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Decolorization of Triphenylmethane Dyes by a Laccase from *Bacillus pumilus*

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The recombinant laccase CotA from *Bacillus pumilus* was evaluated for the decolorization of triphenylmethane dyes Brilliant Blue R and Brilliant Green. The process occurred within the pH range 4–10. Elevated temperatures stimulated the decolorization, the highest level being observed at 75–85 °C. Another attractive property of *B. pumilis* CotA was its resistance to a high concentration of sodium chloride (0.4 M NaCl at alkaline pH). The addition of the redox mediators 2,2-azino-*bis*(3-ethylbenzthiazoline-6-sulfonic acid) and syringaldehyde accelerated the decolorization of the dyes by more than 3 times. The decolorization of Brilliant Green, a toxic dye effective against gram positive bacteria, resulted in its complete detoxification. The ability to decolorize under extreme conditions makes *B. pumillis* laccase a good candidate for dyestuff processing in industry.

Key words: laccase, *Bacillus pumilus,* triphenylmethane dyes, decolorization.

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Laccases (*p*-diphenol: dioxygen oxidoreductases, EC 1.10.3.2) belong to the multicopper oxidase family of enzymes. They are widespread in fungi, higher plants, insects and bacteria. Laccases are able to oxidize a broad range of substrates such as polyphenols, aromatic amines and taking into condiseration a wide range of catalyzed reactions, they are regarded as industrially relevant enzymes. Biotechnological applications of laccases include bleaching in the pulp and paper industry, polymer synthesis and bioremediation of soils and water [1].

Triphenylmethane dyes are widely used in the textile, pharmaceutical, food, paper and cosmetic industries [2]. At present, a number of studies are focused on the degradation, decolorization and detoxification of triphenylmethane dyes by fungal laccases [3, 4]. The potential of bacterial laccases in this respect has not been assessed. In contrast to the majority of fungal laccases, bacterial laccases exhibit high thermostability and alkaline the majority of activity [5].

The goal of this work was to describe the process of decolorizing and detoxifying of triphenylmethane dyes by using a bacterial laccase from *Bacillus pumilus*.

EXPERIMENTAL

Reagents, strains, media and culturing conditions

ABTS, SYD, triphenylmethane dyes BBR and BG were purchased from Sigma-Aldrich. Enzymes were obtained from Sibenzyme. *Escherichia coli* 109 and the vector pUC18 were purchased from Quiagen (Hilden, Germany). *Bacillus pumilus* B-508 (ATCC 7061) and *Bacillus thuringiensis* B-454 (ATCC 10792) obtained from the All-Russian Collection of Microorganisms (VKM) were cultivated in LB medium at 30 °C.

Expression and purification of CotA

The priority of cloning and expression of a bacterial laccase from *B. pumilus* was defended by a patent [6]. In brief, a gene encoding laccase (*cotA*)

Abbreviations: ABTS, 2,2-azino-*bis*(3-ethylbenzthiazoline-6-sulfonic acid); BBR, Brilliant Blue R (Acid Blue 83); BG, Brilliant Green (Basic Green 1); LB medium, Luria-Bertani medium; SYD, syringaldehyde.

was amplified from the genomic DNA of *B. pumilus* B-508 and inserted into the pUC18 expression vector, the resulting recombinant pBR4 plasmid was transformed into the cells of the strain *E. coli* 109; the enzyme was isolated from bacterial biomass by centrifugation, sonication, heat treating at 70 °C and chromatography on anion exchange resins.

Laccase activity assays

Laccase activity was routinely assayed at room temperature. The reaction mixture contained 0.5 mM ABTS and 0.1 M citrate-phosphate buffer, pH 4.0. The oxidation of ABTS was followed by an absorbance increase at 420 nm (ε_{420} = 36000 M^{-1} cm^{-1}). One unit is defined as an amount of the enzyme that oxidizes 1 μmol of substrate per minute.

