CHEMICAL ENGINEERING TRANSACTIONS
VOL. 27, 2012
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at: www.aidic.it/cet
Guest Editors: Enrico Bardone, Alberto Brucato, Tajalli Keshavarz

ISBN 978-88-95608-18-1; **ISSN** 1974-9791
DOI: 10.3303/CET1227025

Decolorization of Dyes by *Aspergillus Ochraceus* Cultivated Under Solid State Fermentation on Sugar Beet Waste

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Solid state fermentation (SSF) is defined as fermentation process performed in the absence of free water on non-soluble materials which can act as physical support and source of nutrient to microorganisms. In this paper, *Aspergillus ochraceus* was cultivated in solid state fermentation using sugar industry waste. In order to decolorize dyes, two different methods were applied using sugar beet waste a) as a substrate for *A. ochraceus* cultivation and as dyes adsorbent; and b) as a substrate for *A. ochraceus* cultivation with the aim of extracellular enzymes production that can be used for dyes decolorization in batch experiments. 100 % conversion of textile violet dye decolorization, 57 % of textile green dye decolorization, 41.1 % of congo red and 51.9 % conversion of metylene blue was reached in batch experiments using produced extracellular enzymes.

1. Introduction

The effluents from textile, leather, food processing, dyeing, cosmetics, paper, and dye manufacturing industries are important sources of dye pollution. Wastewater containing dyes is very difficult to treat, since the dyes are recalcitrant organic molecules stable to light. Different physico-chemical methods such as coagulation, ultra-filtration, electro-chemical adsorption and photo-oxidation can be applied in that purpose. Lots of efforts are lately done regarding the application of biological methods such as biosorption and biodegradation mainly using bacteria and white-rot fungi (Gandolfi Boer et al., 2004; Anjaneyulu et al., 2005; Sarioglu and Atay, 2006; Jalandoni–Buan et al., 2009; Tang et. al., 2011.)
In this work, *Aspergillus ochraceus*, a non-lignolytic fungus, was cultivated in solid state fermentation using sugar industry waste without any additional carbon or nitrogen sources in order to produce enzyme cocktail that can efficiently decolorize different dyes.
The application of solid-state fermentation in food industry for the production of traditionally fermented food is very well known for many centuries. However, its benefits over submerged fermentation are recognized by modern biotechnologists and, therefore, it has been used in different areas in recent years, from the food and drug production until its implementation in environmentally and ecologically friendly processes such as the reuse of agricultural and food processing wastes for the production of microbial products or microorganisms themselves.
Sugar beet waste is a lignocellulosic residue produced during sugar processing. This is a low cost material and it is mainly used as animal feed (Medina et al., 2007). Decolorization of azo dye (Congo red), redox dye (methylene blue) and two textile dyes (violet textile dye and green textile dye) was

Please cite this article as: Tisma M., Komar M., Rajic M., Pavlovic H. and Zelic B., 2012, Decolorization of dyes by aspergillus ochraceus cultivated under solid state fermentation on sugar beet waste, Chemical Engineering Transactions, 27, 145-150
DOI: 10.3303/CET1227025

investigated. Congo red dye (sodium salt of benzidinediazo-bis-1-naphtylamine-4-sulfonic acid, $C_{32}H_{22}N_6Na_2O_6S_2$) is a typical diazo dye with two chromophoric groups (azo group) in its structure. It is highly soluble in water and persistent when once discharged into a natural environment (Tapalad et al., 2008; Jalandoni–Buan et al., 2009; Tang et al., 2011). Methylene blue is redox aniline dye ($C_{16}H_{18}N_3SCl \cdot 3H_2O$). It is not regarded as acutely toxic, but it can have various harmful effects (Sarioglu and Atay, 2006). Textile dyes can vary in their structure and composition and the data about textile dyes used in this study are unknown.
In this work, two different methods were applied in order to decolorize dyes, where sugar beet waste was used a) as a substrate for *A. ochraceus* cultivation and as dyes adsorbent; and b) as a substrate for *A. ochraceus* cultivation with the aim of extracellular enzymes production that can be used for dyes decolorization in batch experiments.

