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LACCASE INSOLUBILIZED AS COMBINATION OF CROSSLINKED ENZYME AGGREGATES: CHARACTERIZATION AND POTENTIAL APPLICATION IN WASTEWATER TREATMENT

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Abstract- Laccases (EC1.10.3.2) are multicopper enzymes capable of oxidizing a large number of organic contaminants in wastewater and soil. In this study, two commercial laccases, *Trametes versicolor* (TvL) and MetZyme (MZL), with pH optima at 4 and 8, respectively, were insolubilized as a combination of cross-linked enzymes aggregates (combi-CLEA) which was active in both acidic and alkaline pHs. The temperature optima were found to be 40°C for free TvL and 50°C for both free MZL and combi-CLEA. Moreover, the combi-CLEA exhibited substantial thermal stability greater than those for the free laccases. The insoluble laccases also kept significant residual activity against both long-term storage and drying effects. When applied to samples of wastewater from pulp and paper mill, 70% of total COD were reduced by the combi-CLEA and 50% of total COD were reduced by the free MZL (active in the pH range of the wastewater). The combi-CLEA was also proven to be recyclable as evidenced by the remaining activity of the biocatalyst after two rounds of application to the treatment of wastewater samples.

Keywords- laccases, combination of cross-linked enzyme aggregates, wastewater treatment.

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Introduction

Pulp and paper (P&P) mills are known to generate large volumes of wastewater containing significant amounts of pollutants including phenolic compounds. These pollutants are known or suspected to have endocrine disrupting properties and their continued presence in the environment pose health and environmental effects and risks [1,2]. Endocrine disrupting compounds (EDCs) have several sources of entry in the environment but are found to be mainly from sewers and wastewater plants systems [3,4]. This indicates that the conventional wastewater treatments such as biological activated sludge systems are inefficient in eliminating these EDCs. A fact that could partly be attributed to the low concentrations (part per billion to part per trillion) of the EDCs in the influent [5].

Beside the conventional treatment systems, several other processes have been investigated for the treatment of these micropollutants in wastewater. For instance, advanced chemical oxidation, activated carbon adsoption and membrane filtration have been used for the (bio)transformation or the removal of some EDCs. The results are very variable ranging from high to poor efficiencies [5].

Moreover, these treatment methods are often expensive for continuous applications (e.g. activated carbon and membrane filtration) [6,7] or generate by-products that are equally or more toxic than the original compounds [8,9]. Due to these disadvantages *inter alia*, enzymes have been investigated as alternative to the physico-chemical and biological treatment processes for EDCs elimination in wastewater [10-12].

However, as free enzymes are currently not economically viable for large scale use in solution, several immobilization and insolubilization techniques have been developed to circumvent this fact. One of the most effective techniques is found to be the simple/combination of cross-linked enzyme aggregates (CLEAs/combi-CLEA) in which the synthesized biocatalyst has enhanced characteristics (activity, stability, etc.) in addition to their economical and environmental potential benefits such as fewer unit operations, less reactor volume, higher volumetric and space-time yields, shorter cycle times and less waste generation [13].

Although several enzymes have been used to synthesize combi-

CLEA for biotechnological [14,15] and bioremediation [16] applications, to our knowledge there is no laccase-based combi-CLEA formed to date. Yet, the use of laccase is very promising for bioremediation purposes because of the nature of its specificity. Indeed, laccases (Lac, E.C. 1.10.3.2) are phenol oxidases able to catalyze the oxidation of a large number of phenolic compounds by simply requiring bimolecular oxygen with concomitant formation of water as by-product and a reactive free radical that can polymerize to a lesser toxic compound than the native contaminant [17].

In this study, fungal laccase from the white rot fungus *Trametes versicolor* with acidic optimum pH and bacterial laccase from MetGen, Oy (Finland) with alkaline optimum pH were synthesized as combi-CLEA using chitosan activated by N-(3- dimethylaminopropyl)-N'ethylcarbodiimide hydrochloride as the crosslinker. The combi-CLEA was characterized and applied to actual wastewater from P&P mill for the removal of chemical oxygen demand (COD) in order to evaluate their potential use in wastewater treatment as a preliminary step to the removal of phenolic compounds in municipal and industrial wastewaters for subsequent experiments.

