnoporus coccineus* are previously demonstrated to be able to efficiently decolourise OOMW via their ligninolytic systems (Joaouani *et al*., 2003, 2005, 2006). Lignin peroxidase was demonstrated to play an important role in OOMW decolourisation by *C. polyzona* (Jaouani *et al*., 2006), while laccase seems to be the key enzyme implicated in effluent dephenolisation by *P. coccineus* (Jaouani *et al*., 2005). The present work focused on the comparison of their performances in OOMW treatment. The main objectives of this work were: (i) to better understand relationships between the treatment variables (inoculum size, initial COD concentration, agitation, nitrogen supplementation and immobilisation) and the colour and COD removals; and (ii) to obtain the optimum conditions for stabilization and treatment of olive oil mill wastewaters.

Materials and Methods

Olive oil mill wastewater

The OOMW used in this study was obtained from an olive oil mill in the Sfax region, home of the olive growing and processing sector, in the southern part of Tunisia. The three-phase centrifugation method (Ahmadi *et al*., 2006b) has been used for the oil extraction. Fresh OOMW was soon transported to the laboratory under refrigeration temperature (<15 h) and in order to carry out all the tests with the same wastewater, 100 ml of OOMW were distributed in 250 ml Erlenmeyer flasks and stored in the freezer at -20°C. At the time of use, the OOMW sample was thawed in a refrigerator and filtered using Whatman filter paper #2. The main characteristics of the OOMW used in this study were pH 5; COD 102±4 g/L; phenolic content 3.7±0.4 g/L; total nitrogen 0.72±0.02 g/L and C/N 53.6±0.6. The optical density of a 100 times diluted sample was 0.435 at 395 nm. The crude OOMW was properly diluted in distilled water in order to obtain initial COD concentrations of 75, 50 and 25 g/L.

Microorganisms and inoculum preparation

C. polyzona (MUCL38443) and *P. coccineus* (MUCL38527) were purchased from the Belgian Coordinated Collections of Microorganisms/Mycothèque de l'Université Catholique de Louvain (BCCM/MUCL) and was maintained at low temperature 4°C, on 2% malt extract agar slants. Subcultures were routinely made every two months. The inoculum was prepared by growing the fungi on a rotary shaker at 150 rpm and 30°C in 125 ml flasks containing 30 ml of 2% malt extract medium pH 5. After 7 days of cultivation mycelial pellets were harvested, washed twice with sterile water (0.9% NaCl) and homogenized two consecutive times (each 10 sec) with a Warring laboratory blender (Jencons-International Medical Products, Brussels, Belgium) (Neifar *et al*., 2009). After drying overnight at 105°C, a mycelium yield of 5.2 g/L was obtained.

Cultivation procedure and OOMW treatment

The OOMW samples obtained and prepared as described above was used in the experiments. Liquid cultures were conducted to monitor colour and chemical oxygen demand (COD), during the growth of *C. polyzona* and *P. coccineus*. OOMW-based media was prepared at four different COD values: media ML100, ML75, ML50, and ML25 for respective COD of 100, 75, 50, and 25 g/L as previously described (Joaouani *et al*., 2003, 2005, 2006). Nitrogen was added as diammonium tartrate at concentrations of 2 and 20 mM for low nitrogen and high nitrogen media respectively. The initial pH of this solution was set at pH 5 using 1 N solution HCl. pH of the medium at the end of the treatment did not change and remained almost constant at about pH 4.5–5.0. The medium was autoclaved at 121°C during 15 min and then inoculated with 0.25, 0.5, and 1‰ (w/v) of fragmented mycelium prepared as previously described. Incubations were carried out at 30°C, statically or on orbital shakers at 50 and 150 rpm. All the experiments were carried out during 21 days. In addition, abiotic controls using non-inoculated

