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Effects of glucose on the decolorization of Reactive Black 5 by yeast isolates

YANG Qingxiang[1,*], TAO Lingxia[1,2], YANG Min[3], ZHANG Hao[1]

1. College of Life Sciences, Key Laboratory of Environmental Pollution Control Technology of Henan Province, Henan Normal University, Xinxiang 453007, China. E-mail: yangqx66@163.com
2. Biology Department, Puyang Vocational and Technical College, Puyang 457000, China
3. State Key Laboratory of Environmental Aquatic Chemistry, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

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Abstract

The cometabolic roles of glucose were investigated in decolorization of an azo dye, Reactive Black 5, by yeast isolates, *Debaryomyces polymorphus* and *Candida tropicalis*. The results indicated that the dye degradation by the two yeasts was highly associated with the yeast growth process and glucose presence in the medium. Color removal of 200 mg dye/L was increased from 76.4% to 92.7% within 60 h to 100% within 18–24 h with the increase of glucose from 5 to 10 g/L, although the activity of manganese dependent peroxidase (MnP) decreased by 2–8 times in this case. Hydrogen peroxide of 233.3 μg/L was detected in 6 h in *D. polymorphus* culture. The cometabolic functions of glucose and hydrogen peroxide could be also confirmed by the further color removals of 95.8% or 78.9% in the second cycle of decolorization tests in which 7 g glucose/L or 250 μg H_2O_2/L was superadded respectively together with 200 mg dye/L.

Key words: decolorization; yeast; manganese dependent peroxidase; Azo dye, Reactive Black 5

Introduction

The serious environmental problems as results of large production and utilization of dyes have been attracted extensive concerns (Wesenberg *et al*., 2003; Fu and Viraraghavan, 2001). Numerous studies were focused on the biological treatment due to the potentially low cost (Borchert and Judy, 2001). Decolorization of various dyes using white rot fungi or their ligninolytic enzymes was widely studied in the past years and several bioreactors in lab-scale were proposed for this purpose (Borchert and Judy, 2001). However, the rigorous conditions for enzyme production in dye containing wastewater and the risk of contamination by bacteria under non-sterile conditions retard the application of white rot fungi for wastewater treatment (Borchert and Judy, 2001). On the other hand, yeast, another kind of fungi, has been successfully applied to treat industrial effluents such as food-, molasses-, and oil manufacturing wastewater as reported by Japanese scientists (Chigusa *et al*., 1996; Moriya *et al*., 1990; Yoshizawa *et al*., 1981) Although several yeasts were reported to be able to remove dyes through the mechanism of biosorption (Kakuta *et al*., 1992; Martins *et al*., 1999; Meehan *et al*., 2000), reports on decolorization by yeast through the above ligninolytic system have been minimal (Hofrichter, 2002). In our previous reports, two yeast isolates, *Debaryomyces polymorphus* and *Candida tropicalis*, which could produce manganese dependant peroxidase (MnP) under simple medium conditions and decolorize six different kinds of dyes showed great potentials in colored wastewater treatment (Yang *et al*., 2003). In the following studies, the optimal conditions for decolorization and MnP production were investigated in detail. The results indicated that the two yeasts could not use dye as a sole carbon or energy source for growth and decolorization. Growth and color removal processes by these two strains were strongly associated with glucose existence in the medium (Yang *et al*., 2005).

In this study, the evidences of glucose as a cometabolic substrate for decolorization by these two yeasts were provided and the possible mechanisms about enzymatic biodegradation of C. I. Reactive Black 5 (RB5), a widely used azo dye, were discussed.

1 Materials and methods

1.1 Dye

C.I. Reactive Black 5 (RB5, Color Index) was obtained from Dystar (Germany) as dye formulation Remazol Schwarz B, containing RB5 in 75% purity. The dye solution was prepared as reported previously (Yang *et al*., 2003).

1.2 Microorganisms and decolorization tests

Debaryomyces polymorphus (Y1-0813) and *Candida*

*Corresponding author. E-mail: yangqx66@163.com.

tropicalis (Y2-0814) were isolated from a municipal wastewater treatment plant in Munich City, Germany and preserved in China General Microbiological Culture Collection Center (CGMCC). These two yeasts were cultivated at 28°C, 140 r/min in the described medium (Yang *et al.*, 2003). Decolorization of RB5 by yeast was detected in 100 ml medium as reported previously (Yang *et al*., 2005). Glucose concentration was changed from 5 to 10 g/L.

