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Efficient microbial degradation of Toluidine Blue dye by *Brevibacillus* sp.

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Abstract

Economical and bio-friendly approaches are needed to degrade dye-contaminated wastewater from various industries. In this study, we report on the isolation and characterization of a strain of *Brevibacillus* that can efficiently degrade Toluidine Blue dye (TB). Eight different bacterial strains which were initially isolated from soil contaminated with Coomassie Brilliant Blue dye (CBB) showed varying degradation rates for CBB. The most efficient isolate was further tested on 7 other dyes; surprisingly, we found this isolate to be much more efficient in degrading TB. Morphological and enzymatic analyses, as well as 16S rRNA sequencing were used to identify this isolate as a species of *Brevibacillus*. We have characterized the efficiency of TB degradation by this isolate as a function of dye concentration, pH, aeration, as well as nitrogen source. Under optimum conditions, most of the TB could be degraded within 24 h. The biodegradation data for TB were fitted to first-order equation with good correlation.

Keywords: Toluidine Blue; Coomassie Blue; Microbial degradation; *Brevibacillus*

1. Introduction

Dyes are an important class of chemicals which are widely used in many industrial processes, like in leather, textile and printing, food and cosmetics industries. Most of these dyes are synthetic in nature and are classified based on their chemical structures into 6 different classes as azo, anthraquinone, sulfur, indigoid, triphenylmethane and phthalocyanine derivatives. Due to the extensive use of these dyes in industries, they become an integral part of industrial wastewater. In fact, of the 450,000 tons of organic dyes annually produced worldwide, more than 11% is lost in effluents during manufacture and application processes [1]. Most of these dyes are toxic and potentially carcinogenic in nature and their removal from the industrial effluents is a major environmental problem [2–4].

Various physical and chemical methods of treatment of industrial wastewater have been suggested; these include adsorption methods, coagulation processes and the ozone and hypochlorite treatment of dye waste effluents [5]. All these methods are either costly, inefficient or result in the production of secondary waste product. Recent progress on this frontier has led to study the feasibility of using advanced oxidation processes, AOP [6]. These AOP methods involve the chemical production of highly reactive hydroxyl radicals, which then react with dyes to break them into small and simpler products. Many studies by us and others have shown the usefulness of these AOP approaches in the degradation of dyes [7,8]. Another attractive approach that has recently shown to be of great promise in the degradation and removal of these toxic dyes is the use of microbes. Literature review on this subject reveals the importance of biodegradation of various wastewater effluents containing dye solutions using microorganisms due to its low cost, ability to produce less sludge and environmental compatibility [9–13]. In this

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Table 1
Structure and classes of dyes used in this study

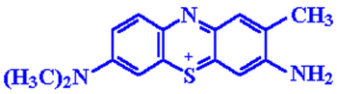
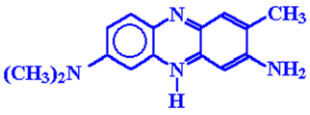
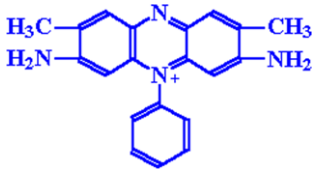
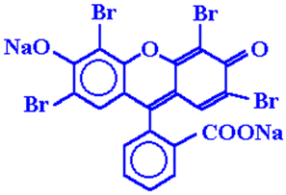
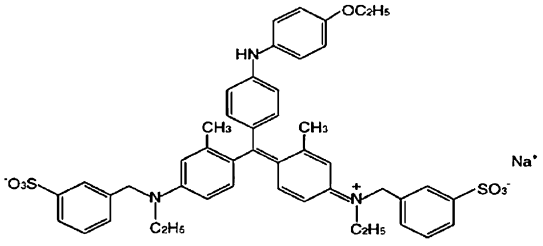
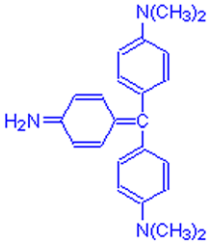
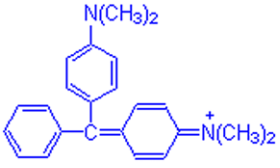
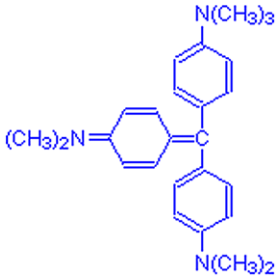
Name	Structure	λ_{max} (nm)	Class
Toluidine Blue		620	Thiazin (quinone-imine)
Neutral Red		540	Azin (quinone-imine)
Safranine O		530	Azin (quinone-imine)
Eosin Yellowish		515	Fluorone
Commassie Brilliant Blue		585	Triarylmethane
Methyl Violet		585	Triarylmethane
Malachite Green		620	Triarylmethane