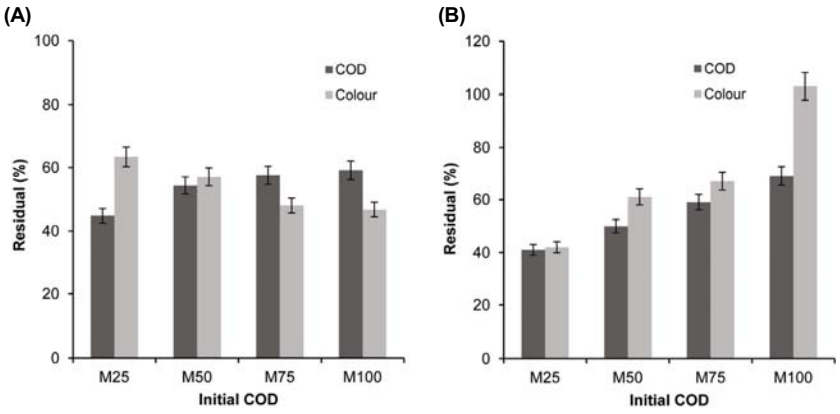

Fig. 1. Effect of initial COD concentration on COD and colour removals of OOMW by (A) *P. coccineus* and (B) *C. polyzona*.

Table 1. Effect of initial COD concentration, nitrogen concentration, inoculum size and agitation on LiP, MnP, and laccase acitivities of *P. coccineus* and *C. polyzona*. This table illustrate the variation of the enzyme yields versus one variable, while fixing the other variables at their constant levels shown in the line 1.

N°	Initial COD concentration (g/L)	Nitrogen concentration (mM)	Agitation (rpm)	Inoculum size (g/L)	Maximum *P. coccineus* activities (U/L)			Maximum *C. polyzona* activities (U/L)		
					LiP	MnP	Lac	LiP	MnP	Lac
1	25	2	0	0.25	ND	ND	48±3 (a)	22±2 (a)	29±2 (a)	38±3 (a)
2	100	2	0	0.25	ND	ND	345±9 (b)	ND	ND	65±3 (b)
3	25	20	0	0.25	ND	ND	112±6 (c)	ND	ND	22±2 (c)
4	25	2	150	0.25	ND	ND	49±3 (a)	12±1 (b)	11±1 (b)	13±1(d)
5	25	2	0	1.00	ND	ND	65±3 (a)	31±2 (c)	38±3 (c)	41±4 (a)

[a-b] differences are not significant at $p<0.05$ level followed by the same letter in each column
ND, not detected

OOMW medium were performed at the same incubation conditions.

The immobilization of *C. polyzona* and *P. coccineus* cells within the polyurethane foam was carried out as described by D'Annibale *et al.* (1998). Polyurethane foam cubes of 1 cm^2 were boiled in water, washed repeatedly with distilled water and were wet sterilized in an autoclave at 121°C. Sterilized polyurethane foam cubes were soaked with OOMW-based media and inoculated with macerated mycelium. Foam cubes without fungal growth and heat-killed immobilized fungus were used as controls. A rather common test was conducted to check polyurethane foam for possibility of having any adsorption capacity toward OOMW: polyurethane foam was soaked in the uninoculated diluted OOMW for 24 h. The polyurethane foam then was placed in the sterilized water for another 24 h. No colour in the distilled water was detected. Same batch of each immobilized fungus was used for different cycles of OOMW treatment and the percentages of color and COD removals were measured with each fresh batch of OOMW.

COD and colour determinations

Before analysis the OOMW samples were filtered on glass microfibres (GF/A Whatman Inc.). Analysis of chemical oxygen demand (COD) of the samples was carried out under the recommendations by APHA (2005). Decolourising activity of the fungi for OOMW was assayed by measuring the decrease in colour intensity spectrophotometrically at 395 nm (Sayadi and Ellouz, 1992). Results were expressed in comparison with non-inoculated and filtered cultures incubated under the same conditions.

Enzyme assays

Ligninolytic enzymes activities, Laccase, Lignin peroxidase (LiP), and manganese peroxidase (MnP) were determined as previously reported (Jaouani *et al.*, 2003). Laccase activity was assayed using 5 mM 2,6-dimethoxyphenol (DMP) in 100 mM sodium tartrate buffer, pH 4.5 (ε_{469}=27,500M^{-1} cm^{-1}, referred to DMP concentration). MnP activity was estimated by the formation of Mn^{3+}-tartrate complex (ε_{238}=6500M^{-1} cm^{-1}) during the oxidation of 0.1 mM Mn^{2+} ($MnSO_4$) in