Experimental

Enzymes and Reagents

T. versicolor laccase (TvL) was purchased from Sigma-Aldrich (St-Louis, MO) and MetZyme laccase (MZL) was provided by MetGen Oy (Turku, Finland). Chitosan from crab shells (65% deacetylation and molecular weight of 750 kDa), N-(3- dimethylaminopropyl)-N'ethylcarbodiimide hydrochloride (EDAC), 2,2'- azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2,6 dimethoxyphenol (DMP) were purchased from Sigma Aldrich. All other chemicals used were of analytical grade.

Enzyme Assay

The activities of the enzymes were determined by monitoring the oxidation of ABTS for TvL and the oxidation of DMP for MZL. The reaction mixture contained 0.5 mM ABTS or 0.5 mM DMP, 0.1 M potassium hydrogen phthalate (PHP)-HCl buffer for pH 4 or 0.1 M Tris buffer for pH 8 and a suitable amount of enzyme. Oxidation of substrates was monitored spectrometrically by absorbance of ABTS at 420 nm (ε=36000 $M^{-1}cm^{-1}$) for TvL and of DMP at 477 nm (ε=14800 $M^{-1}cm^{-1}$) for MZL [18]. Enzyme activity is expressed in units (U) defined as the amount of enzyme required to oxidize 1 µM of substrate (ABTS or DMP) per min.

Preparation of Combi-CLEA and its Yield Estimation

Free MZL (63.5 U/L) and TvL (28311.7 U/L) were combined to a total activity of 1 U/mL and insolubilized as combi-CLEA by precipitating the free enzymes on ammonium sulfate (500 g/L) for 30 min before addition of solutions of chitosan (1 g/L) and EDAC (50 mM) adapted from Arsenault, et al. [19]. Acetate buffer at pH 5 completed the total solution to the desired volume (100 mL). Solution was stored at 4°C for 48 hrs. to allow complete crosslinking reaction followed by combi-CLEA extraction by centrifugation at 10 000g for 5 min and 4°C as conceptually presented in [Fig-1]. The aliquots were then washed 3 times with deionized water and subsequently used for experiment. Combi-CLEA activity was assayed in the same manner as the free enzymes as described above.

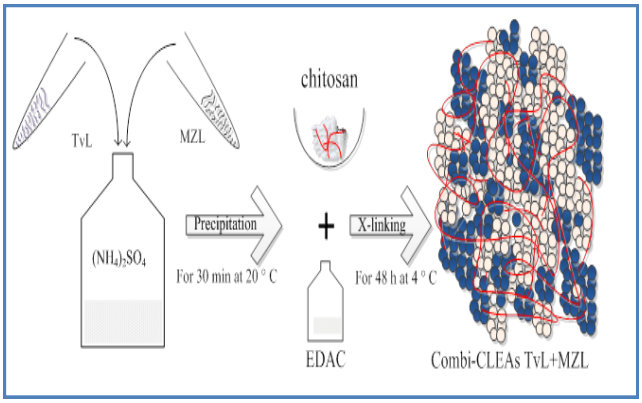

Fig. 1- Preparation of insoluble combi-CLEAs

The yield of the combi-CLEA was estimated based on the activity balance of the amounts of free laccase used and Combi-CLEA produced according to the following equation:

$$Y(\%) = \frac{\text{Total Unit of Insoluble Laccase Produced}}{\text{Total Unit of Free Laccase Used}} \times 100$$

Parallel to combi-CLEA formation, CLEA were formed with each of the two individual laccases in order to be able to draw adequate conclusions.

Effect of pH and Temperature on Enzyme Activity

The effect of pH on enzyme activity was determined in the range of 3-9 at room temperature (RT). In the range of 3-4, 0.1 M PHP-HCl buffer, in the range of 5-6, 0.1 M PHP-NaOH buffer and in the range of 7-9, 0.1 Tris-HCl buffer were used. The optimum temperatures of free and insolubilized laccases were determined by measuring the activities of the biocatalysts in the temperature range of 20-70°C at optimum pH.

