

Application of Antioxidant Indicators to Select Nicotine-Degrading Bacterium for Bioaugmented Treatment of Tobacco Wastewater

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Summary: To select nicotine-degrading bacterium for bioaugmented treatment of tobacco wastewater, the activities of antioxidant indicators such as superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH), and the ability to treat pollutants including nicotine degradation and chemical oxygen demand (COD) removal, were compared between *Acinetobacter* sp. TW and *Sphingomonas* sp. TY. When complicated toxins were present, the activities of SOD induced in strain TY were significantly higher than those in strain TW. However, the activities of CAT were inhibited in strain TY ($CAT/CAT_{LB} < 1$), but upregulated in strain TW ($CAT/CAT_{LB} > 1$). Additionally, the levels of GSH induced in strain TW were significantly higher than those in strain TY. These findings suggest that the antioxidant ability of strain TW was higher than that of strain TY, especially in tobacco wastewater. Moreover, when applied to the treatment of tobacco wastewater, the rate of nicotine degradation at 24 h was 99.50% for TW and 28.76% for TY, while the rate of COD removal at 48 h was 62.69% for TW and 45.80% for TY. Taken together, these findings indicate that the pollution treatment ability of strain TW was stronger than that of TY, and that the stronger the ability of the antioxidant, the higher the potential for treatment of tobacco wastewater.

Keywords: Bioaugmented treatment, antioxidant, indicato, chemical oxygen demand (COD).

Introduction

A considerable amount of waste is generated by the tobacco manufacturing process and some activities that use tobacco [1]. In China, up to 1.3-1.7 million tons of solid waste and several billion tons of liquid waste are discharged annually. A wide variety of toxins including nicotine, benz(a)pyrene and phenylenediamine have been found in tobacco waste [2], all of which are mutagens and carcinogens. Thus, there is a great deal of concern regarding the proper treatment of tobacco waste.

In our previous studies, bioaugmented technology consisting of inoculation of activated sludge with highly effective nicotine-degrading bacteria was developed and successfully applied to tobacco wastewater treatment [3]. The selection of a proper bacterium for inoculation is a key step in the development of successful bioaugmentation techniques [4]. Isolation of nicotine-degrading bacteria has received increasing attention in the past 60 years, and a wide variety of nicotine-degrading bacteria have been reported [5]. Bacteria can metabolize various types of substrates; however, some compounds cannot be catabolized. Isolated nicotine-degrading bacteria should be selected for bioaugmentation based on their comprehensive ability to treat tobacco wastewater; however, methods for the selection of such organisms have not yet been explored.

Antioxidant indicators such as superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) have been proposed as biomarkers for evaluation of the damage pollutants cause to organisms or the impact of pollution on the environment [6]. According to Radić *et al.* [7], when exposed to the same contaminants, the higher antioxidant ability of one organism over another often indicates that the contaminants have less toxicity toward organisms with higher antioxidant abilities. In other words, organisms with higher antioxidant abilities could show higher metabolic activity.

This study was conducted to determine which nicotine-degrading bacterium was most suitable for tobacco wastewater treatment by comparing the antioxidant abilities of two nicotine-degrading bacteria, *Acinetobacter* sp. TW and *Sphingomonas* sp. TY. This is the first trial for selection of a nicotine-degrading strain for bioaugmented treatment of tobacco wastewater.

Results and Discussion

Comparison of the SOD Activities

As shown in Fig.1, there were no differences in the SOD activities of strain TW in different media.

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SOD is considered the first line of the primary system, and is responsible for elimination of the reactive oxygen species (ROS) generating H_2O_2 and O_2 [8]. Accordingly, the presence of SOD in strain TW has the potential to eliminate all ROS induced in different cultures. No toxicity was observed in any of the three cultures containing strain TW. However, as the toxicities of LB+N, N and TWW increased in the media, the SOD activities of strain TY in different cultures increased as follows: $LB+N \leq N < TWW$. The SOD activities of strain TY in TWW were significantly higher than those in LB+N ($p < 0.05$), which indicates that strain TY could struggle to induce SOD activities to eliminate ROS in TWW. There was also toxicity from TWW on strain TY. Thus, based on comparison of SOD activities, the toxicity of nicotine against these strains did not differ ($p > 0.05$), but the adverse effects of many toxins in tobacco wastewater, including benz(a)pyrene and phenylenediamine, were higher on strain TY than on strain TW.

