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Effect of Different Carbon Sources on Decolourisation of an Industrial Textile Dye Under Alkaline–Saline Conditions

Cristiane Ottoni, Luis Lima, Cledir Santos & Nelson Lima

Current Microbiology

ISSN 0343-8651

Curr Microbiol
DOI 10.1007/s00284-013-0441-3

ONLINE FIRST

Current Microbiology

An International Journal

Volume 67 Number 4 October 2013

Colonization of the Mouse Upper Gastrointestinal Tract by *Lactobacillus murinus*: A Histological, Immunocytochemical, and Ultrastructural Study
M. Almirón · G. Traglia · A. Rubio · N. Sanjuan **395**

Carbon Catabolite Repression-Independent and pH-Dependent Production of Indoles by *Rubrivivax benzoatilyticus* JA2
Md. Mujahid · Ch. Sasikala · Ch.V. Ramana **399**

Production of Calcite Nanocrystal by a Urease-Positive Strain of *Enterobacter ludwigii* and Study of Its Structure by SEM
S. Ghashghaei · G. Emtiazi **406**

Microbiota Regulates the TLR7 Signaling Pathway Against Respiratory Tract Influenza A Virus Infection
S. Wu · Z.-Y. Jiang · Y.-F. Sun · B. Yu · J. Chen · C.-Q. Dai · X.-L. Wu · X.-L. Tang · X.-Y. Chen **414**

Investigating the Diversity of *Pseudomonas* spp. in Soil Using Culture Dependent and Independent Techniques
L. Li · W. Abu Al-Soud · L. Bergmark · L. Riber · L.H. Hansen · J. Magid · S.J. Sørensen **423**

Immunologic Mechanism of Patchouli Alcohol Anti-H1N1 Influenza Virus May Through Regulation of the RLH Signal Pathway In Vitro
X.-L. Wu · D.-H. Ju · J. Chen · B. Yu · K.-L. Liu · J.-X. He · C.-Q. Dai · S. Wu · Z. Chang · Y.-P. Wang · X.-Y. Chen **431**

Two Rep Genes in Small Cryptic Plasmid pKST21 of *Escherichia coli*
T. Spaková · L.K. Fecskeová · P. Javorský · P. Pristas **437**

Proteomic Analysis of Protein Expression in the Induction of the Viable But Nonculturable State of *Vibrio harveyi* SF1
J. Jia · Z. Li · J. Cao · Y. Jiang · C. Liang · M. Liu **442**

Effectiveness of Egg Yolk Antibody Against Shiga Toxin II Variant Toxicity In Vitro and In Vivo
Y. Feng · W. Liu · D. Shi **448**

***Alishewanella solinquinati* sp. nov., Isolated from Soil Contaminated with Textile Dyes**
Y.M. Kolekar · S.P. Pawar · S.S. Adav · L.-Q. Zheng · W.-J. Li · Y.S. Shouche · S.G. Dastager · K.M. Kodam **454**

Differential Exoproteome Analysis of Two *Corynebacterium pseudotuberculosis* Biovar *Ovis* Strains Isolated from Goat (1002) and Sheep (C231)
W.M. Silva · N. Seyffert · A. Ciprandi · A.V. Santos · T.L.P. Castro · L.G.C. Pacheco · D. Barh · Y. Le Loir · A.M.C. Pimenta · A. Miyoshi · A. Silva · V. Azevedo **460**

Inoculation with a Mixed Degrading Culture Improves the Pesticide Removal of an On-Farm Biopurification System
P. Verhagen · L. De Gelder · N. Boon **466**

A Three-Scale Analysis of Bacterial Communities Involved in Rocks Colonization and Soil Formation in High Mountain Environments
A. Esposito · S. Ciccazzo · L. Borruso · S. Zerbe · D. Daffonchio · L. Brusetti **472**