Table 1 (*continued*)

Name	Structure	λ_{max} (nm)	Class
Methyl Green		630	Triarylmethane

regard, many studies indicated that microorganisms like *Bacillus subtilis*, *Phanerochaete chrysosporium*, *Aeromonas hydrophila*, *Penicillium* sp., *Klebsiella pneumoniae*, *Proteus mirabilis* and *Pseudomonas cepacia* have shown very promising results for dye degradation [14–18].

In the present study, we were initially interested in isolating and characterizing a bacterial strain that could be used to degrade Coomassie Brilliant Blue (CBB). We report on the isolation of a previously unreported dye-degrading bacterial strain, *Brevibacillus* sp. However, surprisingly we found that this isolate was much more efficient in the degradation of another dye, Toluidine Blue. We have further investigated various factors that affect the degradation of TB by this *Brevibacillus* strain.

2. Materials and methods

2.1. Chemicals

Eight different dyes from various classes (thiazine, azine, flouorone and triarylmethane) were chosen in this study, including Toluidine Blue (TB) (Sigma), Neutral Red (NR) (Sigma), Safranine O (SO) (ICN), Eosin Yellowish (EY) (Fluka), Commassie Brilliant Blue (CBB) (BioRad), Methyl Violet (MV) (Merck), Malachite Green (MalG) (BDH) and Methyl Green (MetG) (Sigma) (Table 1).

2.2. Microorganisms and growth conditions

The strains used in the present study were initially isolated from CBB polluted soil. The bacteria were isolated using the standard procedures using nutrient agar (NA) (Lab M) containing ($g\,l^{-1}$): peptone 5, beef extract 3, sodium chloride 8, and agar 12, with pH 7.3 ± 0.2. The isolates were cultured in incubator at 37 °C.

2.3. Medium for dye degradation

Nutrient broth medium (NB) (BBL) containing ($g\,l^{-1}$): beef extractives 3, gelysate peptone 5 with pH 6.9 ± 0.2 was used. Where needed, pH of the culture media was adjusted using diluted NaOH or HCl. The culture was grown at 25 °C with shaking (200 rpm) unless otherwise mentioned. The medium (50 ml) was supplemented with 5 ml of freshly grown (overnight) bacterial cells, final OD_{600} of the inoculated culture was approximately 0.3.

2.4. Screening of isolates for their ability to degrade CBB

To check the dye decolorizing potential for each bacterial isolate, preliminary batch experiments were carried out using sterile 100 ml conical flasks containing 50 ml of NB in which a sterile solution of 1 ml CBB dye at predetermined

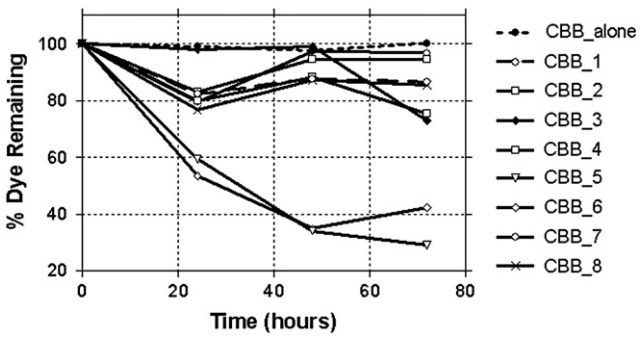


Fig. 1. Screening of 8 bacterial strains for their ability to degrade CBB.