Comparison of the CAT Activities

The activities of CAT are compared in Fig. 2. The activities of CAT in LB+N media did not differ from those in N media, but were significantly higher than those in TWW media. Furthermore, these findings were observed in both TW and TY cells. These findings likely occurred because nicotine was the sole source of oxidant stress in LB+N and N media, while complicated toxins served as sources of

oxidant stress in TWW media (see Table 3). When compared with those in LB media, the activities of CAT in strain TW were induced in TWW media ($CAT/CAT_{LB} > 1$), while those in strain TY were significantly inhibited ($CAT/CAT_{LB} < 1$). Taken together, these findings indicate that the antioxidant system of strain TW was more powerful than that of strain TY when only CAT was considered.

A paired-samples *t*-test indicated significant differences between strain TW and strain TY ($t = 4.242$, $df = 8$, $p = 0.003$). Specifically, the activities of CAT in strain TY were significantly lower than in strain TW. As mentioned above, when nicotine was the only toxin present in LB+N or N media, the activities of SOD did not differ between strain TW and strain TY. These findings imply that equal levels of H_2O_2 were generated by these strains. However, the activities of CAT [9] in strain TY were significantly lower than that in strain TW, indicating that H_2O_2 accumulated in strain TY [10]. Thus, it is suggested that the oxidant stress from nicotine alone on strain TY was higher than that on strain TW.

Comparison of the GSH contents

As shown in Fig. 3, all of the detected GSH contents were significantly upregulated ($GSH/GSH_{LB} > 1$). GSH is conjugated with toxins or their metabolites directly [11]; therefore, these findings imply that GSH effectively eliminated toxins and their metabolites from strains TW and TY.

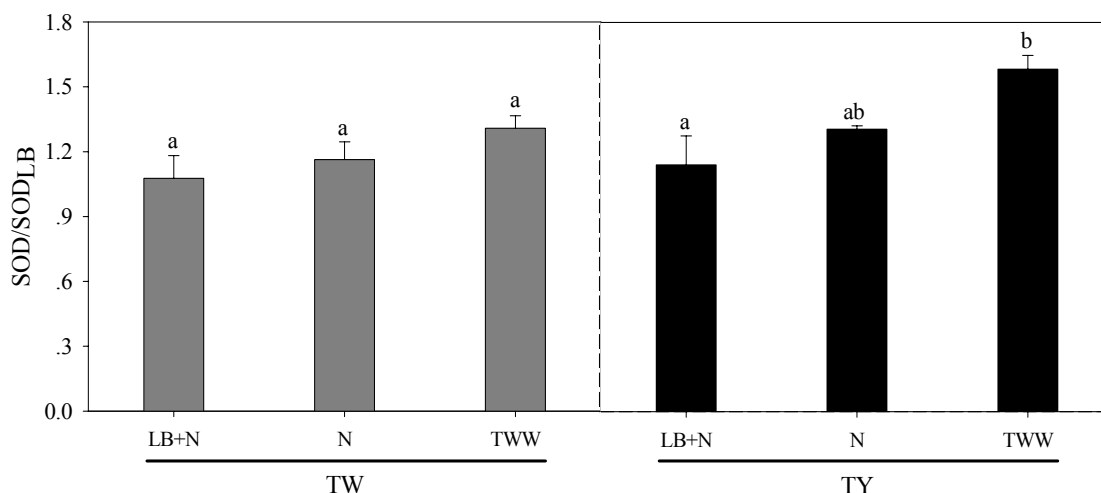


Fig. 1 Comparison of the activities of SOD between strain TW and strain TY

Distinct letters indicate significant differences ($p < 0.05$) according to one-way ANOVA.