Use of an *algD* Promoter-Driven Expression System for the Degradation of Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by *Pseudomonas* sp. HK-6
B.-U. Lee · H. Baek · K.-H. Oh **480**

The rOmp22–HpaA Fusion Protein Confers Protective Immunity Against *Helicobacter pylori* in Mice
X. Huang · B. Xu · G. Duan · C. Song **487**

Molecular Characterization of an Atypical IncX3 Plasmid pKPC-NY79 Carrying *bla*$_{KPC-2}$ in a *Klebsiella pneumoniae*
P.-L. Ho · Y.-Y. Cheung · W.-U. Lo · Z. Li · K.-H. Chow · C.-H. Lin · J.F.-W. Chan · V.C.-C. Cheng **493**

Molecular Characterization of Chitinase Genes from a Local Isolate of *Serratia marcescens* and Their Contribution to the Insecticidal Activity of *Bacillus thuringiensis* Strains
A. Ozgen · K. Sezen · I. Demir · Z. Demirbag · R. Nalcacioglu **499**

Quantitative Analysis of Porcine Endogenous Retroviruses in Different Organs of Transgenic Pigs Generated for Xenotransplantation
U. Mazurek · M.C. Kimsa · B. Strzalka-Mrozik · M.W. Kimsa · J. Adamska · D. Lipinski · J. Zeyland · M. Szalata · R. Slomski · J. Jura · Z. Smorag · R. Nowak · J. Gola **505**

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Curr Microbiol ISSN 0343-8651

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DOI 10.1007/s00284-013-0441-3

Effect of Different Carbon Sources on Decolourisation of an Industrial Textile Dye Under Alkaline–Saline Conditions

Cristiane Ottoni · Luis Lima · Cledir Santos · Nelson Lima

Received: 9 April 2013 / Accepted: 10 July 2013
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Abstract White-rot fungal strains of *Trametes versicolor* and *Phanerochaete chrysosporium* were selected to study the decolourisation of the textile dye, Reactive Black 5, under alkaline–saline conditions. Free and immobilised *T. versicolor* cells showed 100 % decolourisation in the growth medium supplemented with 15 g l^{-1} NaCl, pH 9.5 at 30 °C in liquid batch culture. Continuous culture experiments were performed in a fixed-bed reactor using free and immobilised *T. versicolor* cells and allowed 85–100 % dye decolourisation. The immobilisation conditions for the biomass and the additional supply of carbon sources improved the decolourisation performance during a long-term trial of 40 days. Lignin peroxidase, laccase and glyoxal oxidase activities were detected during the experiments. The laccase activity varied depending on carbon source utilized and glycerol-enhanced laccase activity compared to sucrose during extended growth.

Introduction

Wastewater from textile industries represents a serious concern since it contains different kinds of synthetic dyes, which are toxic to wildlife and humans [21]. The major chemical pollutants present in textile wastewater are dyes containing carcinogenic amines, toxic heavy metals, chlorine bleaching, halogen carriers, free formaldehyde, biocides and softeners [6]. In addition, it contains chelating agents, by-products, surfactants, salts in very high concentrations and a neutral to alkaline pH value ranging from 7.0 to 11.0 [14]. The temperature of various textile and other dyes effluents is relatively high (>40 °C). These characteristics make the effluents difficult to treat.

Several conventional physico-chemical and biological techniques have focused on the decolourisation of wastewater by fungi, particularly the white-rot fungi (WRF), because WRF produce extracellular enzymes such as laccases (Lcc), lignin peroxidases (LiP) and manganese-dependent peroxidases (MnP), which degrade different synthetic chemicals, including textile dyes [11]. However, very few reports described the capability of WRF [14] or marine-derived fungi [18] for the bioremediation of coloured effluents under alkaline–saline conditions.