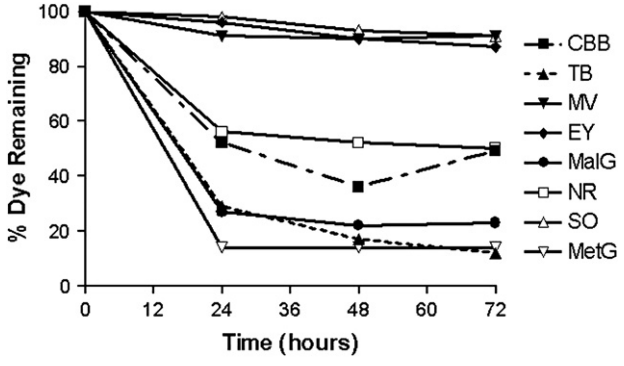


Fig. 2. Examination of biodegradation of various dyes by bacterial isolate #5.

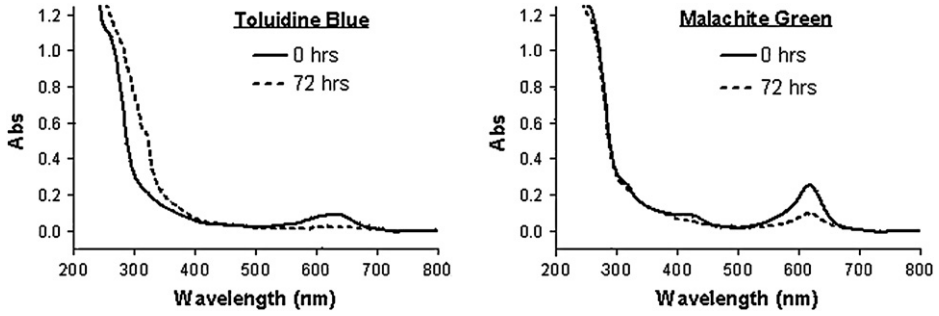


Fig. 3. UV–vis dye spectra of Toluidine Blue and Malachite Green after 72 h incubation with isolate #5.

concentration was added, after which the solution was seeded with an inoculum of freshly grown bacterial cells (5 ml). The final pH was 6.9.

2.5. Identification of selected isolate

The promising bacterial isolate that showed high ability to degrade TB was first examined by Gram staining, and then by enzymatic and morphological diagnostic tests and partial 16S RNA (500 bp) by the Netherlands Culture Collection of Bacteria, NCCB (Netherlands).

2.6. Analyzing instrument and sampling times

All the samples were analyzed for % dye remaining using Cary 50 UV–vis spectrophotometer. All flasks were shaken, as mentioned earlier, for 4 days. Samples from each flask were taken at different times (0 h, 1 day, 2 days, 3 days and 4 days). The samples were briefly centrifuged to pellet the cells, and then the amount of dye in the supernatant was quantified using the Cary spectrophotometer using the λ_{max} for the dyes as listed in Table 1. The data shown represent the mean of duplicate determinations which did not differ by more than 10% from each other.

2.7. Degradation of various dyes

Examination of biodegradation of the eight dyes (CBB, TB, MV, EY, MalG, NR, SO and MetG) by the most effective isolate was carried out by using NB. Each flask was inoculated with 5 ml of freshly grown cells and 125 μM of the selected dye.

2.8. Effect of dye concentration

The effect of culture conditions, such as dye concentration, pH, shaking/aeration and nitrogen source on biodegradation of TB was examined. For dye concentration experiment, three different concentrations (50 μM, 125 μM, 312 μM) of the dyes were used to examine the suitable concentration for biodegradation.

3. Results and discussion

We were initially interested in isolating, identifying, and characterizing a specific bacterial strain that would be capable of efficiently degrading a triarylmethane class of dye, namely, Coomassie Brilliant Blue (CBB), Table 1. Fig. 1 shows the results from the screening of 8 bacterial isolates that initially appeared to grow well in CBB-contaminated soil. As can be seen from the figure, of the 8 that were chosen, only 2 of them could reproducibly degrade the dye, with bacterial isolate #5 appearing to be the best in degrading CBB. During the course of further investigation of this isolate, we tested the ability of this isolate to degrade other dyes. Unexpectedly we found that although isolate #5 could degrade CBB quite well, it was much more efficient in degrading a different dye, Toluidine Blue (TB), Fig. 2. As can be seen from the figure, in addition to CBB and TB, it appears that Neutral Red

Table 2
Characterization of strain isolate #5

Characteristics	Isolate #5: *Brevibacillus* sp.
Morphology	Rod
Gram stain	+
KOH test	−
Aminopeptidase	+
Motility	+
Spores present	−
Catalase	W
Oxidase	+
Growth in air	+
Growth anaerobically	−

W, weakly positive.