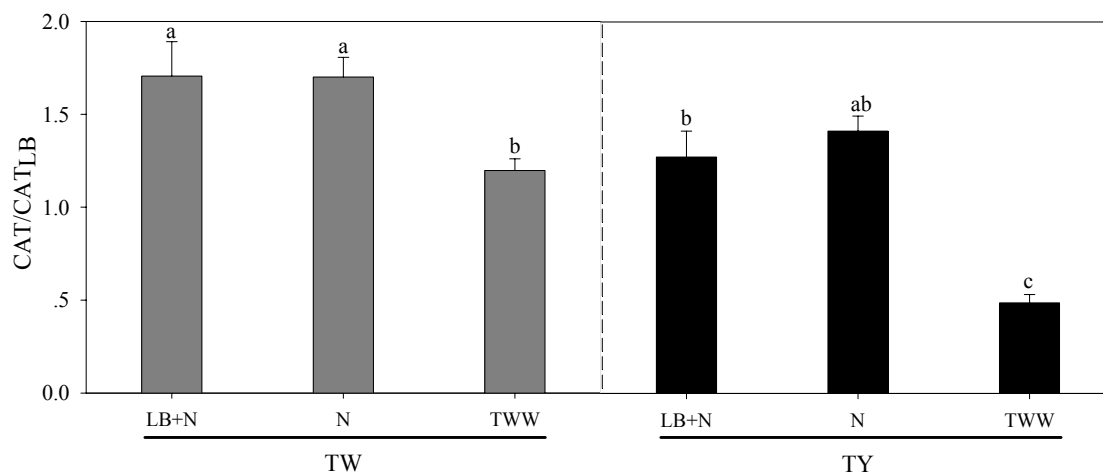


Fig. 2 Comparison of the activities of CAT in strain TW and strain TY

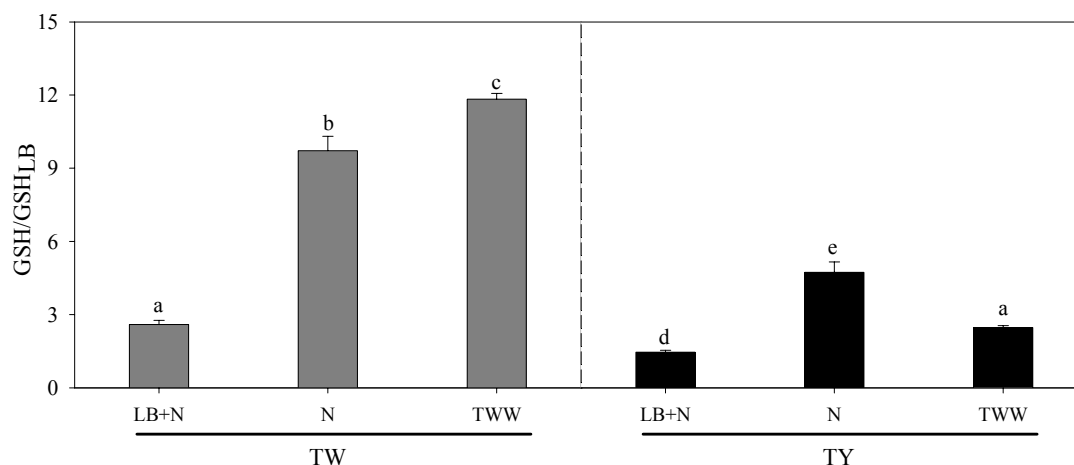
Distinct letters indicate significant differences ($p < 0.05$) according to one-way ANOVA.

Fig. 3 Comparison of the contents of GSH in strain TW and strain TY

Distinct letters indicate significant differences ($p < 0.05$) according to one-way ANOVA.

The contents of GSH increased in strain TW as follows: LB+N < N < TWW. Additionally, the contents of GSH in TWW media were significantly higher than in LB+N media ($p < 0.05$) and lower than in N media when strain TY was cultured ($p < 0.05$). Moreover, a paired-samples t -test indicated significant differences in the GSH content of strain TW and TY ($t = 4.332$, $df = 8$, $p = 0.003$). The contents of GSH in strain TW were significantly higher than in strain TY cultured in the same media ($p < 0.05$). These differences were especially high in TWW media, as indicated by the GSH content of strain TW being 4-fold greater than that of strain TY. Higher GSH contents commonly reflect stronger abilities of detoxification by organisms because more toxins and metabolites are removed by the GSH [12]. Based on the results of this study, the GSH removal

system likely performed better in strain TW than in strain TY; thus, strain TW has stronger antioxidant abilities than TY for nicotine as well as other more complicated toxins.