Synthesis of extracellular enzymes by WRF is influenced by the (a) fungus and its stage of development, (b) type of cultivation and (c) inducers in the culture medium. However, the most critical factors are the sources of carbon and nitrogen and the ratio between these nutrients [8, 17]. Carbon source is usually required for the growth of fungi and enzyme synthesis. Glucose is the most widely used carbon source in research. There are also reports as to the proper assimilation of carbon sources: sucrose, fructose, maltose, xylose, glycerol, starch and xylan [16].

Free and immobilised cells of WRF and their extracellular enzymes for textile effluents treatments have been used in various forms [13], including fixed-bed continuous flow, submerged membrane and biological counter rotating bioreactors, among others. However, fungi are sensitive to high pH and salt concentrations and selecting strains with decolourisation capability under these conditions is crucial [10].

Electronic supplementary material The online version of this article (doi:10.1007/s00284-013-0441-3) contains supplementary material, which is available to authorized users.

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Published online: 27 August 2013

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The current study assessed the decolourisation of Reactive Black 5 (RB5), a recalcitrant azo textile dye model, using batch and fixed-bed bioreactors with free and immobilised cells under alkaline–saline conditions to evaluate the effect of different carbon sources for dye decolourisation.

Materials and Methods

Microorganisms and Medium Composition

Four strains of WRF, *Trametes versicolor* MUM 94.04, 04.100 and 04.101, and *Phanerochaete chrysosporium* MUM 94.15 (ATCC 24725) were obtained from the Micoteca da Universidade do Minho (MUM, Braga, Portugal) fungal culture collection for use in the following experiments. These strains were maintained on Tap Water Agar-cellulose plates (TWA-cellulose, agar 15 g l^{-1} in tap water with a strip of Whatman grade 4 filter paper) and subcultured every month. Liquid culture medium (LCM) (sucrose 5.0 g l^{-1}, ammonium sulphate 0.5 g l^{-1}, Sigma yeast nitrogen base—(YNB)—amino acids and ammonium sulphate free 1.7 g l^{-1}, Sigma L-asparagine 1.0 g l^{-1}, Sigma RB5 0.1 g l^{-1} and different concentrations of NaCl up to 25.0 g l^{-1} with 5.0 g l^{-1} intervals) was used to perform batch and continuous fermentations. The initial pH of the LCM was adjusted to pH 9.5 using 1 M NaOH. The pre-adaption medium (PAM) was LCM containing 15 g l^{-1} Oxoid agar-2 for solidification.

Immobilisation of Biomass and Scanning Electron Microscope (SEM)

Polyurethane foam (PUF) and nylon sponge (NS) supplied by the Scotch Brite 3 M SA Company (Madrid, Spain) were used as support. Cubes of 1 cm^3 were cut to serve as supports. The pre-treatment of PUF and NS was performed according to the method described elsewhere [19]. The morphological structures of the supports selected for cell immobilisation and the fungal mycelium colonisation were obtained by scanning electron microscopy (SEM, Leica/Cambridge Instrument S360, Cambridge, UK). The samples were coated with gold and platinum (80/20 %) before SEM analysis.

Culture Conditions

Batch Decolourisation Using Free Cells

Decolourisation experiments of RB5 (0.1 g l^{-1}) were performed in 250 ml Erlenmeyer flasks containing 100 ml of LCM and conducted for 28 days using the four WRF. Five plug discs of 8 mm diameter, cut with a sterile cork borer from the periphery of a 7-day-old colony grown in PAM plates, were used as inoculum. The cultures were incubated in a Certomat rotary shaker at 150 rpm at 30 °C. This temperature was selected as the optimal common temperature for all strains under studied. Biotic and abiotic controls were carried out under identical conditions but without dye and inoculum, respectively. An additional control was assayed with autoclaved fungal biomass to evaluate the contribution of the fungal cell walls to dye adsorption. Final results represent at least two independent assays.