1.3 Assay of manganese dependant peroxidase

MnP was detected using DMAB/MBTH (3-dimethyl amino benzoic acid/3-methyl-2-benzo-thiazlinoe-hydrazone) as described by Lang (Lang *et al*., 1997). One unit (U) of enzyme activity was defined as the amount of enzyme required producing one micromole of product per minute.

1.4 Detection of H_2O_2 concentration

H_2O_2 concentration in the culture was determined by iodometric titration as described by Harris (1999).

2 Results and discussion

2.1 Correlations between glucose utilization and dye decolorization

In our previous reports, the decolorization process by *D. polymorphus* was divided into two stages, a rapid degradation stage within the first 16 h and a slow decolorization stage thereafter. In the first stage, the dye was biodegraded accompanying with the quick growth of yeast and no color was absorbed on the cells. When the dye could not be removed completely in the fast decolorization stage, the residual color was attached on yeasts giving the cells light pink color (dye concentration of 200–300 mg/L) or blue color (dye concentration over 300 mg/L) in the following cultivation (Yang *et al*., 2005). The decolorization process by *C. tropicalis* exhibited the same trend but with lower color removal rates.

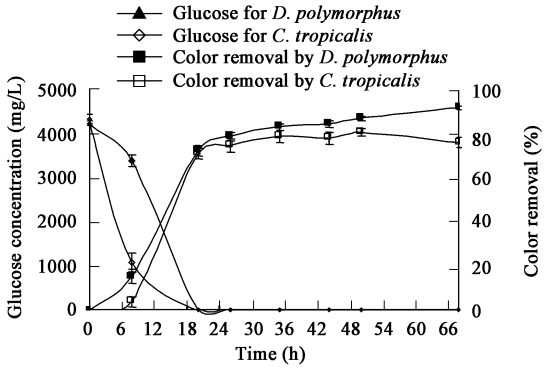

Fig. 1 Kinetic relation between glucose utilization and dye decolorization by *D. polymorphus* and *C. tropicalis* (dye conc.: 200 mg/L). Each point represents mean ± SE of triplication.

In this study, the kinetic process of glucose utilization against cultivation time was detected as shown in Fig.1.

Processes of glucose utilization and dye decolorization by the two yeasts were very similar. Glucose was utilized very fast and exhausted within 20 h cultivation, which was corresponding to the quick decoloriztion stage of biodegradation. After that, the decolorization process went into a slow stage mainly by biosorption and the final color removal rate for *D. polymorphus* and *C. tropicalis* was only 92.7% and 76.4% respectively despite of still high activity of MnP in the culture as shown in Fig.2. It is obvious that color removal process through mechanism of dye biodegradation by these two yeasts was highly associated with the yeast growth and glucose presence in the medium. In our previous report, the yeasts could not use the dye as a sole carbon and energy source for yeast growth and MnP production (Yang *et al*., 2005). Glucose acted as a cometabolic substrate for dye degradation, which was similar with the results obtained in white rot fungi by other authors (Adosinda *et al*., 2001; Swamy and Ramsay, 1999). However, in most white rot fungi, glucose or other carbon sources provided a necessary substrate for cell growth and enzyme production, in which the enzyme production was during their secondary metabolism and was induced by limited nutrient level. The dye degradation process occurred after enzyme production (Wesenberg *et al*., 2003). In this point, the situation in yeasts was different from most white rot fungi, in which the decolorization process occurred during the exponential growth stage of cells and only under the conditions of enough nutrients existence. The phenomena of decolorization by yeasts suggested another side of glucose functions and an unknown decolorization mechanism.