2. Materials and methods

2.1 Microorganisms
Aspergillus ochraceus CBS 589.68 originated from USA (The Centraalbureau voor Schimmelcultures, Utrecht, Netherlands) was cultivated on malt agar medium for 7 days at 28 °C. Mycelial plugs (diameter 6 mm) were used as inoculum.

2.2 Substrates
Sugar beet waste was kindly donated from sugar industry “Sladorana Županja d.d.” (Županja, Croatia)

2.3 Dyes
Metylene blue (Kemika, Zagreb, Croatia), Congo red, (Kemika, Zagreb, Croatia), textile Violet Dye and Textile Green Dye (“Kemoboja Kičić”, Sesvete, Croatia).

2.4 Solid state fermentation
Aspergillus ochraceus growth was tested on sugar beet waste with adjusted water activity level to 0.98 (determined by HygroPalm AW1, Rotronic Instrument Corp., Hauppauge NY, USA). Two different types of experiments were performed: a) Experiments *in vitro*: Six mycelial plugs were transferred into 1000 mL glass flasks containing 50 g of sugar beet waste previously moisturized (a_w = 0.98) and sterilized (121 °C for 20 min); and b) Experiments *in vivo*: five mycelial plugs were transferred into 1000 mL glass flasks containing previously sterilized 30 g of sugar beet waste and 200 ppm of dye dissolved in water (a_w = 0.98). Hrvoje, ovdje ukratko opisati metodu određivnja aw, please.Incubation was carried out at 27 °C. The samples were shaken for 5 min every 24 h. In regular time intervals, samples were harvested and washed with cold Tris-HCl buffer, pH 7 for *in vitro* decolorization experiments and with organic solvents for *in vivo* decolorization experiments.
***In vitro* decolorization experiments**. 3 g of inoculated substrate (after 6, 7, 8, 10 and 21 days of fermentation) was washed with cold Tris-HCl buffer, pH 7 to extract the enzymes. The extract was centrifugated and supernatant was used in all experiments as crude enzyme suspension. 50 mL of dye water solution was mixed with 50 mL of crude enzyme suspension. The reaction mixture was stirred on magnetic stirrer at 30 °C for 24 h. Samples were taken in regular time intervals. Absorbance of the samples during the time course was followed spectrophotometrically (UV 1601, Shimadzu, Kyoto, Japan) at maximum absorbance wavelength for each dye used.
***In vivo* decolorization experiments**. To test the ability of cultures to decolorize azo dyes *in vivo*, each dye was dissolved in water and added into the substrate at the final concentration of 200 ppm. All experiments were performed in triplicates. Reaction mixture was then autoclaved at 121 °C for 20 min. The influence of different solvents (water, MetOH, 50 % MeOH, EtOH, Acetone/Methanol/Water mixture) on extraction of dyes absorbed to substrate and mycelia was investigated. Samples (0.5 g) were withdrawn from each flask, diluted with 10 mL of solvents, shaken in horizontal position in shaker for 1 h / 30 °C and centrifugated at 13,333 rpm for 20 min at 25 °C.
The dye disappearance was determined spectrophotometrically by monitoring absorbance of the color at maximum absorbance wavelength of each used dye (Table 1.). The measurements are done in duplicate. Water was used as a blank sample and decolorized samples represented the 100 % of dye absorption.

Table 1: Characterization of dyes used in experiments

Dye	λ_{max} / nm
Metylene blue	565
Congo red	498
Green textile dye	395
Violet textile dye	515

3. Results

3.1 Measurement of sugar beet waste water activity

Results of sugar beet wastewater activity measurements are presented in Figure 1. Like most biological products, sugar beet wastewater activity follows a sigmoid curve representing the type II isotherm BET classification. The resulting curve is caused by the additive effects of Raoult's law, capillary effects, and surface water interactions. Bend of curve is noted at the value of aw around to 0.998. These are the result of changes in the magnitude of the separate physical-chemical effects. Tested fungal specie of A. ochraceus is capable to grow through whole range of aw values presented in Figure 1. For decolorizing experiments, aw level of 0.98 of sugar beet wastewater activity was applied.