Batch Decolourisation Using Immobilised Cells

Trametes versicolor MUM 04.100 was immobilised in PUF and NS supports. For each support, 21 Erlenmeyer flasks containing 100 ml of LCM were inoculated with 5 cubes of 1 cm^3 and 5 plug discs of 8 mm diameter and incubated at 30 °C and 150 rpm. Three cultures were retrieved daily for determination of biomass quantification, decolourising rate, sucrose concentration and enzymatic activities. After this time, the supports were transferred to a sterile filtration apparatus and washed thoroughly with 150 ml of sterile distilled water to remove the free biomass which was quantified as dried weight to know the importance of this faction on the decolourisation process. In addition, supports with the biomass immobilised were also dried in an oven at 60 °C until constant weight. Then, the biomass quantification was obtained after the subtraction the support weights that in average were for PUF 0.45 $\pm$ 0.09 g and for NS 0.40 $\pm$ 0.07 g. Final results represent at least three independent assays.

Decolourisation of RB5 in a Fixed-Bed Reactor

Continuous RB5 decolourisation was carried out in a fixed-bed reactor using either free or immobilised *T. versicolor* MUM 04.100 cells. Inoculum was prepared as described above. Then, either free or immobilised biomass was filtered using a vacuum system and inoculated into the bioreactor under aseptic conditions. The fixed-bed bioreactor with a volume of 300 ml and a working volume of 260 ml was loaded with fresh medium and free or immobilised biomass (PUF or NS). After total decolourisation in batch mode occurred a continuous fed through a peristaltic pump and continuous aeration was initiated. The continuous system of RB5 solution (0.1 g l^{-1} at pH 9.5 and 15 g l^{-1} of NaCl) was carried out with a fixed flow rate of 2.14 mg·day^{-1}. Details of the fixed-bed reactor are shown elsewhere [7]. During the experiment the biomass was determined as previously described [14]. During the time course of decolourisation when 85 % or less was achieved pulses of sucrose or glycerol (5 g l^{-1} final concentration in LCM) were made at different times in order to maintain fungal metabolism for an extended fermentation time period.

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Analytical Methods

During the experiments the dye and carbon sources concentrations were determined as previously described [14]. pH values were recorded for each sample.

Enzymatic Assays

The enzymatic activities of lignin peroxidases (LiP; $\varepsilon_{310nm} = 9{,}300\ M^{-1}\ cm^{-1}$), laccases (Lcc; $\varepsilon_{525nm} = 65{,}000\ M^{-1}\ cm^{-1}$), manganese-dependent peroxidases (MnP; $\varepsilon_{590nm} = 53{,}000\ M^{-1}\ cm^{-1}$), Glyoxal oxidase—GLOX ($\varepsilon_{436nm} = 18{,}000\ M^{-1}\ cm^{-1}$) and proteases ($\varepsilon_{440nm} = 4{,}600\ M^{-1}\ cm^{-1}$) were determined as described elsewhere [9]. For each enzymatic activity assay, the same reaction mixtures containing boiled supernatant samples were used as blank. One unit (U) of enzyme activity was defined as the amount of the enzyme for changing the absorbance by 0.01 min^{-1}. Enzyme activities of all the samples were expressed as U l^{-1}.

Results and Discussion

Batch Decolourisation

The efficiency of the free cells of four WRF strains to decolourise RB5 under alkaline–saline conditions was assessed in LCM. *T. versicolor* MUM 04.100 displayed the greatest extent of decolourisation (100 %), followed by *T. versicolor* MUM 94.04 (95 %), *T. versicolor* MUM 04.101 (60 %) and *P. chrysosporium* MUM 94.15 (20 %) (Table 1). These results corroborate those of Moreira Neto et al. [10] where *Pleurotus ostreatus* (CCIBT2347) and *Peniophora cinerea* (CCIBT2541) decolourised textile effluent by 93 % with 10 g l^{-1} NaCl and initial pH of 8.0. In the present work, *T. versicolor* MUM 94.04 and 04.100 were able to decolourise RB5 under more stringent alkaline–saline conditions (15.0 g l^{-1} NaCl and pH 9.5). A gradual decrease in pH was observed during RB5 decolourisation and fungal growth. NaCl concentrations above 20.0 g l^{-1} decreased decolourisation indicating physiological limitations due to the high osmotic pressure or enzymatic inhibition. In addition, the fungal cell walls did not adsorb RB5 which means that the dye decolourisation is not a physical process.

