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A New *Stenotrophomonas maltophilia* Strain Producing Laccase. Use in Decolorization of Synthetics Dyes

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Abstract Laccase activity was detected in a soil bacterium *Stenotrophomonas maltophilia AAP56* identified by biochemical and molecular methods. It was produced in cells at the stationary growth phase in Luria Bertani (LB) medium added by 0.4 mM copper sulfate. The addition of $CuSO_4$ in culture medium improved production of laccase activity. However, one laccase enzyme was detected by native polyacrylamide gel electrophoresis. The enzyme showed syringaldazine (K_m=53 μM), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (K_m=700 μM), and pyrocatechol (K_m=25 μM) oxidase activity and was activated by addition of 0.1% (*v*/*v*) Triton-X-100 in the reaction mixture. Moreover, the laccase activity was increased 2.6-fold by the addition of 10 mM copper sulfate; the enzyme was totally inhibited by ethylenediaminetetraacetic acid (5 mM), suggesting that this laccase is a metal-dependant one. Decolorization activity of some synthetic dyes (methylene blue, methyl green, toluidine blue, Congo red, methyl orange, and pink) and the industrial effluent (SITEX Black) was achieved by the bacteria *S. maltophilia AAP56* in the LB growth medium under shaking conditions.

Keywords *Stenotrophomonas maltophilia AAP56* · Laccase · Decolorization · Synthetic dyes · Industrial effluents

Introduction

Extensive research effort has been involved to evaluate the use of enzymes in biotechnological and environmental applications [1]; one of this important potential is the oxidoreductase enzymes such as polyphenol oxidase or phenol oxidase. White-rot fungi have an outstanding capability of producing extracellular oxidative enzymes, mainly involved in lignin

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degradation [2]. In recent years, many studies have demonstrated that several white-rot fungi are capable of oxidizing various types of synthetic dyes, such as azo, anthraquinone-based, metal, triphenyl methane, and phthalocyanine [3–6]. Some researchers demonstrated that oxidizing activity is due to three enzymatic forms: lignin peroxidase, manganese peroxidase, and laccase [7]. In fact, peroxidases and laccases have broad substrate specificities and can catalyze the oxidation of a wide range of toxic organic compounds. The results show that an enzymatic oxidation can decrease the toxicity of some polycyclic aromatic hydrocarbons, phenols, organophosphorus pesticides, and azo dyes in laboratory and some field conditions [8]. Consequently, a very wide variety of bioremediation processes employ laccase in order to protect the environment from damage caused by industrial effluents.

Thus, laccases, EC 1.10.3.2, *p*-diphenol dioxygen oxidoreductase, are part of a larger group of enzymes termed the multicopper enzymes, which includes among others ascorbic acid oxidase and ceruloplasmin. Laccases were widely described and characterized as metal-containing oxidases [9]. This makes them one of the oldest enzymes ever described [10]. Laccases have been detected in many fungi and higher plants [10–13]. However, this enzymatic activity is not widely revealed in bacteria, while there is no report of laccase in *Stenotrophomonas maltophilia*.

For the decolorization assays with bacteria or bacterial laccase, some recent applications were tested and were demonstrated as an important efficacy to decolorize some recalcitrant dyes. *Pseudomonas desmolyticum* NCIM 2112 was able to degrade a diazo dye Direct Blue-6 in static anoxic condition. Induction of oxidative enzymes (lignin peroxidase, laccase) and tyrosinase while decolorization in the batch culture represents their role in degradation [14]. Based on the high stability of laccase activity of *Bacillus subtilis* SF spores compared to the liberated enzyme, only spores and immobilized spores were investigated for dye decolorization. The laccases produced by *B. subtilis* SF spores as well as the spores immobilized on inorganic carrier material were used for the decolorization of the common textile dyes during treatment of process waters contaminated with excess dyestuff. Both types of spore preparations proved to be very efficient in the complete removal of color [15].

This paper is the first report of laccase activity in *S. maltophilia* strain identified by molecular and biochemical markers. We demonstrated the existence of intracellular laccase activity in bacterial cells at stationary growth phase. We showed that the laccase activity is stimulated by copper; we also partially characterized the enzyme and analyzed its affinity towards some substrates. In addition, we report the ability of these bacteria to decolorize some synthetic dyes.

Materials and Methods

Bacterial Strain and Growth Conditions

Several bacterial strains were isolated from different soil from the city of Kelibia (Tunisia) and were analyzed to determine the production of polyphenol oxidase activity. But, so far, only one of this bacterial group was exhibited in an intracellular laccase activity. This bacterial strain was referenced by AAP56 and in fact was isolated from soil containing industrial wine waste. Strain AAP56 was grown and maintained at 30 °C in Luria Bertani (LB) medium which contains 1% (*w*/*v*) peptone, 1% (*w*/*v*) NaCl, and 0.5% (*w*/*v*) yeast extract. Stock culture was stored in 25% glycerol at −20 °C. Laccase production was obtained by growing cells in LB medium supplemented with 0.4 mM $CuSO_4$. This copper molarity was determined as the optimal concentration of laccase induction at stationary growth phase.

Biochemical Identification of AAP56 Strain

After gram's staining test and Drygalski medium test, the identification of bacterial strain AAP56 was monitored by the API 20 NE strip (bioMérieux®sa. REF 20 050). It is a standardized system for the identification of nonfastidious, nonenteric Gram-negative rods (e.g., *Pseudomonas*, *Acinetobacter*, *Flavobacterium*, *Aeromonas*, etc.) combining eight conventional tests, 12 assimilation tests, and a database. Those biochemical tests were performed in triplicate. Reading of the strip is carried out after incubation at 29 °C for 24 h and by referring to API's "Reading reference Table." Identification procedure is achieved by analysis with the software: "Api Lab_BioMérieux." The medium used for preparation of bacterial suspension for API tests is the API NaCl 0.85% medium. This medium is enriched by 200 μL per cup of the API AUX medium that contains 2 g L^{-1} of ammonium sulfate, 1.5 g L^{-1} of agar, 10.5 mL L^{-1} of vitamin solution, 10 mL L^{-1} of trace elements, 6.24 g L^{-1} of monosodium phosphate, and 1.5 g L^{-1} of potassium chloride (pH is adjusted to 7.0–7.2).