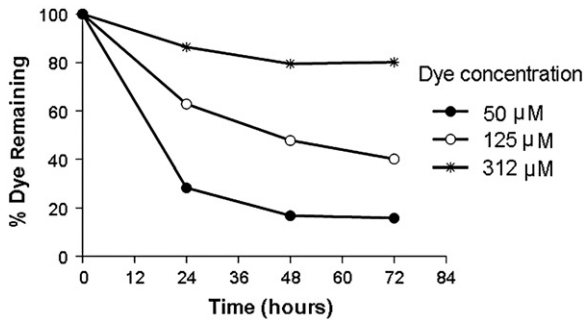


Fig. 4. Effect of dye concentration on the ability of bacterial isolate #5 to degrade Toluidine Blue.

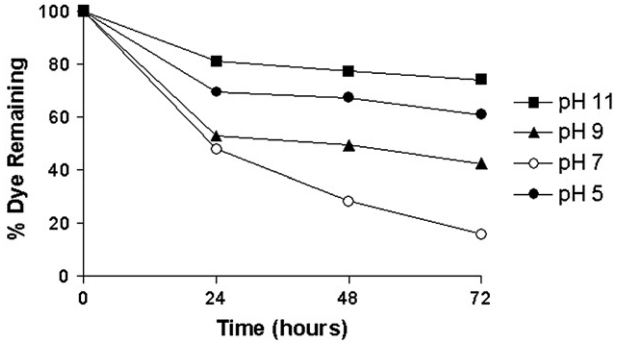


Fig. 5. Effect of pH on the ability of bacterial isolate #5 to degrade Toluiding Blue.

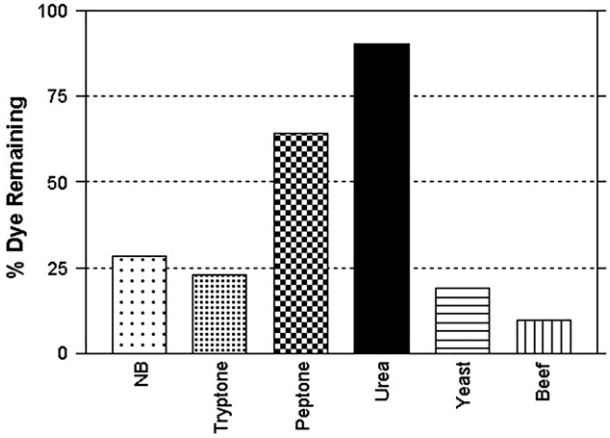


Fig. 7. Effect of nitrogen source on the ability of bacterial isolate #5 to degrade Toluidine Blue dye.

(NR), Malachite Green (MalG), and Methyl Green (MetG) were also decolorized by isolate #5. However, careful examination of cell pellets (after centrifugation of overnight cultures) from these three dyes (NR, MalG, and MetG) showed that there was a very strong adsorption of dyes to the bacterial mass (which was not seen with TB). This is also seen in Fig. 3 which shows the UV–vis spectra of TB and MalG after 72 h of incubation with isolate #5. In the case of TB, one could see new peaks in the 250–350 nm region indicating that the dye is being broken down to new intermediary compounds which absorb light in this region. No such new peaks are observed with MalG or other dyes (not shown). This together with the highly colored bacterial pellets indicate that the decrease in dye concentrations with MalG, NR, and MetG is due to adsorption, whereas in the case of TB and CBB, the decrease in dye concentrations is primarily due to dye degradation. The result that isolate #5 could degrade TB was quite surprising as other dyes similar to CBB (triarylmethane class of dyes) were not degraded by this strain. Similarly, other dyes that belonged to the quinone-imine class (same class as TB) were also not degraded by this strain. Perhaps the strain is very specific to the thiazin sub-class within the quinone-imine class of dyes. This very surprising result shows the usefulness of testing a wide panel of other dyes when one is attempting to isolate a specific dye-degrading bacterial strain. A similar observation has been reported in the literature where the efficiencies of degradation of 8 different dyes by 3 different *Pseudomonas* strains were examined [19].