Among the ligninolytic enzymes produced by *T. versicolor* MUM 04.100, Lcc presented the highest activity (77.2 U l^{-1}) followed by LiP (18.5 U l^{-1}). In contrast, MnP activity was not detected in this assay. The results obtained are consistent with studies conducted elsewhere [2, 23] where it is reported that Lcc is the main enzyme synthesized in various WRF and is responsible for the decolourisation of different dyes and dye effluents. Despite the widespread perception of optimal activity of Lcc under acidic conditions, previous studies (e.g. [12]) have shown a wider flexibility, namely for pH (3.5–8.0) and salinity (0.05–0.6 M). Notwithstanding this, results by Zilly et al. [22] described Lcc expression being inhibited by

Table 1 Dye decolourisation by the white-rot fungi in free cells batch culture for 28 days

MUM strains	Time (days)	Biomass (g l^{-1})	Decolourisation (%)	Lcc (U l^{-1})	LiP (U l^{-1})	pH final
P. chrysosporium	7	0.30 ± 0.10	10 ± 0.09	ND	ND	8.55
94.15	14	0.62 ± 0.09	20 ± 0.13	ND	3.2 ± 0.02	7.13
	21	0.60 ± 0.13	20 ± 0.19	ND	7.8 ± 0.04	6.12
	28	0.61 ± 0.02	20 ± 0.08	ND	3.2 ± 0.01	5.10
T. versicolor	7	1.10 ± 0.08	65 ± 0.01	33.5 ± 0.05	2.7 ± 0.01	8.00
94.04	14	2.60 ± 0.01	80 ± 0.09	49.7 ± 0.07	8.3 ± 0.05	5.10
	21	2.70 ± 0.04	90 ± 0.02	43.1 ± 0.05	4.1 ± 0.02	4.10
	28	2.70 ± 0.01	95 ± 0.02	30.5 ± 0.03	2.2 ± 0.03	4.03
T. versicolor	7	0.60 ± 0.03	85 ± 0.08	18.4 ± 0.07	ND	7.11
04.100	14	2.10 ± 0.01	90 ± 0.02	77.2 ± 0.01	10.7 ± 0.03	5.22
	21	2.82 ± 0.05	100 ± 0.00	63.5 ± 0.06	18.5 ± 0.01	4.73
	28	2.92 ± 0.04	100 ± 0.00	51.3 ± 0.05	3.1 ± 0.01	4.55
T. versicolor	7	0.33 ± 0.04	20 ± 0.13	ND	ND	8.10
04.101	14	0.80 ± 0.02	25 ± 0.20	3.0 ± 0.08	ND	6.23
	21	0.92 ± 0.01	55 ± 0.17	2.5 ± 0.10	ND	6.01
	28	0.99 ± 0.10	60 ± 0.09	1.9 ± 0.05	ND	4.24

Fungi incubated in LCM with initial pH 9.5 and [NaCl] 15.0 g l^{-1}. Average of two independent experiments

ND not detected

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Ganoderma lucidum in the presence of NaCl even at low concentrations (0.02 M). This inhibitory effect appears selective as it was not observed in the presence of sodium sulphate (Na_2SO_4). These authors presented the ability of the enzyme to decolourise the dye Remazol Brilliant Blue R in the presence of 1.0 M Na_2SO_4 and 0.1 M NaCl, which had efficiency of 100 and 50 %, respectively.