2.2 Effects of glucose concentration on MnP production and color removal

To further confirm the effects of glucose on decoloriza-

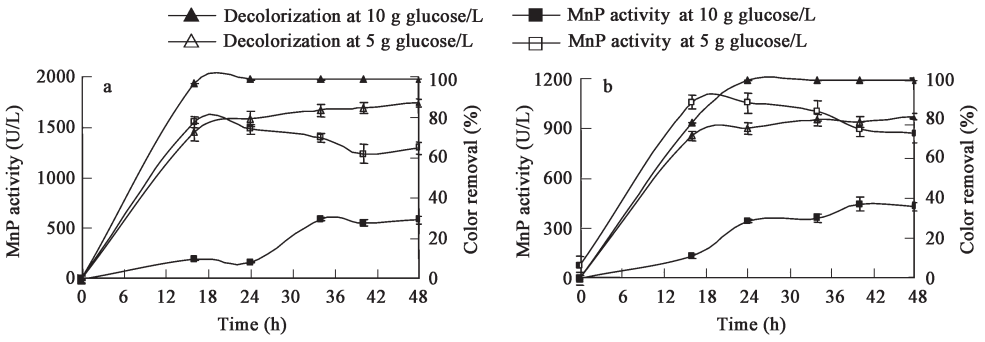

Fig. 2 Comparison of decolorization and MnP activities by yeasts *D. polymorphus* and *C. tropicalis* under different concentrations of glucose (dye conc. 200 mg/L). Each point represents mean ± SE of triplication.

tion process, the concentration of glucose was increased to 10 g/L. Color removals and enzyme activities in these two yeasts were compared under the conditions of 5 and 10 g glucose/L as shown in Fig.2.

When the glucose concentration was increased to 10 g/L, complete color removal (100%) was obtained by *D. polymorphus* and *C. tropicalis* within 16 and 24 h, respectively, which was much quicker than that at the condition of 5 g glucose/L. No slow stage was observed in the decolorization process under higher glucose concentration. However, contrary results were obtained for the relation of MnP activity and glucose concentration. The MnP activities at 5 g glucose/L were about 2–8 times of those at 10 g glucose/L in these two yeasts. In our previous report (Yang *et al*., 2005), production of MnP by *D. polymorphus* was induced by the presence of dye. The possible interpretation for the low level of MnP activity at 10 g glucose/L was that dye degradation was too fast to induce more enzyme production. But it is difficult to elucidate how the yeast cells degrade 200 mg dye/L with so low level of enzyme activity. Besides MnP, there should be other enzymes responsible for color removal of RB5 in the two yeasts.

According to the reports, biodegradation of aromatic hydrocarbon could be catalyzed either by ligninolytic enzyme system including lignin peroxides (LiP), MnP and laccase or by oxygenase in fungi (Cerniglia, 1992). Our previous studies have confirmed that these two yeasts could not produce LiP or laccase (Yang *et al*., 2003).

Further detection of P450 cytochrome oxygenase indicated that there was no activity of this enzyme in *D. polymorphus* or *C. tropicalis* cells at either presence or absence of dye in the culture. However, low level of P450 oxygenase activity (4.07 and 0.8 μmol/g wet cells at presence and absence of dye, respectively) could be detected in another unreported yeast isolate (*Rhodotorula mucilaginosa*) which could also effectively decolorize RB5, but without activity of ligninolytic peroxidase system. The detail mechanism of decolorization by *D. polymorphus* and *C. tropicalis* requires further research. At least, production of MnP was a kind of response to the presence of dye by these two yeasts and should be related with the process of color removal. As our discussion above, the quick decolorization stage by yeasts was strongly accompanied with the cells growth. It suggested that some unknown enzymes producing in the process of cell growth might be responsible for color removal and glucose might play important roles in this process except as a carbon or energy source for yeast growth. According to Hofrichter, H_2O_2 or other peroxide was required to start the reaction when MnP attacked lignose or phenyl compounds (Hofrichter, 2002). Production of H_2O_2 in *D. polymorphus* culture was monitored over 50 h under condition of 10 g glucose/L and 200 mg RB5/L as in Fig.3. The maximum concentration of H_2O_2 (233.3 μg/L) was detected at 6 h and quickly reduced to an undetectable level at 16 h, which coincided with the trends of glucose exhaustion and dye degradation in Fig.1.

According to the literature (Swanmy and Ramsay, 1999), H_2O_2 was normally produced by another enzyme, glucose peroxidase, which was attached on the cell wall when glucose was used as the main carbon source in the culture (Swamy and Ramsay, 1999). Our results strongly supported Hofrichter's supposing on mechanism of MnP catalysis and Swamy's view about H_2O_2 production (Hofrichter, 2002). In this point, glucose played two important roles in biodegradation of dye: providing a carbon and energy source for yeast growth and acting as a substrate for H_2O_2 production.