Molecular Identification Based on the 16s rDNA Sequence

A pure colony of the bacterium was grown in LB medium until log phase growth was obtained. The resulting growing bacteria was obtained with centrifugation. Genomic DNA from this isolate was extracted with the Wizard Genomic DNA purification kit (Promega). Amplification of 16s rDNA fragment of AAP56 strain was performed with 125–1,562 ng of bacterial DNA as template using 0.4 μM of 8f (AGAGTTTGATCCTGGCTCAG) and 0.4 μM of 1492r (GGTTACCTTGTTACGACTT) eubacterial universal primers [16–18] in a reaction mixture which contained 1.5 mM $MgCl_2$, 0.3 mM each of dNTPs, and 2.5 U of Taq polymerase (InvitroGene), in a Whatman Biometra® thermocycler. The polymerase chain reaction (PCR) program included an initial denaturation of 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min, extension at 72 °C for 1.5 min, and a final extension at 72 °C for 15 min. The amplified product was resolved on 1% (*w*/*v*) agarose gel. PCR product elution was carried out with Wizard SV Gel and PCR Cleanup System (Promega). The nucleotide sequence was determined manually using the dideoxynucleotide chain termination method [Thermo Sequenase kit (Amersham)] or automatically using BigDye® Terminator v3.1 Cycle Sequencing Kit in the ABI PRISM 3100-Avant Genetic Analyzer with universal and reverse primers. DNA sequence analyses were performed using the Blast and Bioedit programs.

Optimization of Laccase Production

The production of laccase was optimized by the study of correlation between bacterial growth (strain AAP56) and production of laccase activity. The optimal concentration of $CuSO_4$ added to the growth medium for the induction of laccase activity was determined by using the concentrations 0, 0.1, 0.2, 0.4, 0.6, and 0.8 mM of $CuSO_4$ in LB medium.

Preparation of Crude Extract

Bacteria were grown in LB medium supplemented by 0.4 mM $CuSO_4$ until stationary phase, harvested by centrifugation (10,000 rpm, 15 min), washed, and suspended with phosphate buffer, pH 7.0, 50 mM (pellet cells). The suspension was sonicated in an icebox for 15 min at a relative power output of 30-s duty period in an ultrasonic homogenizer

BioBlock Scientific 88160 (320 W) and then centrifuged at 10,000 rpm for 30 min at 4 °C and the supernatant was stored at −20 °C.

Laccase Activity

Laccase activity was measured at optimal temperature (40 °C) using syringaldazine (SGZ; 8,356 cm^{-1} M^{-1}) as substrate, recording its oxidation at 530 nm in a Beckman DU640 spectrophotometer. The total volume of reaction mixture is 1 ml containing 50-mM phosphate buffer, pH 7.0, 0.1% (*v*/*v*) Triton-X-100, 100 μM SGZ dissolved in absolute ethanol, and an appropriate amount of enzyme. One unit of enzyme activity (U) was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol of SGZ per minute under the standard assay conditions.

The optimum pH for laccase activity was determined as described above in 50-mM buffers between pH 4.0 and 9.0: acetate (pH 4.0–6.0), phosphate (pH 6.0–8.0), and Tris (pH 8.0–9.0).

Similarly, the optimum temperature for laccase activity was analyzed by using different incubation temperatures: 30, 40, 50, 60, and 70 °C under the standard assay conditions.

The K_m value was determined using Lineweaver-Burk plots with SGZ concentration of 10–200 μM, with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; ε_{420nm}= 36,000 M^{-1} cm^{-1}) concentration of 50–1,000 μM and with pyrocatechol (ε_{450nm}= 2,211 M^{-1} cm^{-1}) concentration of 100–5,000 μM. The laccase assay was carried out at 40 °C with 50-mM acetate buffer pH 4.5 when ABTS was used as substrate and with 50-mM phosphate buffer pH 7.0 when pyrocatechol was used as substrate. The enzymatic units were calculated as the amount of the enzymatic oxidation of 1 μmol of substrate per minute under the assay conditions.

Thermal and pH Stabilities of Enzyme

Enzyme stability at various pH values was determined by incubating the enzyme in 50-mM buffer, including the range of pH values from 4.0 to 9.0, for 60 min. The residual activity was measured at optimum pH (pH 7). Thermal stability was determined by incubating the enzyme in phosphate buffer (50 mM, pH 7.0) at a range of temperatures from 20 to 70 °C for 30 min. Sample of enzyme was returned to ambient temperature and the residual enzyme activity was measured in the normal manner as described previously.

Effect of Different Reagents on Laccase Activity

Effect of ethylenediaminetetraacetic acid (EDTA), sodium azide, urea, Cu^{2+}, Fe^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, and Ca^{2+} was studied by incubating enzymatic preparation with each effectors for 5 min at 4 °C prior to substrate (SGZ) addition. The laccase assays were carried out in optimal conditions. The effect of Triton-X-100 concentration in enzymatic assay mixture was analyzed from 0.05% to 1% (*v*/*v*). Control was done with 0% (*v*/*v*) of Triton-X-100. Also, the sodium dodecyl sulfate (SDS) effect was monitored from 0.01% to 0.4% (*v*/*v*). NaCl effect was demonstrated in the range of concentration from 100 to 500 mM. Control was carried out without NaCl in the normal manner.

Electrophoretic Analysis of Laccase Activity in the Gel

In order to identify which type of oxidative enzymes and the number of isoforms involved in oxidation of substrate, the crude extract was subjected to polyacrylamide gel

electrophoresis on nondenaturing conditions. Analytical native polyacrylamide gel electrophoresis (native PAGE) was performed as described by Laemmli [19], using acrylamide concentration of 7% for the resolving gel and 5% for the stacking gel. Electrophoresis was achieved in Tris–glycine buffer under 4 °C at a constant current of 20 mA for 60 min and at 30 mA for 90 min.

To detect enzyme activity by the staining method, the gel was equilibrated during 15 min by immersion in 0.05-M acetate buffer (pH 4.5). After that, the gel was equilibrated in 10 mL of 0.05-M acetate buffer (pH 4.5) supplemented with 5 mM ABTS during 5 min at 40 °C.