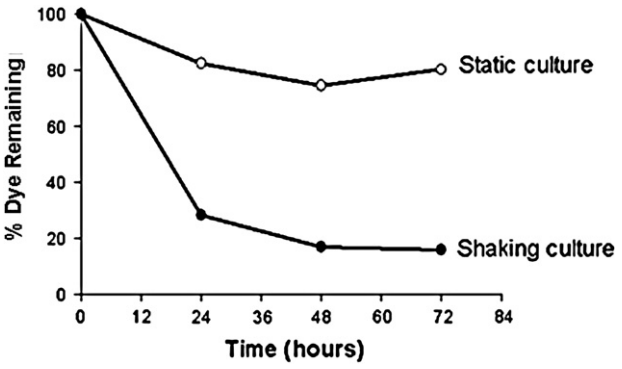


Fig. 6. Effect of shaking/aeration on the ability of bacterial isolate #5 to degrade Toluidine Blue.

All the subsequent works presented here were therefore carried out at characterizing the isolate #5 with regard to its degradation of TB. Identification of the strain was carried out using several biochemical and physiological investigations (Table 2), and it was identified as *Brevibacillus* sp. according to partial sequencing of 16S rRNA gene. Since *Brevibacillus* species have highly homologous 16S rRNA, we were not able to identify the exact species. It is very likely that our isolate is either *B. brevis, B. formosus* or a *B. chosinensis* strain.

In order to further characterize isolate #5, we tested its ability to degrade different concentrations of TB. As can be seen from Fig. 4, the strain could degrade 50 μM as well as 125 μM TB solutions quite efficiently. However, the strain apparently could not handle the higher concentrations of the dye, and as can be seen in Fig. 4, no significant decrease in TB intensity was observed. Therefore the best strategy for degrading TB would be to dilute it to around 50 μM, which could very efficiently be degraded by the strain (>70% degradation within 24 h).

The decoloration of the 50 μM TB solution by *Brevibacillus* sp. could also be fitted to first-order equation, $\ln(A_0/A_t) = -kt$; where k is the apparent rate constant, t is the incubation time and A_0 and A_t are the initial and the final absorbance values of the dye solution, respectively (data not shown). The apparent rate constant for the degradation of 50 μM TB solution was found to be 0.037 h^{-1}.Thus it appears that the bacterial degradation of TB occurs via simple first-order kinetics.

Since waste effluents usually do not have a neutral pH, we were interested in testing the ability of this strain to degrade TB at different pH values. Fig. 5 shows the results from this study, in which the pH of the growth media was altered to pH 5, 9, or 11 (control was pH 7). Fig. 5 shows that neutral pH was best for degrading the dye, but pH 9 appeared to be tolerated well and significant amount of dye could be degraded at that pH. However, to achieve the best rate of degradation, it is suggested that the pH of the textile effluent be neutralized to

around pH 7. Although a small, but significant amount of degradation of TB was observed at pH 5, no significant degradation of the dye could be observed in alkaline media (pH = 11). Although we did not look at bacterial biomass during the experiment, we believe the observed effect of pH could be explained by the inability of the bacteria to grow well in acidic or very alkaline media. Additionally at these two extreme ends, the bacterial strains have higher affinity for the dye resulting in their enhanced color. A similar observation has been reported for other cases in literature [20].

Various groups have reported that bacterial degradation is best under aerobic and shaking conditions [20]. In order to test is this was also true for our isolate, a study was done with TB under static and shaking conditions. The degradation of the dye also appeared to be dependent on the aeration of the culture. As can be seen in Fig. 6, TB is more efficiently degraded when the contents were agitated as compared to static conditions. This implies that when the contents are shaken, the dissolved oxygen content in the sample increased thereby increasing the % degradation of the dye solution.

Lastly, examination of the best nitrogen source for the efficient degradation of TB indicated that beef extract was most efficient in degrading TB, followed by yeast, tryptone, and nutrient broth (NB), all of which were quite efficient as well. Urea and peptone appeared to be the least efficient nitrogen source for the TB-degrading strain, Fig. 7.