The adhesion of *T. versicolor* MUM 04.100 on the supports used during immobilisation was confirmed by SEM (Fig. 1) and the biomass adhesion represented 90.50 and 91.85 % for PUF and NS, respectively (Table 2). A slight variation of immobilised biomass was observed between supports with immobilisation yields 0.18 ± 0.08 and 0.23 ± 0.09 $g.g_{support}^{-1}$ for PUF and NS, respectively. Both supports with immobilised fungus promoted a complete decolourisation of the LCM within 2 days of incubation (data not shown) and the maximum values of Lcc and LiP activities were 70.2 and 10.7 U l^{-1} in PUF, and 57.6 and 8.9 U l^{-1} in NS (Table 2). The pH of the LCM was initially adjusted to 9.5, and during the experiment, the pH decreased continuously. The higher values of Lcc activity were detected in both supports when the pH ranged between 5.1 and 4.9 (Table 2).

Our results are in accordance with Park et al. [15] who described the average of decolourisation obtained with immobilised cells of *Funalia trogii* as being superior when compared with free cells. These authors described the stability of the decolourisation for a period of at least 12.5 days. Enayatizamir et al. [3] related that the decolourisation ability of *P. chrysosporium* was greatly influenced by the support (NS and sunflower seed shells) used. This is likely due to the support, which influences both the type and proportion of the produced enzymes responsible for dye decolourisation. According to these authors, this underlines the importance of a suitable selection of supports for this type of process.

Decolourisation of RB5 by *T. versicolor* in a Fixed-Bed Bioreactor

A set of experiments in a fixed-bed bioreactor was performed using free and immobilised cells. The results using *T. versicolor* MUM 04.100 free cells are presented in Fig. 2. After the complete dye decolourisation at 3 days, the process of continuous dye solution feeding was initiated. During the fermentation process pulses of sucrose at 8 and 15 days and glycerol at 20 days promoted high cell growth. Pulse of sucrose and glycerol increased the rate of decolourisation to values higher than 90 %. After the introduction of glycerol, a maximum value of Lcc activity (102.2 U l^{-1}) was detected. Higher values of LiP activity (10.2 U l^{-1}) was observed at 11 day after pulse of glucose. Residual values of GLOX were determined. MnP and proteases activities were not detected.

Using the fungus immobilized in PUF and NS (Fig. 3; Supplementary Fig. 1) similar profiles of dye decolourisation and enzymatic activities were obtained. After the first 24 h of batch growth total decolourisation occurred for both experiments and the constant feed input of dye