Dye Decolorization Assay

The decolorization experiments were also carried out in culture medium, into 250-mL Erlenmeyer flasks containing 25 mL of the Luria Bertani medium (contained 10 g L^{-1} of NaCl, 10 g L^{-1} of pancreatic peptone, and 5 g L^{-1} of yeast extract). The culture medium and the dye solutions have been adjusted to pH 7.0 with 1 M NaOH and autoclaved separately at 120 °C for 30 min. Individual dye was added to Erlenmeyer flasks which were inoculated with bacterial cells. Five-hundred microliters (2%; *v*/*v*) of each dye was added to 25 mL of LB medium and preliminary decolorization assays were carried out by bacteria incubation at 30 °C in LB medium with different dye concentration: 0.6 $OD_{665\ nm}$ per milliliter for methylene blue, 0.4 $OD_{665\ nm}$ per milliliter for toluidine blue, 0.5 $OD_{630\ nm}$ per milliliter for methyl green, 0.35 $OD_{485\ nm}$ per milliliter for methyl orange, 0.85 $OD_{530\ nm}$ per milliliter and 2.3 $OD_{470\ nm}$ per milliliter for Congo Red. The bacterial culture of *S. maltophilia AAP56* was incubated in 30 °C at 150 rpm for shaking. Decolorization was monitored spectrophotometrically using a Beckman DU640 spectrophotometer. The samples from bacterial growth medium at respective time intervals were centrifuged at 10,000 rpm for 15 min. The supernatant of the centrifuged samples was read at optimal length wave of the appropriate dyes. Experiments were performed in triplicate. Values shown are representative of at least three experiments. The decolorization efficiency was expressed as per the following equation:

$$\text{Residual coloration}(\%) = [\text{AF}/\text{AI}] \times 100.$$

where AI=initial absorbance and AF=absorbance of decolorized medium.

Results

Identification of the Bacterial Strain

Api20NE Strip

Drygalski medium plate assays were performed to determine the glucose fermentation by the isolates. Physiological and biochemical characters of the bacterial strain AAP56 were examined according to methods described above by the Api20NE strip (Table 1) like many nonfastidious Gram-negative rods [20]. The isolates were characterized for the following traits: shape and color of bacterial colonies [21]. Morphology was evaluated by performing phase contrast microscopy. The Gram reaction was performed as per standard procedures (Table 1). Results of Api strip were explored by soft system "Api Lab_BioMérieux." The analytical profile was found on percentage of identification and factor *T* which is the typical character of the strain onto taxon. The quality of identification was found with this two

Table 1 Some morphological and physiological properties of *S. maltophilia AAP56* and the results of Api 20 NE strip for enzymatic, assimilation, and oxidase tests.

Test	Property of	Reaction/enzymes	Assimilation of	Results
Shape	Bacterial colonies			Round small
Color	Bacterial colonies			Yellow
Morphology	Bacterial cells			Rod
Gram staining	Bacterial cells			−
Drygalski medium	Oxidase test			+
	Glucose fermentation			−
NO_3		Reduction of: nitrates to nitrites nitrates to nitrogen		+
TRP		Indole production		−
GLU		Fermentation		−
ADH		Arginine dihydrolase		−
URE		Urease		−
ESC		Hydrolysis with β-glucosidase		+
GEL		Hydrolysis with protease		+
PNPG		β-galactosidase		+
GLUa			Glucose	+
ARAa			Arabinose	−
MNEa			Mannose	+
MANa			Mannitol	−
NAGa			*N*-acetyl-glucosamine	+
MALa			Maltose	+
GNTa			Potassium gluconate	−
CAPa			Capric acid	−
ADIa			Adipic acid	−
MLTa			Malic acid	+
CITa			Trisodium citrate	+
PACa			Phenylacetic acid	−
OX		Cytochrome oxidase		+

The sign (−) indicated that the biochemical test had shown a negative result and (+) indicated that the biochemical test showed a positive result.

parameters, the soft system expressing: "acceptable identification" if 80%<percentage of identification≤90% and $0<T\leq0.25$; "Good identification" if 90%<percentage of identification≤99% and $0.25<T\leq0.5$; "Very good identification" if percentage of identification>99% and $T>0.5$; "Excellent identification" if percentage of identification>99.9% and $T>0.75$ [20]. The analytical profile of the strain AAP56 showed 99.9% of identification and $T=0.78$, indicating an excellent identification of *S. maltophilia*.

16s rRNA Gene Sequencing

To determine phylogenetic relationships between AAP56 and other representative strains of the genus *Stenotrophomonas*, approximately 1,500-bp fragments of its 16s rDNA were PCR-amplified using universal eubacterial 16s rDNA primers and only 565 bp are sequenced. Partial 16s rRNA gene sequence was compared with those available in the GenBank database of the National Center for Biotechnology Information (NCBI) by BLAST search and multiple-alignment analysis. For identification, the following criteria were used: (1) when the determined sequence yielded a similarity score of 99% with a reference sequence of a classified species, the unknown isolate was assigned to this species; (2) when the score was between 95% and 99%, the unknown isolate was assigned to the corresponding genus; and (3) when the score was 95%, the unknown isolate was assigned to a family. If the 16s rRNA gene sequence had identical similarity to two or more sequences of different species listed in GenBank, the unknown isolate was considered as unidentifiable to the species level. The partial 16s rRNA gene sequence of strain AAP56 (referenced by EU 152129 in the GenBank of the NCBI database) has similarity score of 99% with those of the bacterial specie *S. maltophilia* (Fig. 1). This result confirmed the biochemical identification with the Api20NE strip.

Production of Laccase by *S. maltophilia* AAP56

In time course experiments, laccase activity were detected in the bacterial cells after 6-h growth in cultures supplemented with 400 μM copper sulfate and reached peak levels after 48 h (Fig. 2a). The effect of copper concentration on laccase expression in cultures of *S. maltophilia AAP56* was shown in Fig. 2b. Laccase production was augmented with increasing $CuSO_4$ concentrations in the culture medium up to 400 μM. Higher copper concentrations were inhibitory and approximately 13% as enzyme activity were maintained when bacteria were grown in the presence of 800 μM copper sulfate. No laccase activity was detected in bacterial cells grown in the absence of copper.

Specific staining of laccase activity in the native PAGE showed a single band in the presence of protein extract from bacteria growing in LB medium supplemented by $CuSO_4$ (Fig. 3). Protein extract from bacteria growing in the absence of $CuSO_4$ does not reveal any band and consequentially any laccase activity. The *S. maltophilia AAP56* cultivated in experiment condition described previously (in "Materials and Methods") seems to produce one laccase enzyme.