The thermal stability study was carried out by incubating samples of the biocatalysts in a thermostatic bath (Isotemp 2100 water bath, Fisher Scientific) over a period of 75 h. Each biocatalyst was incubated at its optimum temperature (40°C for free TvL and 50°C for both free MZL and combi-CLEA).

The results of activity for pH and temperature studies were expressed in relative form with the highest value being assigned 100% activity for pH and temperature optima and initial measurement assigned 100% activity for thermal stability.

Long-Term Storage and Drying Effects on the CLEAs and Combi-CLEA Activity

The biocatalysts were monitored for residual activity after 10 months of storage at 4°C in deionized water. Drying effect was performed on the residual activity of samples of each biocatalyst by reacting 100 µl of biocatalyst and substrates (ABTS and DMP) in Eppendorf tube and spun solution pipetted on a 96-well plate.

Application of the Biocatalyst to Wastewater Treatment

Samples of wastewater (WW) from a typical thermo-mechanical P&P mill were used for COD test with the biocatalysts as assessment for their applicability in wastewater treatment. Hundred microliters (100 µl) of TvL-CLEA (484.2 U/L), MZL-CLEA (39.8 U/L),

combi-CLEA (777.1 U/L with ABTS and 55.0 U/L with DMP) and free MZL, respectively, were applied on 4.4 mL of WW samples of initially measured chemical oxygen demand (COD). First, each mixture of biocatalyst and wastewater was spun at 20°C for 5 min at 5000 rpm. Subsequently, 2 mL of the supernatant was taken from each sample and the total COD monitored. Next, 2 mL of MiliQ water is added to each sample and continued agitation for 24 hrs. before measuring the total COD. The results presented are those of total COD remaining taking into account the total COD induced by the biocatalysts.

However, it is noteworthy that the effect of the biocatalysts on the COD reduction should be discriminated given the different activity content in 100 µl of each of the biocatalyst. In fact, the test aimed at providing an indication of the capability of each biocatalyst to reduce the COD content in the WW rather than to compare the efficacy between the biocatalysts to do so.

Additionnally, the continued recyclability of the combi-CLEA was qualitatively checked for residual actvity by adding 100 µL of ABTS to the mixtures of biocatalysts and WW.

Results and Discussion

Production of Combi-CLEA and Yield

TvL and MZL were successfully crosslinked to insoluble biocatalyst *via* the use of chitosan, a hydrophilic biodegradable and renewable biopolymer and EDAC. The combi-CLEAs generated have specific activities of 67.8 U/g and 4.8 U/g using ABTS and DMP, respectively [Table-1]. These results are similar to specific activities of CLEAs produced with laccase from the white-rot fungus *Coriolopsis Polyzona* using chitosan and EDAC as crosslinker [19] although the 4.8 U/g is lower than the minimum (14.7 U/g) found by these authors. The conditions of insolubilization gave rise to highly substrate-dependent yields. While the yield with ABTS resulted to 23%, that of DMP was found to be 115% [Table-1]. This latter value should be taken cautiously given the fact that the activity contribution from MZL much lower than the TvL (i.e. a ratio of 1:70 activity). Otherwise, it may also be assumed that the covalent binding of MZL to chitosan results in hyperactivation of this laccase activity similarly to result found by Cabana, et al. [20] in which conjugation of laccase from the white rot fungus *T. versicolor* to chitosan resulted in biocatalysts with hyperactivated laccase. In this case, further investigation is needed to determine what would prevent the TvL from being hyperactivated by the chitosan unlike the MZL.

Table 1- Apparent laccase activity and yield of combi-CLEA produced in function of ABTS and DMP.