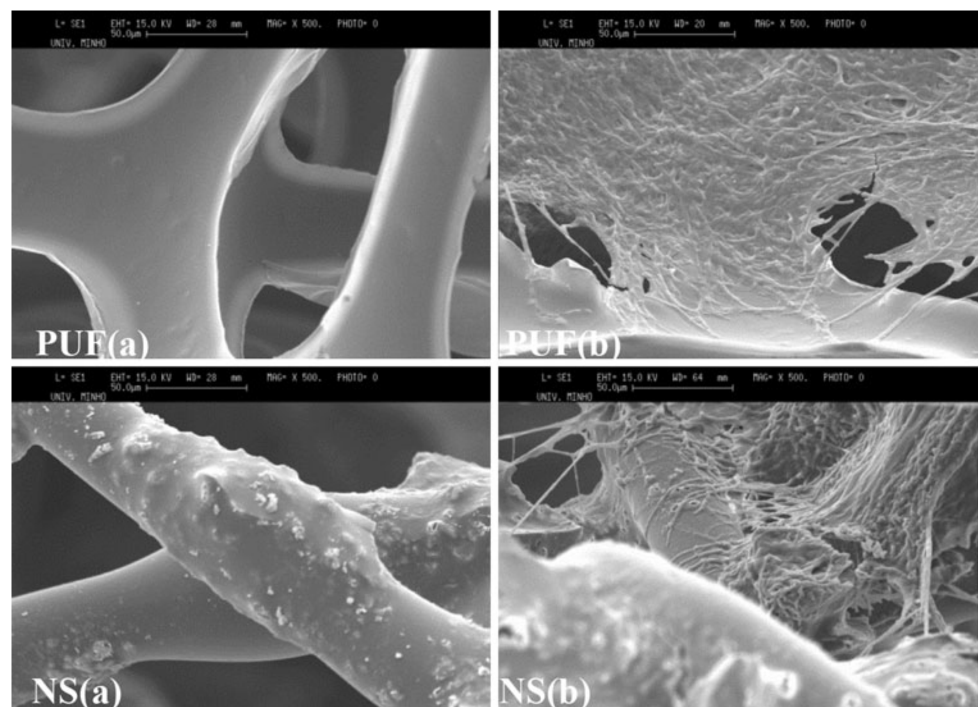

Fig. 1 Microphotographs obtained by SEM for PUF and NS supports: **a** without fungi and **b** colonised by *T. versicolor* strain MUM 04.100 after 7 days of incubation at 30 °C. *Bars* 50 µm

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Table 2 Biomass and enzyme activities of *T. versicolor* MUM 04.100 immobilised in PUF and NS in batch culture for 7 days

Support	Time (days)	Immobilised biomass (g)	Free biomass (g)	Lcc activity (U l^{-1})	LiP activity (U l^{-1})	pH
PUF	1	0.92 ± 0.10	0.05 ± 0.02	49.7 ± 0.01	8.7 ± 0.02	5.2
	2	1.10 ± 0.20	0.07 ± 0.01	70.2 ± 0.02	10.7 ± 0.01	4.9
	3	1.39 ± 0.25	0.08 ± 0.06	9.4 ± 0.04	2.7 ± 0.06	4.2
	4	1.83 ± 0.30	0.12 ± 0.02	6.6 ± 0.03	2.1 ± 0.01	4.0
	5	2.24 ± 0.05	0.19 ± 0.08	3.0 ± 0.01	ND	3.7
	6	2.26 ± 0.08	0.22 ± 0.07	2.7 ± 0.04	ND	3.3
	7	2.28 ± 0.10	0.24 ± 0.01	2.2 ± 0.02	ND	3.0
NS	1	1.21 ± 0.20	0.03 ± 0.01	53.3 ± 0.22	7.2 ± 0.01	5.3
	2	1.89 ± 0.16	0.03 ± 0.01	57.6 ± 0.17	8.9 ± 0.02	5.1
	3	2.29 ± 0.05	0.04 ± 0.01	9.4 ± 0.12	5.7 ± 0.02	3.7
	4	2.45 ± 0.10	0.06 ± 0.02	6.8 ± 0.08	5.7 ± 0.02	3.3
	5	2.46 ± 0.08	0.10 ± 0.02	2.9 ± 0.03	2.0 ± 0.02	3.3
	6	2.48 ± 0.07	0.17 ± 0.05	ND	ND	3.1
	7	2.48 ± 0.02	0.22 ± 0.04	ND	ND	3.1

Immobilised fungi incubated in LCM with initial pH 9.5 and [NaCl] 15.0 g l^{-1}. Average of three independent experiments

ND not detected

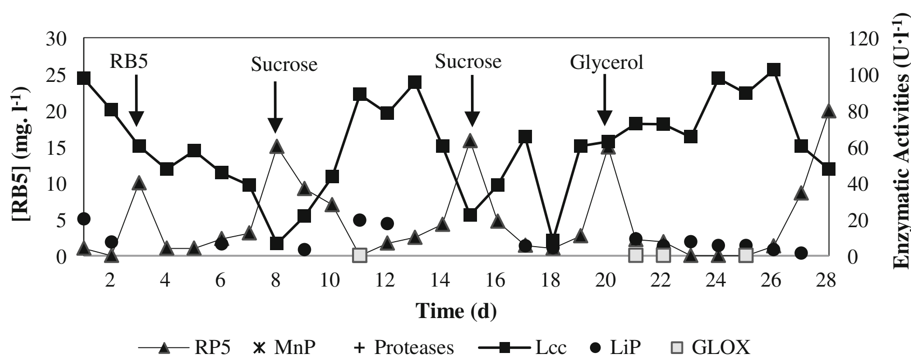

Fig. 2 RB5 decolourisation and enzymatic activities using free cells of *T. versicolor* MUM 04.100 in a fixed-bed reactor