Decolorization of dyes

The enzymatic treatment of the dyes was performed in the presence or absence of redox mediators ABTS (2 μM, in 0.1 M citrate-phosphate buffer, pH 4.4) or SYD (200 μM, in 0.1 M bicarbonate buffer, pH 8.4) at 0.1 mg mL^{-1} of the dye (260 μM of BG and 125 μM of BBG) and 0.05 U mL^{-1} of laccase. The reaction mixtures were incubated at 50 °C, and the decolorization activity of laccase was determined as the relative decrease in the visible light absorbance with BG and BBR at 625 and 560 nm, the absorbance maxima of the dyes, respectively. The control samples contained the reaction mixture without the enzyme. The influence of pH was evaluated in 0.1 M citrate-phosphate (pH 4–6), tris-HCl (pH 7–9) and glycine-NaOH (pH 9–10) buffers at 50 °C. The temperature optimum was measured at temperatures ranging from 50°C to 90 °C in 0.1 M citrate-phosphate (pH 4.4) and 0.1 M bicarbonate (pH 8.4) buffers with BG and BBR, respectively. The influence of salts on decolorization was determined in the presence of 50, 100, 200 and 400 mM NaCl at 50 °C using 0.1 M citrate-phosphate (pH 4.4) or 0.1 M bicarbonate (pH 8.4) buffer with BG and BBR, respectively. All reactions were carried out in triplicate, and the data were presented as means of triplicates.

Antibacterial activity of BG

The antibacterial activity of BG was determined by the agar difuusion method from the growth inhibition of B. thuringiensis [7]. Sterile LB-agar in Petri dishes was inoculated with bacterial cells ($2 \cdot 10^7$ cells on a dish). After incubation of the reaction mixture containing 0.1 M citrate-phosphate buffer (pH 4.4), 0.4 mg mL^{-1} of the dye and 1 U mL^{-1} laccase at 37 °C, the control and enzyme-treated BG reaction mixtures (3 μL) were put on sterile paper discs, which were slightly dried and plated on inoculated agar. Petri dishes were incubated for 24 h at 30 °C. The relative antibacterial activity was calculated as $C_s/_c \cdot 100\%$, where C_c and C_s are BG concentrations in the reaction mixture untreated or treated by the enzyme. C_s was calculated from the linear regression equation determined for a known BG concentration: $y = 7.09x + 10.98$ (y is the zone of the inhibition diameter and x is log BG concentration, $r = 0.996$). All experiments were performed in triplicate.

Statistical analysis

All experiments were conducted in three replicates. The results were statistically analyzed by program SigmaPlot 11 and their mean values were presented with standard deviations.

RESULTS AND DISCUSSION

In this study, we examined the potential applicability of the recombinant laccase CotA from *B. pumilus* for the decolorization and detoxification of triphenylmethane dyes. The alkalic dye Brilliant Green (BG, Basic Green 1) and acidic dye Brilliant Blue R (BBR, Acid Blue 83) were investigated (Fig. 1).

The effect of pH on decolorization of the dyes was measured in the pH range of 4.0–10. As shown in Fig. 2*a*, the optimum pH was 4.4 with BG and 8.3 with BBR. The results on alkaline decolorization of BBR corresponded to the data on alkaline decolorization of anthraquinonic and azo dyes by CotA laccase of *B. subtilis* [8]. The results on acidic decolorization of BG were in agreement with those for fungal laccase of *Trametes versicolor* [3]. The effect of temperature on the decolorization rate was assayed in 0.1 M citrate-phosphate (pH 4.4) and 0.1 M bicarbonate (pH 8.4) buffers with BG and BBR, respectively. Elevated temperatures stimulated the decolorization; the highest level was observed at 75–85 °C (Fig. 2*b*). Another attractive property of CotA of *B. pumilus* was its resistance to a high concentration of sodium chloride. As shown in Fig. 2*c*, the effect of NaCl on the BBR decolorization at pH 8.4 was negligible at a concentration of up to 0.2 M NaCl and decreased by only 9% at 0.4 M NaCl. However, for BG the decolorization was inhibited at pH 4.4 in the presence of NaCl in the reaction mixture. The level of decolorization at 0.2 M NaCl was reduced by 72% and was totally inhibited at 0.4 M NaCl. Among bacterial laccases, halide tolerance and thermostability in laccases of the marine bacterium *Marinomonas mediterranea* and *Streptomyces viridochromogenes* have been described [9, 10].