Substrate	Activity of Combi-CLEA U/g	Yield (%)
ABTS, pH 4	67.8	23.3
DMP, pH 8	4.8	115.6

Effect of pH on Activity of the Biocatalysts

The effect of pH on activity of free and insolubilized laccases is given in [Fig-2]. The pH optimum of free TvL was found at 4.0 (most of the activity is between pH 3-5) and was shifted to pH 5 after insolubilization as combi-CLEA using ABTS as substrate. A fact attributed to the secondary interactions between the enzyme and the crosslinking reagents as all the available amino groups on the surface of the enzyme used. Therefore, the acidic groups remaining on the surface of the enzyme renders it negatively charged, which shifts the optimum pH to the alkaline side [21]. The optimum pH for free MZL was found at pH 8. Unlike the free TvL, no shift was noticed towards higher alkaline side as a consequence of the insolubilized MZL as combi-CLEA when using DMP as substrate. This may be due to the much lower activity level of the MZL compare to the TvL in the mixture of the two laccases during insolubilization unless this shift occurred to a fraction of the pH scale (i.e. less than 1) between pH 8 and 9. In fact, a peak activity at pH 5, essentially due to the TvL, is still detected for the combi-CLEA with DMP. However, it is noteworthy that despite this low activity ratio of the MZL in the combi-CLEA activity balance, about 10% activity is detected at pH 8 for combi-CLEA with DMP entirely due to the MZL as zero TvL activity is detected at pH 7 and beyond. Therefore, it is in our belief that laccase-based combi-CLEA with a broader pH range can be synthesized should we combine alkaline and acidic laccases with more or less even activity ratio in the mixture. Having laccase-based combi-CLEA active in both acidic and alkaline pH range would increase the versatility of the biocatalyst in the application of wastewaters from various sources (municipal, industrial, etc.) with different characteristics.

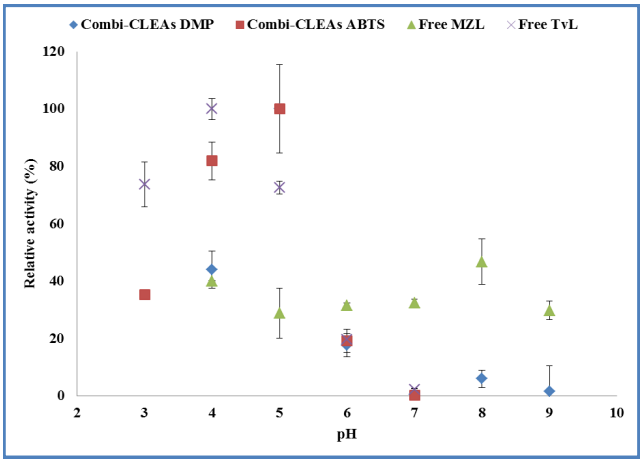

Fig. 2- Effect of the pH on the activity of the biocatalysts at 20°C. The results are means of triplicates ± standard deviation

Effect of Temperature on Enzyme Activity

The activity of soluble enzyme is known to be strongly dependent on temperature. As temperature increases, the activity increases to its optimum before starting to decrease due to the thermal denaturation of the free enzyme as a result of configuration distorsion or damage by heat exchange which appears to be preventable by insolubilization [21]. From [Fig-3], the activity of the free TvL when oxidizing ABTS gradually increased from 20°C to its optimum at 40°C (100% relative activity) before steeply decreasing (50% activity at 60°C to less than 20% at 70°C). The combi-CLEA exhibited a slightly different trend when oxidizing ABTS as the activity remains nearly constant from 20°C before peaking at 50°C instead of 40°C. The shift of the optimum temperature to a higher value could originate from the rigidification of the enzyme tertiary structure. This is also promoted by the multipoint covalent crosslinking of the enzyme surface residues to reduce conformation change and so increasing the optimum temperature [22]. On the other hand, both free MZL and combi-CLEA oxidizing DMP exhibited their temperature optima at 50°C with a significant higher activity for the latter

over the former in the temperature range of 20-70°C. At the extremes of this temperature range, the activities of free MZL and combi-CLEAs are about 20% and 70% at 20°C, respectively and about 40% and 50% at 70°C, respectively. Thus, an improvement of thermal activity due to the insolubilization of the MZL can be concluded although the trend of the combi-CLEA with DMP as susbtrate is similar to that of the curve of the free TvL which, however, expresses poor activity with DMP (more suitable for alkaline enzyme). Also, beyond the optimum temperature (50°C), the combi -CLEA with DMP exhibited activity greater than both those of the free TvL and MZL.