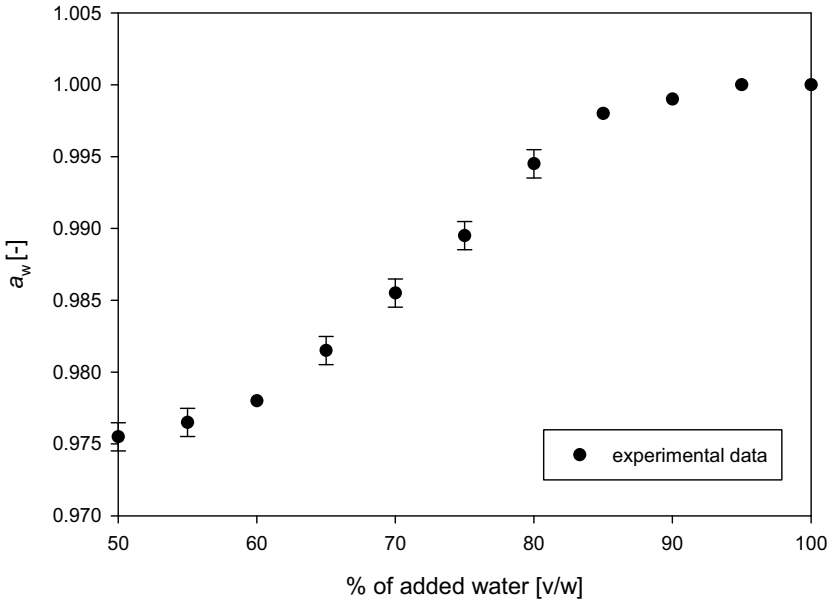

Figure 1: Dependence of sugar beet waste water activity on percentage of added water

3.2 Decolorization experiments *in vitro*

Conversion of methylene blue and congo red by crude enzyme suspension produced by *A. ochraceus* cultivation during 7 days on sugar beet waste is presented in Figure 2. 41.1 % conversion of congo red and 51.9 % conversion of metylene blue were achieved after 24 h. Samples were then filtrated through the filter paper (Whatman Grade No. 1, Voigt Global Distribution Inc., Lawrence, USA) and absorbance of the filtered samples was measured again and no difference in conversion was noticed.

Conversion of methylene blue by crude enzyme suspension of *A. ochraceus* cultivated during 10 days on sugar beet waste is presented in Figure 3. In this experiment, 46.10 % conversion of metylene blue was achieved, which is less than in comparison to previous experiment. In this experiment, the pH value was also monitored but it didn't change significantly, it decreased from initial pH 7.03 untill pH

6.8) In order to exclude the spontaneous decolorization of dyes dissolved in buffer, additional experiments were conducted under the same conditions but without the addition of crude enzyme suspension. No conversion was noticed in experiments performed without the addition of enzyme cocktail.

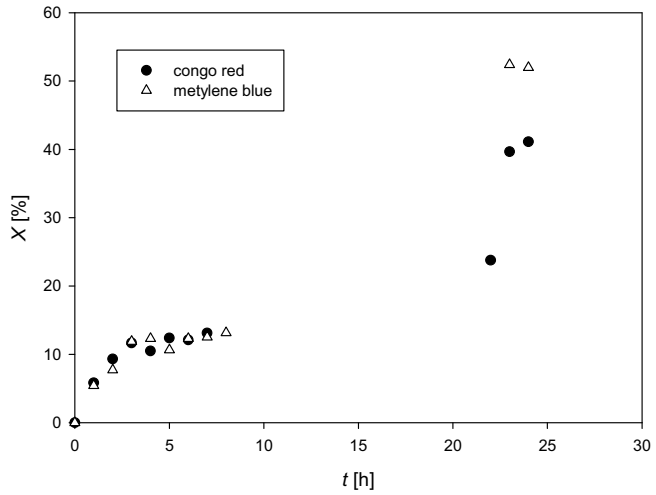

Figure 2. Decolorization experiments in vitro - conversion of methylene blue and congo red by enzyme suspension of A. ochraceus after 7 days of cultivation on sugar beet waste (Initial process conditions: $\gamma_{methylene\ blue}$: 3.57 µg/mL, pH = 7.02, $\gamma_{congo\ red}$: 6.01 µg/mL, pH = 7.03, n = 330 rpm)