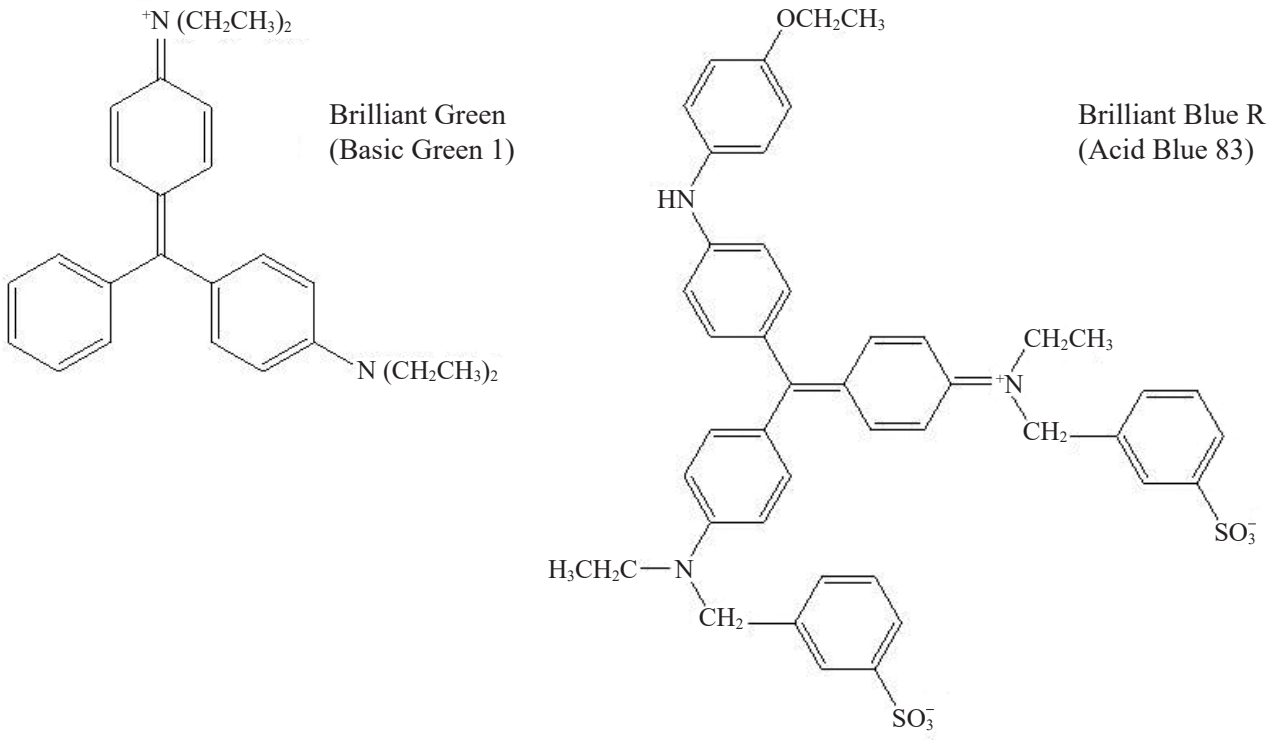


Fig. 1. Chemical structure of triarylmethane dyes

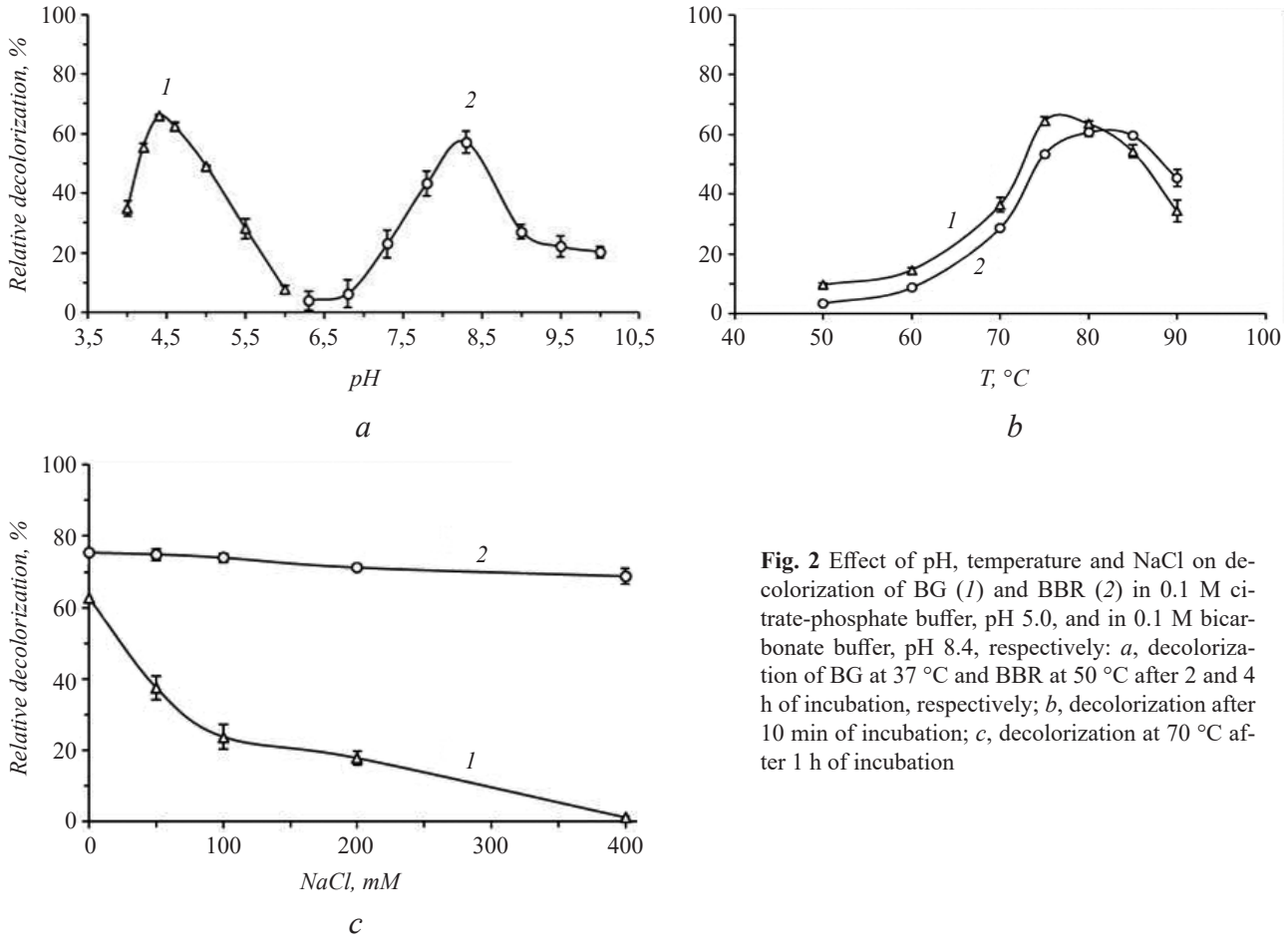


Fig. 2 Effect of pH, temperature and NaCl on decolorization of BG (*1*) and BBR (*2*) in 0.1 M citrate-phosphate buffer, pH 5.0, and in 0.1 M bicarbonate buffer, pH 8.4, respectively: *a*, decolorization of BG at 37 °C and BBR at 50 °C after 2 and 4 h of incubation, respectively; *b*, decolorization after 10 min of incubation; *c*, decolorization at 70 °C after 1 h of incubation