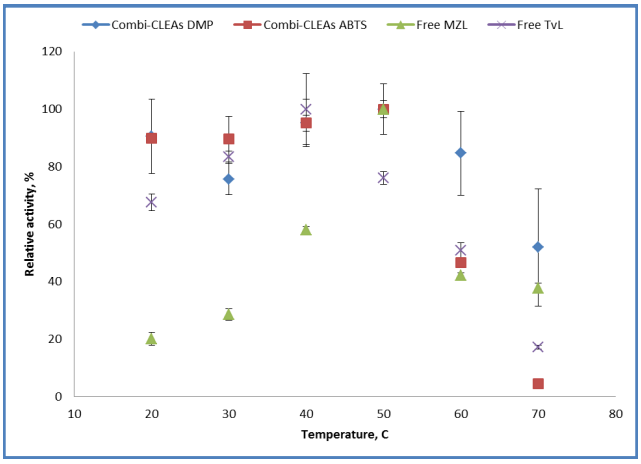

Fig. 3- Temperature optima for biocatalysts at pH 4 (free TvL and combi-CLEAs ABTS) and pH 8 (free MZL and combi-CLEAs DMP). The results are means of triplicates ± standard deviation.

The thermal stability curves for free and insolubilized laccases are given in [Fig-4]. Both free TvL and combi-CLEA using ABTS as substrate exhibited rapid decrease in their activities with about 70% and more than 80% loss after 57 hrs. of incubation at their temperature optima, respectively. Also there is a very good overlap of the curves of both biocatalysts which surprisingly imply that the insolubilization did not improve the thermal stability of the free TvL.

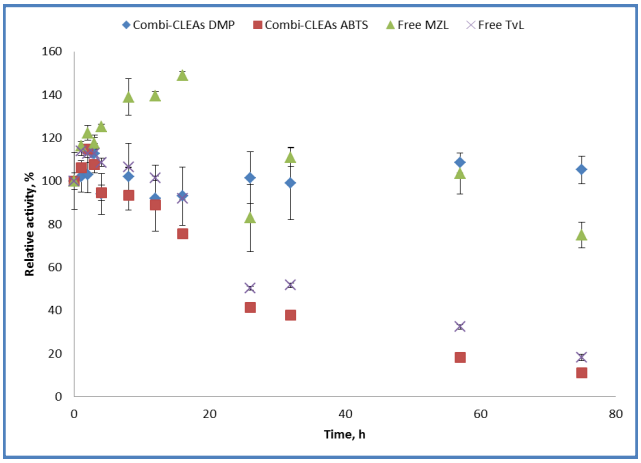

Fig. 4- Thermal stability for the biocatalysts at pH 4 (free TvL and combi-CLEAs ABTS), pH 8 (free MZL and combi-CLEAs DMP), 40°C (free TvL) and 50°C (free MZL, combi-CLEAs ABTS and combi-CLEAs DMP). The results are means of triplicates ± standard deviation.

This finding is in contradiction with results found in the literature for other insolubilized enzymes as combi-CLEA or CLEA [14,21,23]. In contrast, the free MZL and the combi-CLEA using DMP as substrate showed high thermal stability over the course of 75 hrs. of incubation time. Indeed, during the first 20 hrs. of incubation time, the free MZL exhibited a spike in its relative activity up to 150% before subsequently decreasing and remaining stable between 80-75%. The combi-CLEA showed an even better stability as its relative activity remained stable at around 100% over the course of the whole incubation time after a small spike to 112% during the first 4 hrs. followed by a decrease down to 90% before the stable increase. Although part of this stability can be attributed to the MZL (not to the TvL), it clearly appears that the insolubilization had a positive effect on the thermal stability. Indeed, while the free MZL had decreasing activity between 20-75 hrs. (20 to 25% activity decrease) the combi-CLEA activity increased by 1 to 5% over the same period of time. This increased thermal stability may due to both the rigidification of the three-dimensional structure of the insoluble molecules, as explained earlier and some addtional ionic and hydrophobic intermolecular contacts of the combi-CLEA [21].