Thermal and pH Stabilities

Some physicochemical and kinetic properties of laccase activity were analyzed in this work and the standard specific substrate for laccase: Syringaldazine [22] was used for all activity assays. The enzyme optimum pH was 7.0 in 50-mM phosphate buffer with syringaldazine as substrate (Fig. 4a). The activity was stable in the range between pH 6.0 and 9.0 within

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AAP56  1     AAACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCAGCAA
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Steno  1400  AAACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCAGCAA

AAP56  61    TGCTGATCTGCGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCC
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Steno  1340  TGCTGATCTGCGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCC

AAP56  121   GGACTGAGATAGGGTTTCTGGGATTGGCTTACCGTCGCCGGCTTGCAGCCCTCTGTCCCT
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Steno  1280  GGACTGAGATAGGGTTTCTGGGATTGGCTTACCGTCGCCGGCTTGCAGCCCTCTGTCCCT

AAP56  181   ACCATTGTAGTACGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGACGTCATCCCC
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Steno  1220  ACCATTGTAGTACGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGACGTCATCCCC

AAP56  241   ACCTTCCTCCGGTTTGTCACCGGCGGTCTCCTTAGAGTTCCCACCATTACGTGCTGGCAA
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Steno  1160  ACCTTCCTCCGGTTTGTCACCGGCGGTCTCCTTAGAGTTCCCACCATTACGTGCTGGCAA

AAP56  301   CTAAGGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGA
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Steno  1100  CTAAGGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGA

AAP56  361   CGACAGCCATGCAGCACCTGTGTTCGAGTTCCCGAAGGCACCA-TCCATCTCTGGAAAGT
             ||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Steno  1040  CGACAGCCATGCAGCACCTGTGTTCGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGT

AAP56  420   TCTCGACATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATACTC
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Steno  980   TCTCGACATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATACTC

AAP56  480   CACCGCTTGTGCGG-CCCCCGTCA-TTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCC-
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Steno  920   CACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCC

AAP56  537   AGGCGGCGAACTTAACGCGTTAGCTTCGA  565
             ||||||||||||||||||||||||||||
Steno  860   AGGCGGCGAACTTAACGCGTTAGCTTCGA  832
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(*)**AAP56** : Partial sequence of 16s rRNA gene of unknown strain AAP56
Steno : Partial sequence of 16s rRNA gene of *Stenotrophomonas sp*. 4_0_7 (code number in GenBank: EF540516.1)

Fig. 1 Alignment sequences of partial 16s ribosomal RNA sequence gene of unknown strain AAP56 and of *Stenotrophomonas sp*. 4-0-7 (code number in GenBank: EF540516.1)

1 h (Fig. 4c); Rosconi et al. [23] reported that CE52G laccase of *Sinorhizobium meliloti* was stable at a range of pH between 4.0 and 8.0. The optimum temperature was 40 °C (Fig. 4b) as reported for CotA of *B. subtilis* oxidase which has a maximum activity at 45 °C [22]. The enzyme thermal stability study revealed a high sensibility to temperature; the laccase maintained 48% of its activity at 40 °C and 25% at 50 °C after 30-min incubation and it was totally inactivated at 70 °C (Fig. 4d).

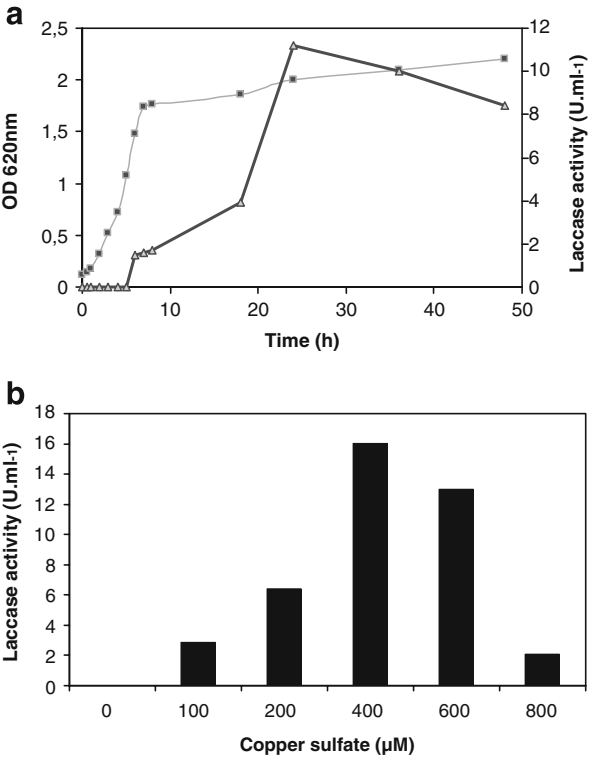

Fig. 2 **a** Production of laccase activity (*triangles*) relative to bacterial growth (OD 620 nm, *squares*) with 400 μM copper sulfate. **b** Induction of laccase activity in bacterial cell growth in LB medium preliminary supplemented with different concentrations of copper sulfate. Until stationary phase, bacterial culture was harvested by centrifugation (10,000 rpm, 15 min), washed, and suspended with phosphate buffer, pH 7.0, 50 mM. The suspension was sonicated in an icebox for 15 min and then centrifuged at 10,000 rpm for 30 min at 4°C and the supernatant was used for the laccase assay within normal manner described previously

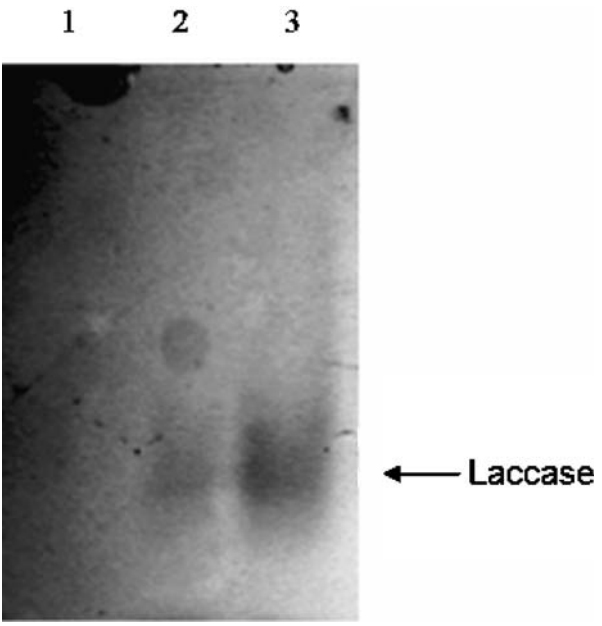

Fig. 3 Native PAGE was performed as described by Laemmli [29], using acrylamide concentration of 7% for the resolving gel and 5% for the stacking gel. Electrophoresis was achieved in Tris–glycine buffer under 4°C at a constant current of 20 mA for 60 min and at 30 mA for 90 min. Laccase activity in the gel was revealed by incubation at 40°C with 5 mM ABTS as substrate for 5 min. *1*: 15 μg of proteins (without Cu induction), *2*: 7.5 μg of proteins (with Cu induction), *3*: 15 μg of proteins (with Cu induction). Induction was carried out with 400 μM $CuSO_4$ supplemented to the growing medium (LB)