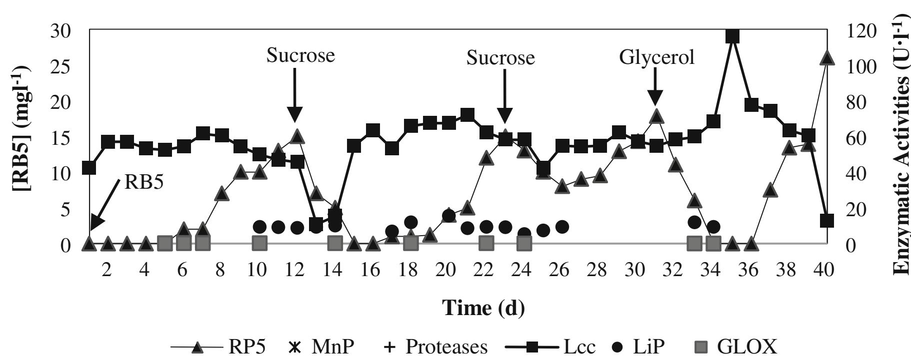

Fig. 3 RB5 decolourisation and enzymatic activities using *Trametes versicolor* MUM 04.100 immobilised in PUF in a fixed-bed reactor

solution was commenced. Sucrose and glycerol promoted dye decolourisation from 90 to 100 % and maximum Lcc activity occurred after glycerol addition as also described in the free cells experiment. Maximum Lcc activities were 119.8 and 112.8 U l^{-1} when the fungus was immobilised in PUF and NS, respectively.

In Asgher et al. [1], the stimulatory effect of starch to MnP expression was described with the concomitant effluent decolourisation by *Coriolus versicolor* IBL-04. Kanwal and Reddy [5] obtained maximum Lcc activity for mannose, followed by rhamnose, galactose, ribose, sorbose and mannitol as carbon source using the WRF *Morchella*

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crassipes. These authors concluded that Lcc activity is highly influenced by carbon source. In Jonstrup et al. [4] the capability of *Bjerkandera* sp. BOL13 to decolourise Remazol Red RR in a packed-bed bioreactor was described where high dye decolourisation and Lcc and MnP enzymatic activities were detected when wood was used as packing. Decolourisation efficiencies of 65–90 % were maintained for 12 days when glucose was supplied as growth substrate. Rodríguez Couto et al. [20] added glucose, cellulose, fructose and glycerol in an air-lift bioreactor at different times to investigate the effect of carbon source on Lcc activity of *T. hirsuta*. The highest Lcc activity (19,394 U l^{-1}) was detected when using glycerol. This corresponded to 1.32-, 1.55- and 2.44-fold higher than the results obtained for glucose, cellulose and fructose, respectively.

Conclusion

Trametes versicolor MUM 04.100 completely decolourised RB5 under alkaline–saline condition in Erlenmeyer flasks and is a potential fungus to be employed industrially for this process. The immobilised fungus provided a rate of decolourisation equal or higher than 85 % for a period of 40 days in a fixed-bed bioreactor again indicating the utility of the fungus. Lcc is the main enzyme involved in the breakdown of the reactive dye and glycerol is an important substrate for oxidative metabolism which promotes higher Lcc production hence increasing the decolourisation process and the addition of which could contribute to an effective process, although the co-metabolism process needs more work for better comprehension.

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