Long-Term Storage and Drying Effects on the Combi-CLEA Activity

Samples of each of the biocatalysts, TvL-CLEA, MZL-CLEA and combi-CLEA, were stored in 50 mL centrifuge tubes at 4°C and used when needed. After ten months of storage, both the CLEA and combi-CLEA kept residual activity [Fig-5] to be tested for drying effect.

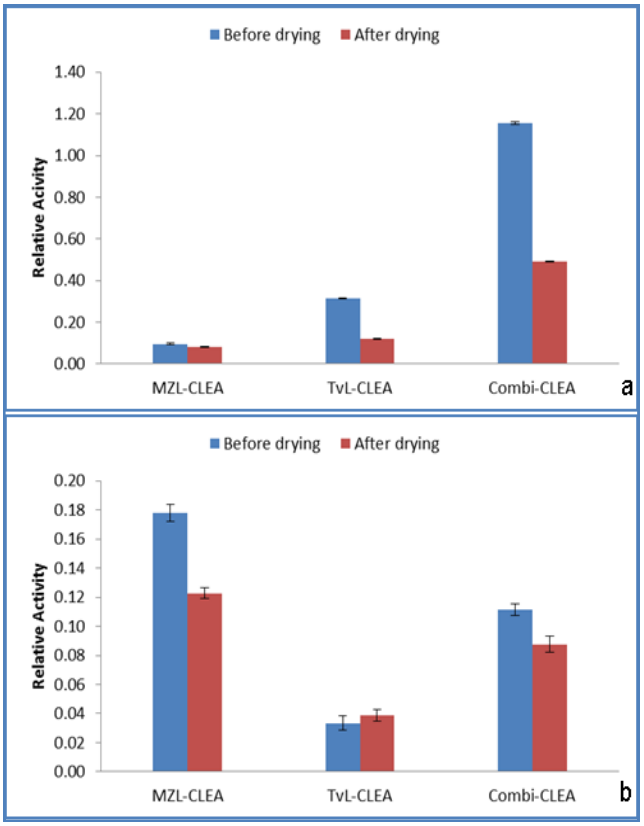

Fig. 5- Effect of drying on activities of insoluble laccases using ABTS (a) and DMP (b) as substrates. The results are means of triplicates ± standard deviation.

CLEA and combi-CLEA were small particles in liquid and when dried by evaporation for 3 days at room temperature they formed flakes which were subsequently crushed to powder, homogenized in deionized water and shaken. The results of activity measurements before and after drying are presented in [Fig-5a and 5b]. The drying appears to have greater effect on the combi-CLEA and TvL-CLEA (57% and 62% of activity reduction) than the MZL-CLEA (only 14% of activity reduction) when using ABTS as substrate [Fig-5a]. These results should however be taken with caution given that the MZL, a more alkaline bacterial laccase, does not meaningfully oxidize ABTS for its optimum activity expression.

On the other hand, after drying and using DMP as substrate, the residual activities for MZL-CLEA and combi-CLEA resulted in 31% and 22% loss, respectively. This indicates that the biocatalysts were not significantly affected by the drying effect, perhaps due to the thermal stability of the MZL and the rigidification of the three-dimensional structure of the protein. Such result is somewhat coherent with the results of the thermal stability study [Fig-3] where the free and insoluble MZL showed good thermal stability and positive correlation. In the case of the TvL-CLEA, an 18% increase of the residual activity was observed. But, this small jump in the activity may simply be due to either the poor affinity of the DMP with the acidic biocatalyst or an error of measurement because of the low residual activity before drying.

The ability to withstand long-term storage and drying effects of the insolubilized biocatalysts provide promising properties for their commercial use.