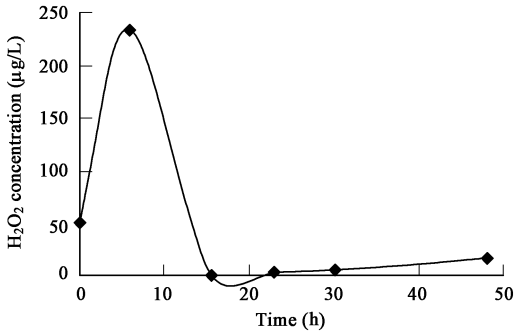

Fig. 3 Production of H_2O_2 by *D. polymorphus* over 50 h under the conditions of 200 mg RB5/L and 10 g glucose/L.

Further research indicated that 7 g glucose/L was enough for decolorization of 200 mg RB5/L by *D. polymorphus* as shown in Fig.4. The same result was obtained in *C. tropicalis* (data not shown here).

2.3 Effects of superadded glucose and H_2O_2 on color removal

To confirm the cometabolic effects of glucose and H_2O_2, three sets of tests were designed in the above flasks containing 7 g glucose/L and 200 mg RB5/L after the first cycle of decolorization (22 h). Another dosage of 200 mg RB5/L was superadded into the cultures separately (case 1), together with 7 g glucose/L (case 2) or together with 250 μg H_2O_2/L (case 3). The results indicated that effective and much quicker color removal was observed only under the condition of cases 2 and 3 (95.8% or 78.9% at 12 h, respectively). The culture flasks in case 1 did not exhibit obvious color change, which suggested that the decolorization reaction could not be effectively initiated without the presence of glucose or H_2O_2 despite of still high MnP activity and enough amount of biomass

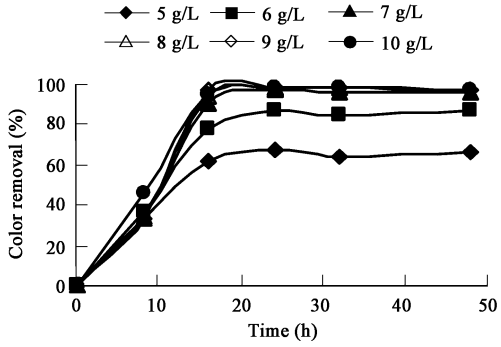

Fig. 4 Effects of glucose concentration on color removal of Reactive Black 5 by *D. polymorphus* (dye conc., 200 mg/L).

in the culture. The function of H_2O_2 in the decolorization processes by the two yeast isolates was obvious although further research was required about the optimal conditions.

A decreased color removal rate of 85.2% and 74.7% was obtained respectively in the third and forth cycle of decolorization tests when case 2 was successively repeated every 24 h. Decrease of enzyme activity, inhibition of metabolic products as well as exhaustion of other nutrients might be the reasons for this trend and have already been solved by designing continuous decolorizing bioreactors.

Considering the future application of these two yeasts in real wastewater treatment, other carbon sources such as sucrose, maltose and starch were also tested in batch experiments and only starch could not support the yeasts growth and decolorization (Yang *et al*., 2005). In our present biofilm reactor inoculated with these two yeasts, municipal wastewater also showed a very effective carbon and nitrogen source for decolorization (The results will be reported in another paper).

3 Conclusions

The quick decolorization processes of RB5 through mechanism of biodegradation by yeasts, *D. polymorphus* and *C. tropicalis*, were highly associated with glucose existence in the medium. For the decolorization of 200 mg dye/L, increase of glucose concentration from 5 to 10 g/L obviously increased the color removal efficiency although the production of MnP exhibited reverse trends. Compared with our previous results about the MnP production by *D. polymorphus*, the low activity of enzyme at higher glucose concentration was possibly due to scarce of efficient induction by dye or its intermediate products.

In the process of decolorization, glucose acted as a carbon source for yeast growth and a substrate for H_2O_2 production. The latter was considered to start the decolorization and catalytic reaction of MnP. The effective function of glucose and H_2O_2 in the second cycle of decolorization tests strongly supported Hofrichter's hypothesis about mechanism of MnP catalysis (Hofrichter, 2002). For complete color removal of 200 mg RB5/L, at least 7 g glucose /L was required by these two yeasts. The detailed mechanism of dye decolorization by these two yeasts and the key enzymes responsible for dye degradation are still required further study.

Acknowledgements

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