The addition of the redox mediators ABTS and SYD accelerated the decolorization. The enzyme alone was unable to decolorize BBR at pH 4.4, but in the presence of 2 μM ABTS, the decolorization reached up to 86% after 2 h of incubation. In contrast, the enzyme alone was able to decolorize BBR at alkaline pH. After 2 h incubation at pH 8.4, 37% of BBR color was found to be removed. In the presence of a redox mediator SYD, a 3.3-fold increase in BBR decolorization level was observed within the initial 0.5 h at pH 8.4, and a 76% BBR color was removed after 2 h of incubation (Fig. 3). BG was decolorized at acidic pH by the laccase alone by 75% within 2 h. Laccase from *Bacillus vallismortis* spores alone decolorized BG at acidic pH only by 15% within 24 h [11]. In the presence of a redox mediator ABTS, a 3-fold increase in the BG decolorization level was observed within the initial 30 min and a 90% color removal occurred after 2 h of incubation (Fig. 3).

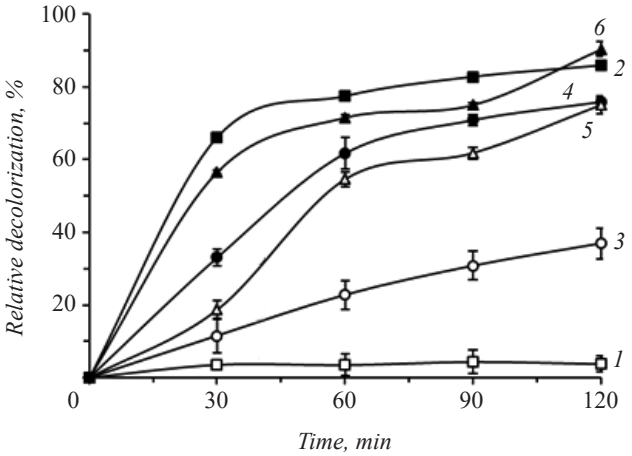
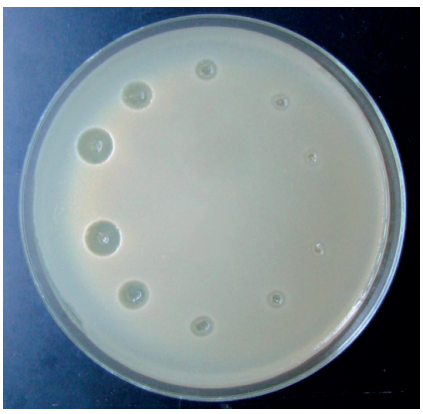


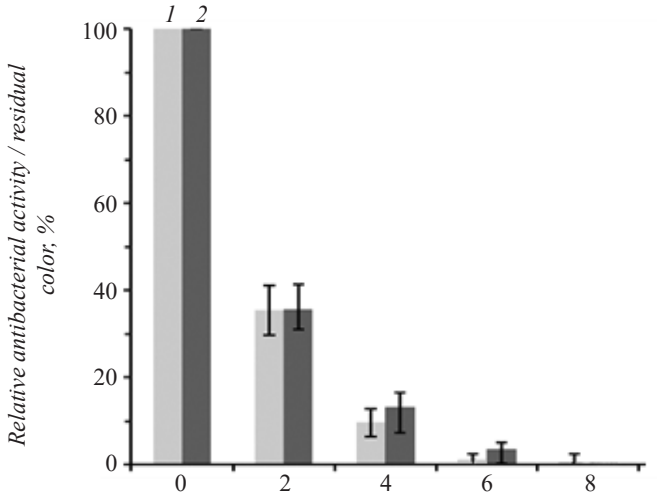
Fig. 3 Effect of redox mediators on decolorization of dyes. Decolorization of BBR and BG in the absence and presence of ABTS (2μM) or SYD (200 μM) at 50 °C: *1*, BBR, *2*, BBR + ABTS at pH 4.4; *3*, BBR, *4*, BBR + ABTS at pH 8.4; *5*, BG, *6*, BG + SYD at pH 4.4

BG is a toxic dye effective against gram positive bacteria that is used in medicine as a topical antiseptic. We tested the growth inhibition activity of a laccase-treated and -untreated reaction mixture against *B. thuringiensis* and observed a direct correlation between the reduction of toxicity and the degree of color removal (Fig. 4). These results corresponded to the data on the decolorization and detoxification of the triphenylmethane dye malachite green by the laccase of *Ganoderma lucidum* [4].

a



b



c

Fig. 4 Antibacterial activity of BG against *B. thuringiensis*: *a* – control dish with known dilutions of the dye; *b* – the experimental dish; *c* – histogram of the correlation between the degree of decolorization of the dye (*1*) and the antimicrobial activity of the reaction products (*2*).