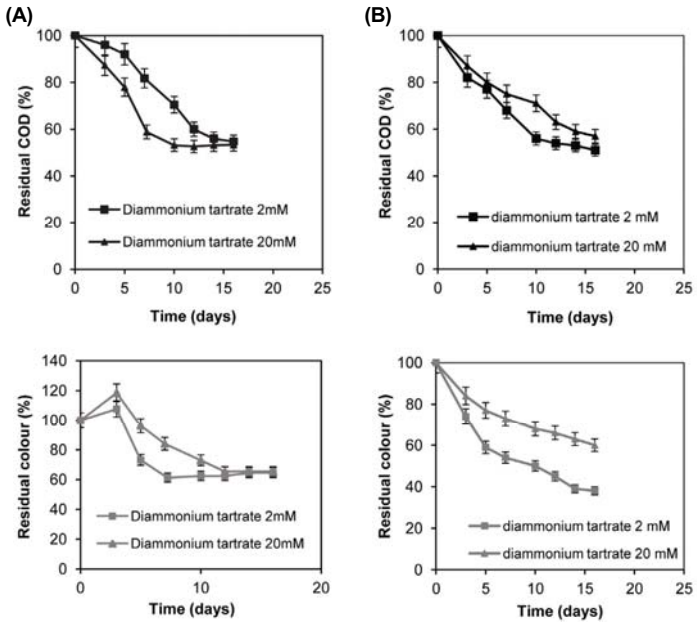

Fig. 2. Effect of nitrogen source concentration on COD and colour removals of OOMW by (A) *P. coccineus* and (B) *C. polyzona*.

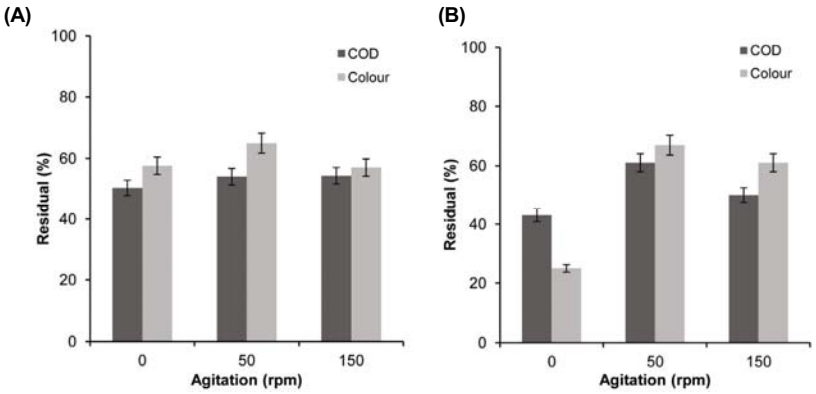

Fig. 3. Effect of agitation on COD and colour removals of OOMW by (A) *P. coccineus* and (B) *C. polyzona*.

100 mM sodium tartrate buffer, pH 5, in the presence of 0.1 mM H_2O_2. LiP activity was determined by the H_2O_2-dependent veratraldehyde (ε310=9300 M^{-1} cm^{-1}) formation from 2 mM veratryl alcohol in 100 mM sodium tartrate buffer, pH 3, in the presence of 0.4 mM H_2O_2. The enzymatic reactions were carried out at room temperature (22–25°C) and one unit of enzyme activity was defined as the amount of enzyme oxidizing 1 µmol of substrate per min.

Statistical analysis

Triplicate experiments were run for comparison and samples were analyzed in duplicate. The values in the figures correspond to mean values. Differences between means were compared using the ANOVA function in Microsoft Excel at the 0.05 significance level.

Results and Discussion

In the present study, C. *polyzona* and *P. coccineus*, which were different in their ligninolytic system were tested for their ability to biodegrade OOMW. The relationships between two important criteria of the pollutant removals (COD and colour) and five controllable factors namely agitation, inoculum size, OOMW concentration, nitrogen source addition and cell immobilisation were evaluated.