4. Conclusion

In summary, we describe here the isolation and characterization of a strain of *Brevibacillus* capable of efficiently degrading Toluidine Blue. This identity of the strain was confirmed using partial 16S rRNA sequencing as well as morphological/enzymatic assays. The dye degradation was found to be dependent on dye concentration, aeration, pH, as well as N source. Interestingly, this strain was isolated from soil contaminated with a different dye, but was also found to have relaxed substrate specificity and could degrade other dyes, namely Toluidine Blue. Thus, our study also points out to an unexpected benefit of testing a wide panel of other dyes when one is attempting to isolate a specific dye-degrading bacterial strain.

References

[1] Lewis DM. Coloration for the next century. Review of Prog in a Color Relat Topics 1999;29:23–8.
[2] Golka K, Kopps S, Myslak ZW. Carcinogenicity of azo colorants: influence of solubility and bioavailability. Toxicol Lett 2004;151:203–10.
[3] Reife A, Freeman HS, Freeman HC. Environmental chemistry of dyes and pigments. 1st ed. USA: Wiley-Interscience; 1995.
[4] Pinheiro HM, Touraud E, Thomas O. Aromatic amines from azo dye reduction: status review with emphasis on direct UV spectrophotometric detection in textile industry wastewaters. Dyes Pigments 2004;61:121–39.
[5] Forgacs E, Cserhati T, Oros G. Removal of synthetic dyes from wastewaters: a review. Environ Int 2004;30:953–71.
[6] Lv X, Xu Y, Lv K, Zhang G. Photo-assisted decolorization of anionic and cationic dyes over iron(III)-loaded resin in the presence of hydrogen peroxide. J Photochem Photobiol A Chem 2005;173:121–7.
[7] Rauf MA, Ashraf S, Alhadrami SN. Photolytic oxidation of Coomassie Brilliant Blue with H_2O_2. Dyes Pigments 2005;66:197–200.
[8] Ashraf SS, Rauf MA, Alhadrami SN. Decolorization of Methyl Red using Fenton's reagent and the effect of various salts. Dyes Pigments 2006;69:80–4.
[9] Balan DSL, Monteiro RTR. Decolorization of textile indigo dyes by ligninolytic fungi. J Biotechnol 2001;89:141–5.
[10] Jianlong W, Xiangchun Q, Liping H, Yi Q, Hegemann W. Microbial decolorization of quinoline by immobilized cells of *Burkholderia pickettii*. Water Res 2002;36:2288–96.
[11] Coughlin MF, Kinkle BK, Bishop PL. Decolorization of Acid Orange 7 in an aerobic biofilm. Chemosphere 2002;46:11–9.
[12] Supaka N, Juntongjin K, Damronglerd S, Delia ML, Strehaiano P. Microbial decolorization of reactive azo dyes in a sequential anaerobic–aerobic system. Chem Eng J 2004;99:169–76.
[13] Pranab PK, Ghosh K, Philip L. Atrazine decolorization in anaerobic environment by a mixed microbial consortium. Water Res 2004;38:2277–84.
[14] Chung KT, Stevens Jr S. Decolorization of azo dyes by environmental microorganisms and helminthes. Environ Toxicol Chem 1993;12:2121–32.
[15] Wong PK, Yuen PY. Decolorization and biodecolorization of Methyl Red by *Klebsiella pneumoniae.* Water Res 1996;30:1736–44.
[16] Tatarko M, Bumpus JA. Biodecolorization of Congo Red by *Phaerochaete chrysosporium.* Water Res 1998;32:1713–7.
[17] Zheng Z, Levin RE, Pinkham JL, Shetty K. Decolorization of polymeric dyes by a novel *Penicillium* isolate. Process Biochem 1999;34:31–7.
[18] Zissi U, Lyberatos G, Pavlou S. Biodecolorization of *p*-aminoazobenzene by *Bacillus subtilis* under aerobic conditions. J Ind Microbiol Biotechnol 1997;19:49–55.
[19] Chen KC, Huang WT, Wu JY, Houng JY. Microbial decolorization of azo dyes by *Proteus mirabilis.* J Ind Microbiol Biotechnol 1999;23:686–90.
[20] Chen KC, Wu JY, Liou DJ, Hwang SCJ. Decolorization of the textile dyes by newly isolated bacterial strains. J Biotechnol 2003;101:57–68.