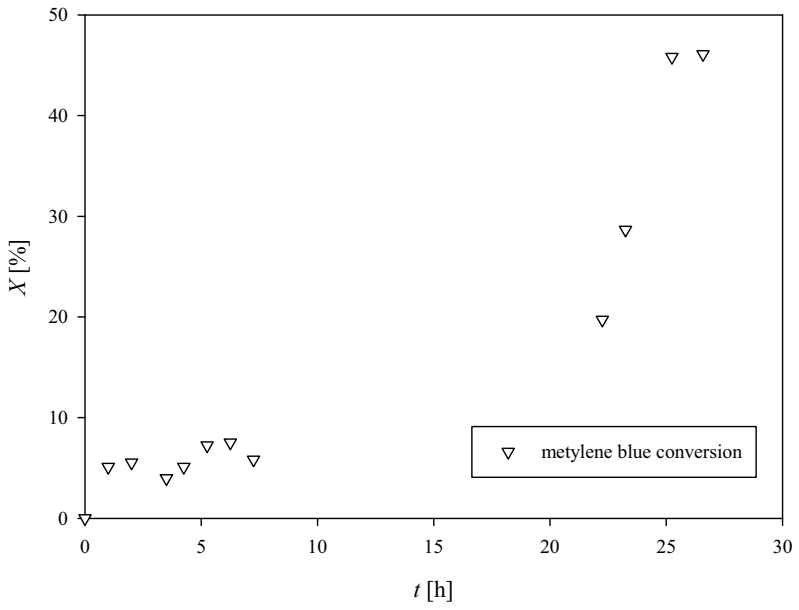

Figure 3. Decolorization experiments in vitro - conversion of methylene blue by enzyme suspension of A. ochraceus after 10 days of cultivation on sugar beet waste (Initial process conditions: $\gamma_{methylene\ blue}$: 5.24 µg/mL, pH = 7.03, n = 330 rpm)

When *A. ochraceus* was cultivated in solid state fermentation longer than 10 days, enzyme suspension extracted from the media using buffer solution became slightly green due to the fungus sporulation. Different batch experiments of methylene and congo red decolorization were performed (with and without the spore suspension), but conversions were lower than in previously performed experiments (data not shown).

Conversion of textile dyes by crude enzyme suspension of *A. ochraceus* cultivated during 6 and 8 days on sugar beet waste is presented in Figures 4. A (violet textile dye) and 4. b (green textile dye).