Effect of initial COD concentration

The performances of *C. polyzona* and *P. coccineus* in decolourisation and COD removal of OOMW were evaluated on four different liquid media: ML25, ML50, ML75, and ML100 corresponding, respectively to 25, 50, 75, and 100 g/L as initial COD (Fig. 1). The effectiveness of the two fungi to decolourise the effluent and to decrease COD was affected when the initial COD of the effluent increased. *P. coccineus* removed 55.3 and 39.9% of the COD after 21 days of cultivation on ML25 and ML100, respectively (Fig. 1A). Respective values of 59.1 and 31.3% were obtained for C. *polyzona* (Fig. 1B). This finding indicates a probable concentration dependent toxic effect of the OOMW soluble fraction, phenolics in particular, as also suggested by other studies (Vinciguerra *et al.*, 1995; Martirani *et al.*, 1996; D'Annibale *et al.*, 2004). Figure 1A shows that the COD concentration exhibits a significant ($p<0.05$) positive effect on the colour removal by *P. coccineus.* However, for *C. polyzona*, the increase in COD concentration has resulted in lower decolourisation yields (Fig. 1B). Also, we have concluded from Fig. 1, that both fungi were active in the color removal of diluted OOMWs (25–75 g/L COD) and only *P. coccineus* was effective in the decolourisation of crude OOMW (100 g/L COD). This high degradation potential of *P. coccineus* may be due to the production of high amount of laccase during the fungal cultivation on ML100 (Table 1, Experiments N° 1 and 2).

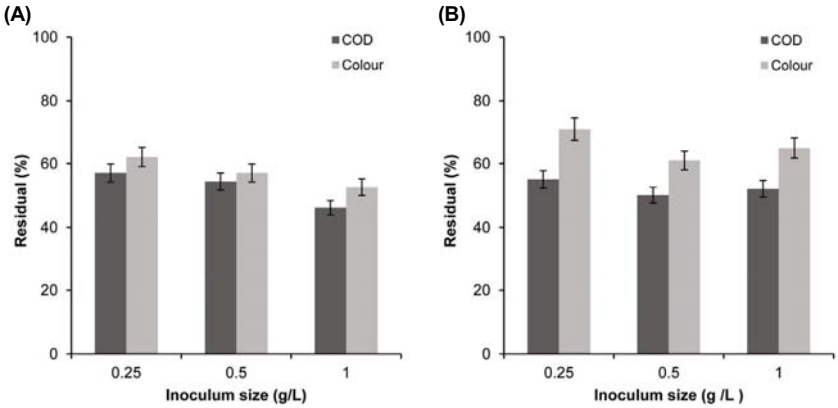

Fig. 4. Effect of inoculum size on COD and colour removals of OOMW by (A) *P. coccineus* and (B) *C. polyzona*.

These results are relevant since few white-rot species are able to withstand such initial organic loads maintaining their degradative potential and the capacity to produce lignin-modifying enzymes (Sayadi and Ellouz, 1993; Joaouani *et al.*, 2003; D'Annibale *et al.*, 2004). In many other OOMW studies, diluting of this wastewater was found essential and initial COD concentration in most cases, provided basis for the dilution (Sayadi and Ellouz, 1995).

Effect of nitrogen source concentration

Extensive research works are carried out and reported in the literature regarding the relationship between the amount and type of either nitrogen and/or carbon source and expression of the ligninolytic enzymes as an important physiological event in the well-studied white-rot fungi (Joaouani *et al.*, 2003; Ahmadi *et al.*, 2006). The capacity of *P. coccineus* in the COD removal of OOMW increased as the concentration of the nitrogen source increased from 2 to 20 mM (Fig. 2A). However, the increase of diammonium tartrate concentration showed significant negative effects on COD and colour removals by *C. polyzona* (Fig. 2B). As shown in table 1 (Experiments N° 1 and 3), the regulation of ligninolytic enzyme production in these two fungal genera was quite distinct, occurring either under N-limitation in *C. polyzona* or under N-sufficiency in *P. coccineus*. This is in agreement with previous findings about white rot fungi (Eriksson *et al.*, 1990; Kaal *et al.*, 1995). For examples, ligninolysis and ligninolytic enzyme production in liquid cultures of *P. chrysosporiurn* are suppressed and delayed by high concentrations of N (Kirk and Farrell, 1987). However, ligninolytic peroxidase production in *Bjerkandera* sp. was greatly stimulated by high N (Kaal *et al.*, 1993). Our results confirm that the addition of readily utilizable nitrogen sources supports wastewater treatment by selective WRF, as observed in other studies (Bergabuer *et al.*, 1991; Vinciguerra *et al.*, 1995; Dias *et al.*, 2004; Ahmadi *et al.*, 2006) even though supplementation did not improve the biodegradation ability of some species, such as *Coriolus versicolor* and *Funalia trogii* (Yesilada *et al.*, 1998).