Thus, the *B. pumilus* laccase was able to decolorize triphenylmethane dyes in the acdic to alkaline range at high temperatures (up to 90 °C); the decolorization of the acidic dye BBR in an alkaline medium was weakly sensitive to high salt concentrations; the decolorization reaction of the alkalic dye BG was accompanied by the loss of its antimicrobial activity. Because of this kind of properties the CotA laccase might be effective when used to decolorize triphenylmethane dyes in industrial applications.

REFERENCES

1. Couto S.R., Herrera J.L.T. Industrial and biotechnological applications of laccases: a review. *Biotechnol. Adv*., 2006, 24, 500–513. doi: 10.1016/j.biotechadv.2006.04.003
2. Azmi W, Sani R.K., Banerjee U.C. Biodegradation of triphenylmethane dyes. *Enzyme Microbiol. Technol*., 1998, 22, 185–191. doi: 10.1016/S0141-0229(97)00159-2
3. Casas N., Parella T.,Vicent T., Caminal G., et al. Metabolites from the biodegradation of triphenylmethane dyes by *Trametes versicolor* or laccase. *Chemosphere*., 2009, 75, 1344–1349. doi: 10.1016/j.chemosphere.2009.02.029
4. Murugesan K., Yang I.H., Kim Y.M., Jeon J.R., et al. Enhanced transformation of malachite green by laccase of *Ganoderma lucidum* in the presence of natural phenolic compounds. *Appl. Microbiol. Biotechnol*., 2009, 82, 341–350. doi:10.1007/s00253-008-1819-1
5. Chauhan P., Goradia B., Saxena A. Bacterial laccase: recent update on production, properties and industrial applications. *3 Biotech*., 2017, 7(5), 32–53. doi:10.1007/s13205-017-0955-7
6. Glukhov I.L., Shmatchenko V.V., Leont'evskij A.A. Method for photometric measurement of total bilirubin in blood serum by use of oxidase from *Bacillus pumilus.* Patent (RU) 2418072 C1, C 12 Q 1/26. 2011
7. Balouirin M., Sadiki M., Ibnsouda S. K. Methods for *in vitro* evaluating antimicrobial activity: a review. *J. Pharm. Anal*., 2016, 6, 71–79. doi: 10.1016/j.jpha.2015.11.005
8. Pereira L, Coelhoa A.V., Viegas C.A., et al. Enzymatic biotransformation of the azo dye Sudan Orange G with bacterial CotA-laccase *J. Biotechnol*., 2009, 139, 68–77. doi:10.1016/j.jbiotec.2008.09.001
9. Jiménez-Juárez N., Román-Miranda R., Baeza A., et al. Alkali and halide-resistant catalysis by the multipotent oxidase from *Marinomonas mediterranea*. *J. Biotechnol*., 2005, 117, 73–82. doi:10.1016/j.jbiotec.2005.01.002
10. Trubitsina L.I., Lisov A.V., Zakharova M.V., et al. Structural and functional characterization of two-domain laccase from *Streptomyces viridochromogenes*. *Biochimie*., 2015, 112, 152–159. doi: 10.1016/j.biochi.2015.03.005
11. Zhang C., Diao H., Lu F., et al. Degradation of triphenylmethane dyes using a temperature and pH stable spore laccase from a novel strain of *Bacillus vallismortis*. *Bioresour. Technol*., 2012, 126, 80–86. doi: 10.1016/j.biortech.2012.09.055