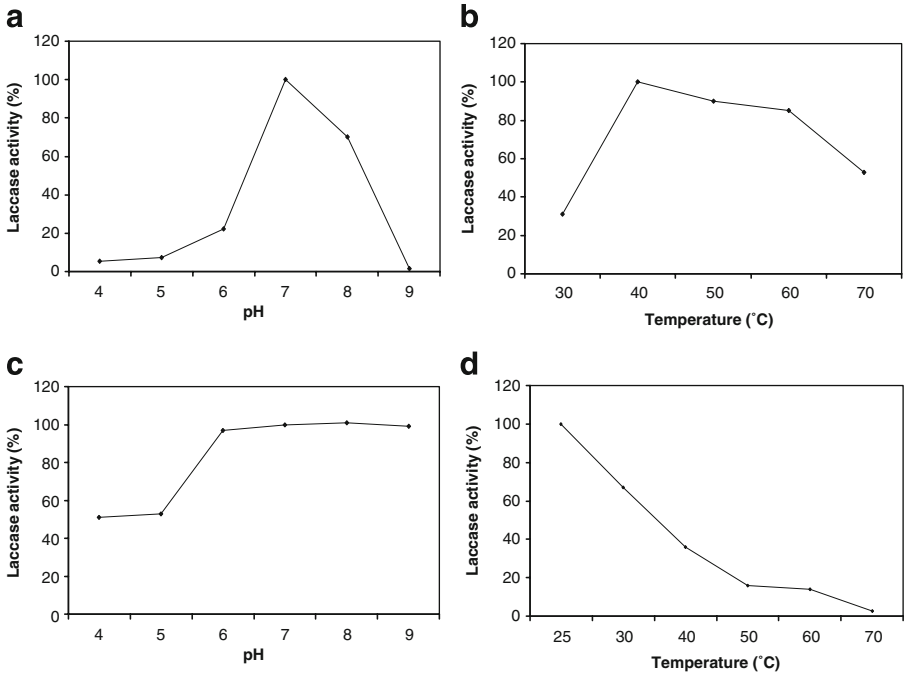

Fig. 4 **a** pH effect, **b** temperature effect, **c** pH stability, **d** temperature stability on or of laccase activity tested with syringaldazine as substrate

The K_m was 50 μM when syringaldazine was used as a substrate, 0.7 mM with ABTS, and 25 μM with pyrocatechol (Table 2). For bacterial laccase CotA, K_m is determined at 30 μM for SGZ and 110 μM for ABTS [24].

Effect of Different Reagents on Laccase Activity

Effect of chloride sodium and Triton-X-100 was studied; laccase activity was optimal at 100 mM NaCl and 0.1% Triton-X-100 (*v*/*v*); the enzyme activity was increased fivefold when 0.1% (*v*/*v*) of Triton-X-100 was added to the reaction mixture (Fig. 5a). Also, the addition of 0.05% (*v*/*v*) SDS enhanced the laccase activity 2.5-fold (Fig. 5b). EDTA, sodium azide, and urea were tested as possible inhibitors. Laccase activity was totally inhibited by EDTA (5 mM) and by urea (0.4 mM) but less with 5 mM sodium azide (40%). Since the laccase activity was a metal-dependant enzyme, some metal ion was added to the reaction mixture and the results shown in Fig. 5c demonstrated that the copper ion was

Table 2 Some kinetic parameters of the bacterial laccase of *S. maltophilia AAP56*.

Substrates	K_m (μM)	Vm (10^{-3} U)
Syringaldazine	53	2.9
ABTS	700	3.35
Pyrocatechol	25	6.9

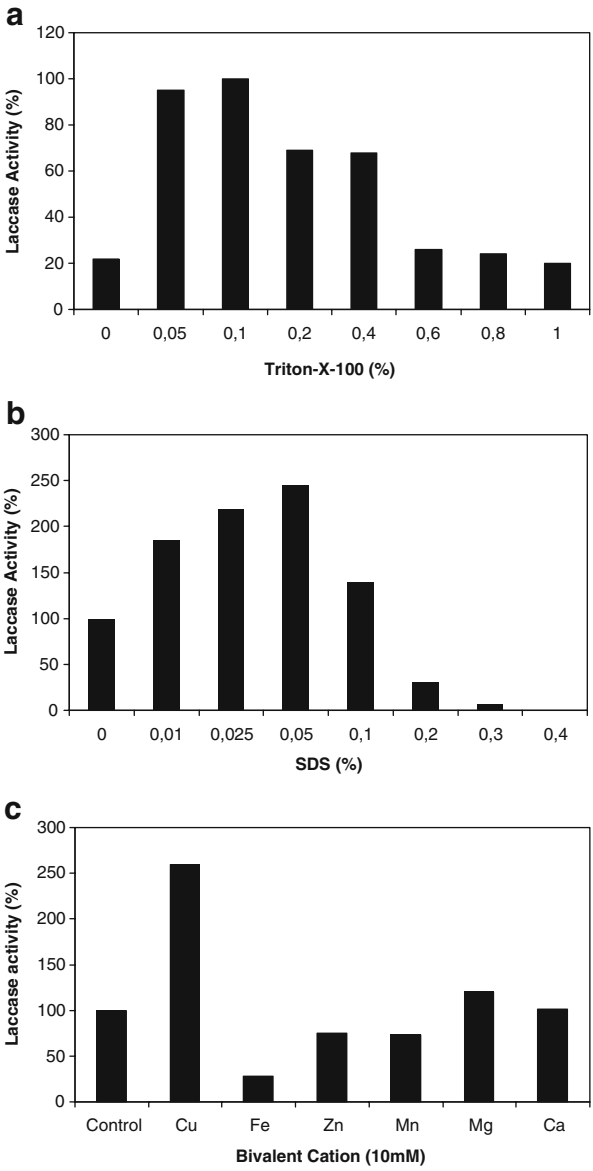

Fig. 5 Effects of **a** TritonX100, **b** SDS, and **c** bivalent cation (10 mM) on laccase activity

implicated in the enhancement of laccase activity at 2.6-fold; Mg^{2+} and Ca^{2+} ions have a weak activated effect and Zn^{2+}, Mn^{2+}, and Fe^{2+} have an inhibitory effect on laccase activity.