Effect of agitation

Agitation speed is a very important factor in the fermentation process since it will increase the amount of dissolved oxygen in the cultivation medium (Purwanto *et al.*, 2009). Agitation speed of the culture broth has a variety of effects on microorganisms, including rupture of the cell wall, change in the morphology of filamentous microorganisms, variation in the efficiency and rate of growth and also variation in the rate of formation of the desired product (Porcel *et al.*, 2005; Purwanto *et al.*, 2009). In this study, the OOMW degradation by *C. polyzona* and *P. coccineus* was found to be influenced by the agitation speeds viz. 0, 50, and 150 rpm. As shown in Figs. 3A and 3B, the agitation has a significant ($p<0.05$) negative effect on the colour and COD removals by *C. polyzona* whereas it has a slightly effect on OOMW treatment by *P. coccineus.* The COD and color removals in static cultures of *C. polyzona* were 57.3 and 75.2%, respectively, whereas for cultures agitated at 150 rpm the corresponded values were 50.1 and 39.4%. These biodegradation results could be supported by the ligninolytic enzyme profiles obtained under static and agitated conditions (Table 1, Experiments N° 1 and 4). The laccase production by *P. coccineus* was not affected by agitation whereas *C. polyzona* showed higher LiP and MnP production under static condition. Apparently, mechanical stress gave greater impact on *C. polyzona* cells and could alter the cell internal structures, thus lower the enzyme activity. Kim and Song (2009) also found that fungal pellet morphology and enzyme production were strongly affected by the agitation.

Effect of inoculum size

The effect of inoculum size has a significant effect on the OOMW degradation efficiency by *C. polyzona* and *P. coccineus* (Figs. 4A and 4B). With an optimum inoculum size of 0.5 g/L, *C. polyzona* removed approximately 50% COD and 40% color (Fig. 4A). *P. coccineus* removed approximately 55% COD and 50% colour of the OOMW when an inoculum size of 1.0 g/L was used (Fig. 4B). There have been many reports on the effect of inoculum size in fungal growth and productivity (Yesilada *et al.*, 1998; Shahvali *et al.*, 2000). When the inoculum size is small, longer cultivation time is required. A large inoculum size on culture will lead rapidly to crowded conditions and nutritional deficiency (Neifar *et al.*, 2009). The effect of fungal inoculum size on ligninolytic enzymes production by *C. polyzona* and *P. coccineus* was also investigated (Table 1, Experiments N° 1 and 5). An

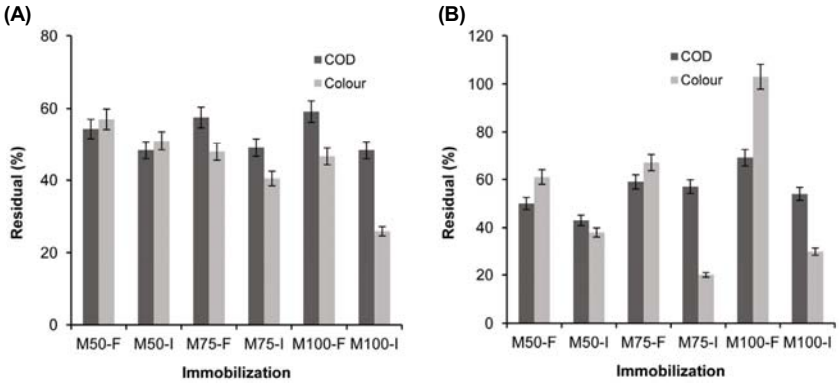

Fig. 5. Effect of (A) *P. coccineus* and (B) *C. polyzona* immobilisation on COD and colour removals of OOMW. Abbreviations I and F represent immobilized and free cells, respectively.