Treatment of P&P Mill Effluent Using Insolubilized Laccase

The addition of free and insolubilized laccases in samples of wastewater from P&P produced distinct results. After 5 min of agitation, a spike of COD was observed in wastewater treated with free laccase whereas a reduction of COD content in wastewater treated with insolubilized laccases was found [Fig-6]. Also, this COD reduction (40%) was higher for combi-CLEA than for TvL-CLEA (27%) and MZL-CLEA (5%). However, after 24 hrs. of treatment, both free and insolubilized laccases significantly reduced COD content in the wastewater (combi-CLEA and CLEA reduced 70-75% COD, free laccase reduced 50% COD). These results may indicate that the insolubilized enzymes are potentially more efficient in wastewater treatment than their free counterparts without concealing the fact that the free enzyme, CLEA and combi-CLEA have different activity per 100 µL. Moreover, after 48 hrs. and two rounds of wastewater treatment (results for round-2 COD reduction not shown), there still was residual enzymatic activity in the insolubilized laccases qualitatively expressed by the green-colored samples resulting from oxidation of ABTS with the CLEA and combi-CLEAs [Fig-7]. The remainder of residual activity suggests that the synthesized CLEA and combi-CLEA could possibly be reused for additional treatment cycles of wastewater. In this case, the insoluble biocatalysts are thus recyclable in contrast with the free enzyme which required additional fresh free MZL for each treatment cycle. This is because the free enzyme lacks stability in solution and is not recoverable. Indeed, previous studies have already shown high loss of activity and difficult recovery of free enzymes as limiting factors to their operational stability and reusability in contrast with immobilized or insolubilized enzymes [24, 25]. The darker green-color observed for the TvL-CLEA and combi-CLEA samples in the [Fig-7] is mainly due to the fact that the samples of these two biocatalysts have higher activities of laccase with the ABTS that is more favorable for the TvL than the MZL.

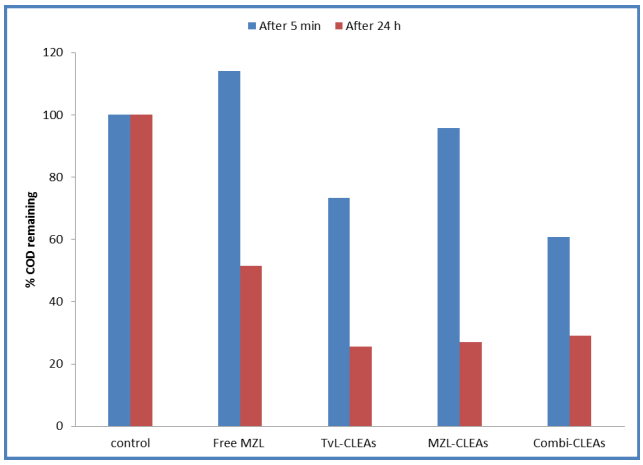

Fig. 6- Percentage of total COD content observed at two time points of application of biocatalysts in P&P wastewater at 20°C and pH 7.

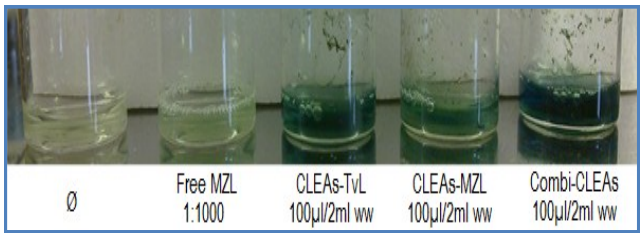

Fig. 7- Evidence of remaining enzymatic activity in biocatalysts after 2 rounds of waste water treatment using ABTS as substrate

Conclusion and Future Work

Fungal laccase with acidic pH and bacterial laccase with alkaline pH were successfully insolublized as combi-CLEA which turned out to be active in both pHs range. The combi-CLEA exhibited considerable thermal stability in more than three days of incubation at its optimum temperature which could be an important property for application in some continuous treatment processes. The biocatalyst also adequately withstood the effects of long-term storage and drying which are important factors for its use in large-scale. Furthermore, the combi-CLEA proved to be able to remove COD from wastewater. Finally, these preliminary results demonstrated promising outcome toward the formation of combi-CLEA which can be optimized to produce a robust biocatalyst applicable in the biotransformation of phenolic compounds in wastewater.

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