Decolorization of Some Synthetic Dyes

Laccase activity is usually used for biotechnological application and the most important one was decolorization of textile wastewater [10]. To explore this important biotechnological

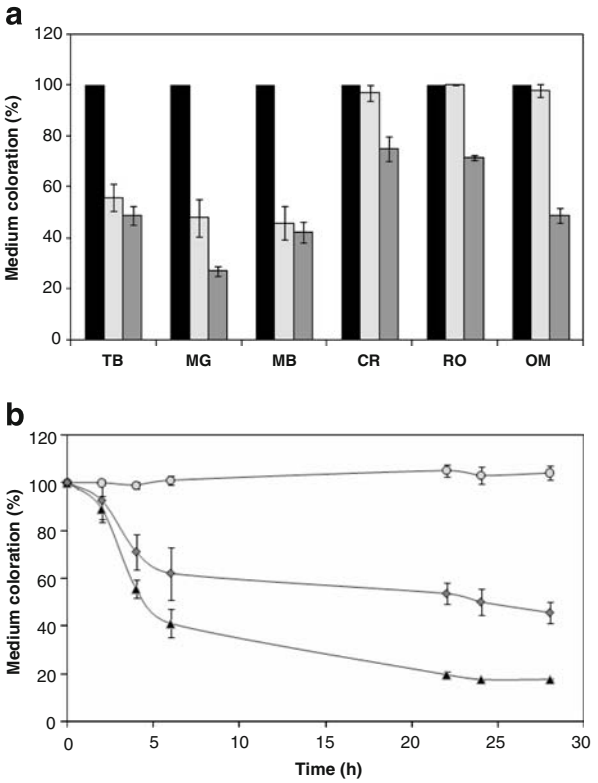

Fig. 6 **a** Decolorization rate of methylene blue (*MB*), toluidine blue (*TB*), methyl green (*MG*), methyl orange (*MO*), Congo red (*CR*), and pink (*RO*) at 0 h (*black bars*), 24 h (*light gray bars*), and 48 h (*dark gray bars*). **b** Kinetic decolorization of industrial dye SITEX Black (0.4 $OD_{630\ nm}$ per milliliter) by incubation of bacteria in LB medium with 400 μM $CuSO_4$ (*triangles*) and without (*diamonds*) copper. Abiotic control was monitored with colored LB medium supplemented with 400 μM of copper sulfate (*circles*)

aspect, the bacterial strain AAP56 producing laccase activity was tested towards some synthetic dyes. We used three distinct classes of dyes: (1) triphenyl methane dyes (methyl green, methyl orange), (2) heterocyclic dyes (methylene blue, toluidine blue), and (3) other dyes (pink, SITEX black) with unknown classes. Different dye concentrations were considered to determine the optimal one for an improved decolorization rate, the results indicated that the strain AAP56 exhibited an activity for color removal (Fig. 6a). Bacteria can finally avoid 53% of methylene blue, 50% of toluidine blue, 71% of methyl green, 48% of methyl orange, 30% of Congo red, and 28% of pink. To valorize the decolorization activity, some finalizing studies with industrial effluent SITEX black were achieved. Amounts of this concentrated effluent were added to the LB medium and the decrease of decolorization ability was found with the increase of dye concentration from 0.2 to 0.6 OD per milliliter; an optimal concentration was found at 0.4 OD per milliliter. Decolorization ability was set up by Cu^{2+} addition (Fig. 6b).

Discussion

The Api 20NE analytical profile of the strain AAP56 showed 99.9% of identification and T=0.78, so there is an excellent identification of the unknown isolate as specie *S. maltophilia*. Moreover, the molecular identification of the AAP56 strain was considered. So, the partial sequence of 16s rRNA gene (565 bp) confirmed the result of Api20NE strip to the strain identification as *S. maltophilia* specie. We concluded that 16s rRNA gene sequencing and Api 20NE were two effective means for strain identification [25].

This bacterial strain was able to produce an intracellular laccase (Sm laccase) when copper was added at 0.4 mM to the growth medium. Absence of copper in the LB medium caused loss of activity; this result showed that the Sm laccase is inductible and its production is not constitutive. The strict dependency of laccase production to the presence of copper showed that presence of this enzyme is a copper-dependent one. Cu^{2+} is communally employed for laccase expression in many fungi and bacteria [26, 27]. In fact, copper is originated of oxidative stress or metal response which caused the gene expression of multicopper oxidase such laccase [28, 29]. The physiological role of laccase was reported to taking a copper tolerance to *Escherichia coli* [29] or in melanin production by *S. meliloti* [30]. Copper ion enhances Sm laccase production in *S. maltophilia AAP56* culture. In vivo observation showed a curious dark color of *S. maltophilia AAP56* growing with 400 μM $CuSO_4$. This color development is also observed for soil bacterial strain *S. meliloti* [30]. Sm laccase may be implicated in the bacterial colonies' dark pigmentation.

Copper ion has also an effect on Sm laccase activity (2.6-fold). Many works reported that the copper ion is the cofactor implicated in the active site of laccase enzymes in *B. subtilis* [22], *Bacillus sphaericus*, *Azospirillum lipoferum*, *Marinomonas mediterranea*, and *E. coli* [31]. Implication of copper ion in the Sm laccase activity site and induction suggests that this enzyme is a copper-dependant one.

EDTA at 5 mM caused a total inhibition of Sm laccase activity, confirming that a metal is involved in laccase activity. The entire inactivation of *A. lipoferum* and *S. meliloti* laccases by addition of EDTA has been reported by Diamantidis et al. [32] and Castro-Sowinski et al. [30], respectively. Sodium azide is also the Cu-chelating agent [33] but does not cause a total inhibition (40%) of laccase activity at 5-mM concentration, whereas azide caused total inhibition of *Pleurotus florida* laccase and *Lentinus edodes* laccase at 0.1-mM concentration [34, 35].

Laccase activity was tested by syringaldazine (SGZ) as substrate. The optimal pH of Sm laccase was 7.0. In fact, this is the usual functional pH for the organic substrate such SGZ; the same optimum pH activity was described for CotA of *B. subtilis* [24], laccase of *Rhizoctonia solani* [36], and laccase of *Melanocarpus albomyces* [37]. Sm laccase has sensibility against the temperature. In contrast, CE52G laccase was stable at 70 °C after 3 h of incubation; in addition, CotA has a half-life of 2–4 h in 80 °C [24] and the thermostable laccases of *Streptomyces griseus* and *Streptomyces lavendulae* retained 40% and 100% activity after 1 h incubation at 70 °C. Fungal laccases from *Agaricus bisporus* [38] and from *Botrytis cinerea* [39] have respective half-lives of 10 min at 70 °C and 220 s at 53 °C. Persistence of Sm laccase activity with higher concentration of NaCl (100 to 500 mM) is particularly interesting since this salt is widely used in different procedures and may favor the potential use of this laccase in various industrial processes. Moreover, laccase activity was enhanced twofold by the addition of 100 mM NaCl. Triton-X-100 is a nonionic detergent and it is widely used to solubilize the protein fraction in the extraction procedures; in fact, Triton-X-100 enhances fivefold the laccase activity when it is added at 0.1% (*v*/*v*) in the reaction mixture. However, the addition of 0.05% SDS improves the laccase activity only 2.5-fold. The enhanced effect of Triton-X-100 was mostly better than the SDS one.