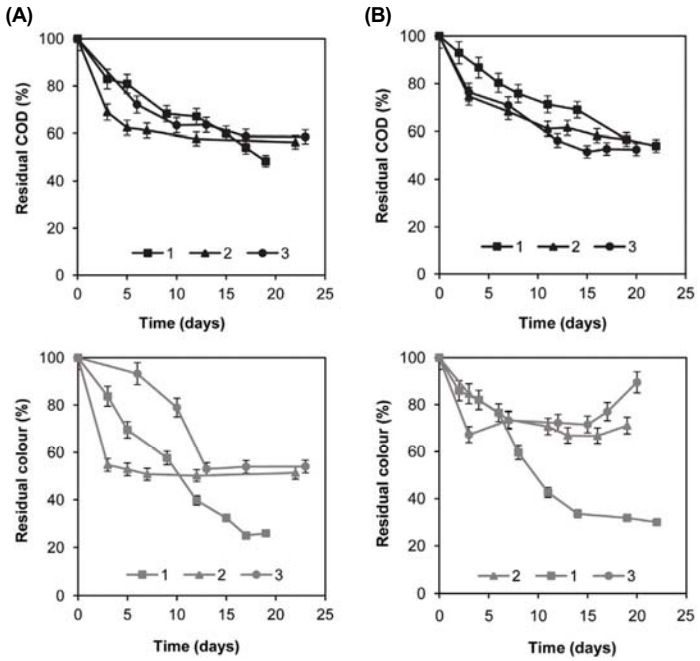

Fig. 6. **Repeated (three cycles) COD and colour removals by immobilized cells of (A) *P. coccineus* and (B) *C. polyzona*.**

increase in inoculum size enhanced the utilization of the nutrients, thereby improving Lac, LiP, and MnP activities. However, with further increase in inoculums above 1.0 g/L, ligninolytic enzyme production by *C. polyzona* and *P. coccineus* was decreased because of fast depletion of the nutrients, resulting in a decrease in metabolic activities (data not shown). Similar effects of inoculum size on lininolytic enzyme production and OOMW degradation were reported for *Phanerochaete chrysosporium* and *Coriolus versicolor* (Yesilada *et al*., 1998; Shahvali *et al*., 2000).

COD and colour removals of OOMW by immobilized cells

Immobilized cells were grown in OOMW with different initial COD concentration under the optimum conditions. The results of the effect of COD concentration on colour and COD removals by immobilized cells are shown in Fig. 5. The immobilized cultures of *P. coccineus* had higher degradation effeciencies of COD and color than free cultures (Fig. 5A). The free and immobilized cultures of *C. polyzona* did not show a significant difference on COD degradation of ML50. The COD removal by the immobilized cells increased with the increasing of the initial COD concentration from 75 to 100 g/L. This was in opposite with the effect of COD concentration on OOMW degradation by free cells (Fig. 5B). Indeed, COD removals of ML75 and ML100 by free cells were 34±2 and 29±2 g/L, respectively, whereas for immobilized cells the corresponded values were 32±2 and 46±3 g/L. Decolourisation yields of ML100 by free and immobilized *P. coccineus* were 0 and 69%, respectively. These results suggested that the immobilized *C. polyzona* was more effective than free cells for the treatment of OOMW, which had high COD concentration. All these results were in agreement with the literature date. For example, Yesilada *et al.* (1998) reported high degradation yields of OOMW by immobilized cultures of *Coriolus versicolor* and *Funalia trogii*.

Repeated OOMW treatment by reuse of the immobilized cells

The reuse of immobilized cells might be advantageous because it can decrease waste of cells, save time, and cut down cultivation cost (Lan *et al*., 2009). The repeated colour and COD removals of OOMW at 100 g/L by reuse of the immobilized cells were performed under the optimum conditions. The results in Fig. 6 indicated that cycle number significantly affected colour removal of M100 by immobilized cells whereas the COD degradation was unaffected. The immobilized *P. coccineus* could be reused for 3 cycles and approximately 40% COD and 50% colour were degraded. For *C. polyzona*, the immobilized cells could be reused for a maximum of 2 cycles and 45% COD and 25% colour were removed (Fig. 6).

In summary, the results obtained in this study suggested that free and immobilized *C. polyzona* and *P. coccineus* might be applicable to OOMW treatment system for the removal of colour and COD. The following conclusions were reached: (i) The optimum conditions for this treatment was achieved by setting the experiment with high initial COD of OOMW (100 g/L) and 20 mM of nitrogen for *P. coccineus*; (ii) *C. polyzona* was more effective on diluted OOMW under static condition (iii) the COD and colour removals were comparatively higher from immobilized cells in polyurethane foam than that by free cells. (iv) the reusability of immobilized cells of *P. coccineus* for wastewater decolourisation was higher than *C. polyzona*. These features are important since they demonstrate the capacity of WRF to degrade OOMW in a sequencing batch mode over long periods of time without need for supplementation of new mycelium.

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