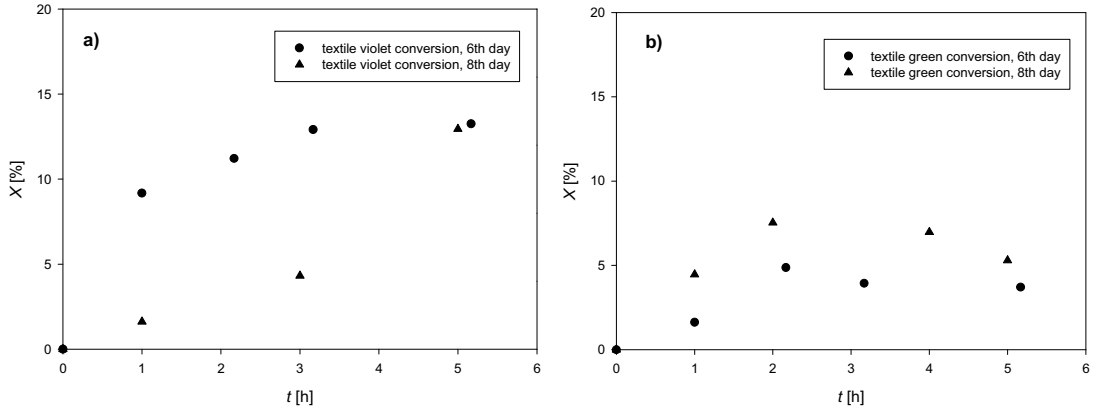

Figure 4. Decolorization experiments in vitro - conversion of textile dyes by enzyme suspension of A. ochraceus after 6 and 8 days of cultivation on sugar beet waste. Initial process conditions: a) $\gamma_{violet\ textile}$: 0.24 mg/mL, pH = 6.68, n = 330 rpm, b) $\gamma_{green\ textile}$: 0.24 mg/mL, pH = 6.9, n = 330 rpm

In comparison to the results of conversion of methylene blue and congo red, Figure 4. presents the results of textile dyes conversion during the first five hours of batch experiments duration, because in these experiments absorbance started to increase after five hours. After 24 h of process duration, the reaction mixture was filtrated and absorbance was measured again. In comparison to experiments with methylene blue and congo red decolorization, the absorbance of both textile dyes decreased after filtration process. 100 % of violet dye decolorization and 57 % of green dye decolorization were detected. Additional experiments were conducted under the same conditions but without the addition of crude enzyme suspension. The reaction mixtures were filtrated after 24 h but no decolorization occurred. UV–Vis spectrum scan (300–800 nm) of reaction mixture after filtration process in experiment with violet dye showed that absorption peak at 515 nm vanished and no new peaks in this spectrum appeared. However, in experiment with green dye, shift in λ max was noticed from 395 nm toward higher values. According to the obtained results, it can be assumed that dye decolorization processes are influenced by the dye structure. More sophisticated analytical methods will be used to identify the molecular mass of the products (HPLC-MS) and to calculate the remaining organic carbon values (TOC).

3.3 Decolorization experiments *in vivo*

In experiments performed *in vivo* visible disappearance of the dyes (methylene blue and congo red) started to occur after 7 days of *A. ochraceus* cultivation and continue until the almost complete dye decolorization at the 21st day of fermentation. Mycelia mats of *A. ochraceus* started to growth after 7 days. Dyes strongly absorbed to the substrate and the mycelia mats. Different organic solvents were used in order to extract tested dyes. Similar methodology and observation were noticed by Gandolfi Boer et al. (2004) who were using solid state fermentation to cultivate *Lentinula (Lentinus) edodes* on with corn cob as a substrate with the aim of decolorization of different dyes. Regarding the extraction of adsorbed dyes, the solid state system containing sugar beet waste, dye and microorganism appeared to be very complex. Numerous experiments were performed with the aim to find the optimal conditions for dye extraction. The influence of a) time of the extraction, b) position of the tube in bath during the process of extraction, c) centrifugation and d) solvent was investigated in preliminary analysis. Even though some authors published that some dyes can be easily extracted by ethanol from corn cobs (Gandolfi Boer et al., 2004), the process of extraction was not successful in all experiments performed in this study. However, it is well known that in order to achieve the maximum dye-desorption, the selection of the solvents should be done for the type of substrate used as sorbent (Robinson et al., 2002). According to the obtained results it can be concluded that sugar beet waste is a good adsorbent for methylene blue and congo red. This is somehow expected while it is previously known that

methylene blue can easily adsorb to some solid materials (Sarioglu and Atay, 2006). Therefore the adsorption experiments using sugar beet waste inoculated with *A. ochraceus* should be carried out in a batch process by using aqueous solution of dyes to check if *A. ochraceus* could be used in biological waste water treatment.

4. Conclusion

Nonlignolytic strain *Aspergillus ochraceus* and its extracellular enzymes can be potential candidates for wastewater treatment of dye effluents. Further experiments are already in progress in order to optimize the process conditions with the aim of the production of enzymes cocktail that can be successfully used in decolorization processes of a great variety of dyes as well as dye mixtures.

Acknowledgment

This research was financially supported through The National Foundation for Science, Higher Education and Technological Development of the Republic of Croatia

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