Many investigations demonstrate that laccases and peroxidases may be responsible for the decolorization process of different dyes [1, 7, 10, 31, 40, 41, 43]. Fungal laccases is a subject of many applications for the decolorization. The white-rot fungus *Bjerkandera adusta* is capable of decolorizing a number of chemically different synthetic dyes, even at relatively high concentrations. Ligninolytic enzyme production of such laccase by this strain explains its ability to decolorize [42]. *Ganoderma lucidum KMK2* produced laccase as the dominant ligninolytic enzyme during solid-state fermentation of wheat bran, a natural

lignocellulosic substrate. Crude enzyme shows excellent decolorization activity (~90%) to anthraquinone dye Remazol Brilliant Blue R without redox mediator [43]. Partial decolorization of two azo dyes and complete decolorization of two triphenyl methane dyes were achieved by cultures of *Pycnoporus sanguineus* producing laccase as the sole phenol oxidase. Laccase purified from *Trametes hirsuta* was able to degrade triaryl methane, indigoid, azo, and anthraquinonic dyes used in dyeing textiles as well as 23 industrial dyes [10].

Some bacteria such as *A. lipoferum* [44, 45] *M. mediterranea* [46], and *S. meliloti* [23, 30] present the laccase activities. *A. lipoferum* is the first bacteria exhibiting an intracellular laccase activity [32]. *M. mediterranea* is a melanogenic marine bacterium expressing both an enzyme having an SDS-activated tyrosinase and a laccase activity. *S. meliloti* is a melanin-producing strain producing a periplasmic laccase. Moreover, the presence of the multicopper oxidase like CotA in *B. subtilis*, CueO in *E. coli*, CumA in *Pseudomonas putida,* CopA in *Pseudomonas syringae*, and PcoA in *Ralstonia metallidurans* was previously described to exhibit phenol oxidase activity and was implicated in intrinsic copper or manganese resistance in many bacteria [47–50].

Decolorization activity of *S. maltophilia AAP56* was demonstrated under several dyes. Some synthetics dyes (methylene blue, toluidine blue, methyl green, methyl orange, Congo red, and pink) were decolorized by bacteria culture in LB medium supplemented with dyes (Fig. 6a). In the aim to explore this significant activity, the industrial effluent SITEX black was added to the bacteria growth medium in the presence and absence of Cu^{2+}. After 24 h, the decolorization is clear in both cases. However, as seen in Fig. 6b, copper improved the discoloration rate and kinetic of SITEX black.

In addition, we reported previously in Fig. 2b that $CuSO_4$ caused laccase induction in bacteria. So, interestingly, the increase of dye decolorization rate seems to be correlated with laccase induction; thus, the result suggested the implication of laccase activity in the industrial effluent SITEX black treatment.

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References

1. Karam, J., & Nicell, J. A. (1997). *Journal of Chemical Technology and Biotechnology (Oxford, Oxfordshire)*, *69*, 141–153. doi:10.1002/(SICI)1097-4660(199706)69:2<141::AID-JCTB694>3.0.CO;2-U.
2. Palmieri, G., Giardina, P., & Sannia, G. (2005). *Biotechnology Progress*, *21*, 1436–1441. doi:10.1021/bp050140i.
3. Libra, J. A., Borchert, M., & Banit, S. (2003). *Biotechnology and Bioengineering*, *82*, 736–744. doi:10.1002/bit.10623.
4. Blanquez, P., Casas, N., Font, X., Gabarrell, X., Sarra, M., Caminal, G., et al. (2004). *Water Research*, *38*, 2166–2172. doi:10.1016/j.watres.2004.01.019.
5. Asgher, M., Bhatti, H., Ashraf, M., & Legge, R. (2008). *Biodegradation*, (March 30). doi:10.1007/s10532-008-9185-3.
6. Asgher, M., Kausar, S., Bhatti, H. N., Hassan Shah, S. A., & Ali, M. (2008). *International Biodeterioration & Biodegradation*, *61*, 189–193. doi:10.1016/j.ibiod.2007.07.009.
7. Maximo, C., Pessoa, T., Amorim, M., & Costa-Ferreira, M. (2003). *Enzyme and Microbial Technology*, *32*, 145–151. doi:10.1016/S0141-0229(02)00281-8.
8. Torres, E., Bustos-Jaimes, I., & Le Borgne, S. (2003). *Applied Catalysis B Environmental*, *46*, 1–15. doi:10.1016/S0926-3373(03)00228-5.
9. Messerschmidt, A., & Huber, R. (1990). *European Journal of Biochemistry*, *187*(2), 341–352. doi:10.1111/j.1432-1033.1990.tb15311.x.

10. Mayer, A. M., & Staples, R. C. (2002). *Phytochemistry*, *60*, 551–565. doi:10.1016/S0031-9422(02)00171-1.
11. Thurston, C. F. (1994). *Microbiology*, *140*, 19–26.
12. Solomon, E. I., Sundaram, U. M., & Machonkin, T. E. (1996). *Chemical Reviews*, *96*, 2563–2605. doi:10.1021/cr950046o.
13. Harvey, B. M., & Walker, J. R. K. (1999). *Journal of Biochemistry, Molecular Biology and Biophysics*, *3*, 45–51.
14. Kalme, S. D., Parshetti, G. K., Jadhav, S. U., & Govindwar, S. P. (2006). *Bioresource Technology*, *98*(7), 1405–1410. doi:10.1016/j.biortech.2006.05.023.
15. Held, C., Kandelbauer, A., Schroeder, M., Cavaco-Paulo, A., & Guebitz, G. M. (2005). *Environmental Chemistry Letters*, *3*, 74–77. doi:10.1007/s10311-005-0006-1.
16. Mohana, S., Desai, C., & Madamwar, D. (2006). *Bioresource Technology*, *98*(2), 333–339. doi:10.1016/j.biortech.2005.12.024.
17. Eden, P. A., Schmidt, T. M., Blakemore, R. P., & Pace, N. R. (1991). *International Journal of Systematic Bacteriology*, *41*, 324–325.
18. Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. (1991). *Journal of Bacteriology*, *173*, 697–703.
19. Laemmli, U. K. (1970). *Nature*, *227*, 680–685. doi:10.1038/227680a0.
20. Ferroni, A., Sermet-Gaudelus, I., Abachin, E., Quesnes, G., Lenoir, G., Berche, P., et al. (2003). *Pathologie Biologie*, *51*, 405–411. doi:10.1016/S0369-8114(03)00021-X.
21. Monteil, H., & Harf-Monteil, C. (2002). In *Pseudomonas et apparentés, Dossier scientifique, Bactériologie: Taxonomie et autres actualités. Revue Française des laboratoires Vol. 2002 (343)* (pp. 31–40).
22. Hullo, M. F., Moszer, I., Danchin, A., & Martin-Verstraete, I. (2001). *Journal of Bacteriology*, *183*(18), 5426–5430. doi:10.1128/JB.183.18.5426-5430.2001.
23. Rosconi, F., Franco Fraguas, L., Martinez-Drets, G., & Castro-Sowinski, S. (2005). *Enzyme and Microbial Technology*, *36*(5), 800–807. doi:10.1016/j.enzmictec.2005.01.003.
24. Martins, L. O., Soares, C. M., Pereira, M. M., Teixeira, M., Costa, T., Jones, G. H., et al. (2002). *The Journal of Biological Chemistry*, *277*, 18849–18859. doi:10.1074/jbc.M200827200.
25. Bosshard, P. P., Zbinden, R., Abels, S., Böddinghaus, B., Altwegg, M., & Böttger, E. C. (2006). *Journal of Clinical Microbiology*, *44*(4), 1359–1366. doi:10.1128/JCM.44.4.1359-1366.2006.
26. Collins, P. J., & Dobson, A. D. W. (1997). *Applied and Environmental Microbiology*, *63*(9), 3444–3450.
27. Kilaru, S., Hoegger, P. J., & Kües, U. (2006). *Current Genetics*, *50*, 45–60. doi:10.1007/s00294-006-0074-1.
28. Crowe, J. D., & Olsson, S. (2001). *Applied and Environmental Microbiology*, *5*(67), 2088–2094. doi:10.1128/AEM.67.5.2088-2094.2001.
29. Grass, G., Thakali, K., Klebba, P. E., Thieme, D., Müller, A., Wildner, G. F., & Rensing, C. (2004). *Journal of Bacteriology*, *186*(17), 5826–5833. doi:10.1128/JB.186.17.5826-5833.2004.
30. Castro-Sowinski, S., Martinez-Drets, G., & Okon, Y. (2002). *FEMS Microbiology Letters*, *209*, 119–125. doi:10.1111/j.1574-6968.2002.tb11119.x.
31. Claus, H. (2004). *Micron (Oxford, England)*, *35*, 93–96. doi:10.1016/j.micron.2003.10.029.
32. Diamantidis, G., Effosse, A., Potier, P., & Bally, R. (2000). *Soil Biology & Biochemistry*, *32*, 919–927. doi:10.1016/S0038-0717(99)00221-7.
33. Chefetz, B., Chen, Y., & Hadar, Y. (1998). *Applied and Environmental Microbiology*, *9*(64), 3175–3179.
34. D'Annibale, A., Celletti, D., Felici, M., Di Mattia, E., & Giovannozzi-Sermanni, G. (1996). *Acta Biotechnologica*, *16*, 257–270. doi:10.1002/abio.370160408.
35. Das, N., Chakraborty, T. K., & Mukherjee, M. (2001). *Journal of Basic Microbiology*, *41*, 261–267. doi:10.1002/1521-4028(200110)41:5<261::AID-JOBM261>3.0.CO;2-V.
36. Wahleithner, J. A., Xu, F., Brown, K. M., Brown, S. H., Golightly, E. J., Halkier, T., et al. (1996). *Current Genetics*, *29*, 395–403. doi:10.1007/BF02208621.
37. Kiiskinen, L. L., Viikari, L., & Kruus, K. (2002). *Applied Microbiology and Biotechnology*, *59*, 198–204. doi:10.1007/s00253-002-1012-x.
38. Wood, D. A. (1980). *Journal of General Microbiology*, *117*, 327–338.
39. Gigi, O., Marbach, I., & Mayer, A. M. (1981). *Phytochemistry*, *20*, 1211–1213. doi:10.1016/0031-9422(81)80006-4.
40. Zouari-Mechichi, H., Mechichi, T., Dhouib, A., Sayadi, S., Marinez, A. T., & Martinez, M. J. (2006). *Enzyme and Microbial Technology*, *39*, 141–148. doi:10.1016/j.enzmictec.2005.11.027.
41. Lopez, M. J., Guisado, G., Vargas-Garcia, M. C., Suarez-Estrella, F., & Moreno, J. (2006). *Enzyme and Microbial Technology*, *40*, 42–45. doi:10.1016/j.enzmictec.2005.10.035.
42. Eichlerova, I., Homolka, L., & Nerud, F. (2007). *Dyes and Pigments*, *75*, 38–44. doi:10.1016/j.dyepig.2006.05.008.

43. Murugesan, K., Nam, I. H., Kim, Y. M., & Chang, Y. S. (2007). *Enzyme and Microbial Technology*, *40*, 1662–1672. doi:10.1016/j.enzmictec.2006.08.028.
44. Givaudan, A., Effosse, A., Faure, D., Potier, P., Bouillant, M. L., & Bally, R. (1993). *FEMS Microbiology Letters*, *108*, 205–210. doi:10.1111/j.1574-6968.1993.tb06100.x.
45. Faure, D., Bouillant, M. L., & Bally, R. (1995). *Applied and Environmental Microbiology*, *61*(3), 1144–1146.
46. Sanchez-Amat, A., Lucas-Elio, P., Fernandez, E., Garcia-Borron, J. C., & Solano, F. (2001). *Biochimica et Biophysica Acta*, *1547*, 104–116.
47. Claus, H., & Filip, Z. (1997). *Microbiological Research*, *152*, 209–216.
48. Chris, A. F., & Bradley, M. T. (2001). *Applied and Environmental Microbiology*, *67*(9), 4272–4278. doi:10.1128/AEM.67.9.4272-4278.2001.
49. Grass, G., & Rensing, C. (2001). *Biochemical and Biophysical Research Communications*, *286*, 902–908. doi:10.1006/bbrc.2001.5474.
50. Sitthisak, S., Howieson, K., Amezola, C., & Jayaswal, R. K. (2005). *Applied and Environmental Microbiology*, *71*(9), 5650–5653. doi:10.1128/AEM.71.9.5650-5653.2005.