Comparison of the abilities of pollution treatment

The abilities of pollution treatment by the two strains are shown in Table 1. Strain TW showed no difference in the ability to degrade nicotine when cultured on the three different media evaluated herein. However, COD removal by strain TW in LB+N media was significantly higher than that in TWW media at 24 h ($p < 0.05$), but not at 48 h ($p > 0.05$). Nicotine degradation by strain TY occurred in the order of N > LB+N > TWW at 24 h ($p < 0.05$), while no differences were observed at 48 h ($p > 0.05$). The COD removal in LB+N media was significantly

higher than that in TWW media at both 24 h and 48 h ($p < 0.05$). When the abilities for pollution treatment of strains TW and TY were compared, both nicotine degradation and COD removal by strain TW were higher than those by strain TY at 24 h. As the treatment time was increased to 48 h, the COD removal by strain TW was still higher than that by strain TY, but the nicotine degradation did not differ between strains.

Table-1: Comparison of the abilities to treat pollution between strain TW and TY.

Bacteria / Media		Strain TW (%)		Strain TY (%)	
		24 h	48 h	24 h	48 h
Nicotine	LB+N	99.79±0.17 ^a	100.00±0.00 ^a	73.10±5.16 ^b	100.00±0.0 ^a
	N	99.91±0.09 ^a	100.00±0.00 ^a	99.79±0.29 ^a	100.00±0.0 ^a
	TWW	99.50±0.07 ^a	100.00±0.00 ^a	28.76±3.45 ^c	99.82±0.18 ^a
	LB+N	40.38±2.22 ^a	63.08±2.30 ^c	21.80±1.54 ^d	52.03±3.38 ^e
COD	N	-	-	-	-
	TWW	35.00±1.63 ^b	62.69±1.92 ^c	11.97±1.20 ^e	45.80±0.50 ^c

-: non-tested in the study.

Different letters indicate significant differences ($p < 0.05$). Small letters indicate the comparison of nicotine degradation rate, while capital letters indicate the comparison of COD removal rate.

When nicotine was the sole source of carbon, nitrogen and energy, there was no difference in nicotine degradation between strain TW and TY in N media. However, when nutrients other than nicotine were present in the LB+N media, the rate of nicotine degradation by strain TW was higher than that by strain TY. These findings suggested that the presence of other easily catabolized carbons could interfere with nicotine degradation by strain TY, but showed less interference with that by strain TW. This phenomenon has been reported in many pollutant-degrading strains and explains why some strains are not suitable for application during engineering [13]. When cultured in TWW media, nicotine degradation by strain TW occurred faster than that by strain TY. There are many toxins in tobacco wastewater besides nicotine, and strain TW has better resistance to complicated toxins than strain TY. Thus, strain TW was more suitable than strain TY for tobacco wastewater treatment. The COD removal by strain TW was also faster than that by strain TY in TWW media. Overall, these findings indicate that higher antioxidant ability was associated with greater potential for tobacco waste treatment.

Correlation between Treatment Abilities and Antioxidant Abilities

The correlation between pollutant treatment abilities and antioxidant abilities of the two strains is shown in Table 2. There were significant correlations between nicotine degradation and the activities of CAT in the two strains ($r=0.771$, $p = 0.015$ for strain TW; $r=0.917$, $p = 0.001$ for strain TY), while no

correlation was observed between nicotine and the activities of SOD or the contents of GSH. Organisms possess complete anti-oxidative systems to cope with free-radicals induced by a variety of toxins and alleviate their toxic effects [14, 15]. SOD is responsible for elimination of reactive oxygen species (ROS), while the bulk of H_2O_2 is scavenged by CAT and GSH is conjugated with toxins or their metabolites. Blockage of the scavenging activity of SOD and CAT can be compensated for by the induction of GSH to a certain extent [16-18]. In this study, even though CAT activities had significant effects on nicotine degradation by the strains tested, the toxic effects of nicotine could also be reduced by SOD and GSH. Thus, when the culture time was increased to 48 h, the nicotine degradation in all treatments exceeded 99%. As a result, the use of only one antioxidant indicator could not serve as a biomarker to evaluate whether the bacterium was suitable for pollutant treatment.

No correlation was observed between COD removal and each of the antioxidant indicators in strain TW. However, there were significant correlations between COD removal and the antioxidant activities in strain TY ($r=-0.870$, $p = 0.024$ for SOD; $r=0.989$, $p = 0.000$ for CAT; $r=-0.941$, $p = 0.005$ for GSH). Based on our previous studies, when the toxicity was removed from tobacco wastewater, there were no correlations between antioxidant indicators and COD removal, and vice versa. Therefore, these findings implied that the antioxidant indicators in strain TY were induced to remove the toxicity from tobacco wastewater to the greatest extent. Tobacco wastewater had a toxic effect on strain TY; thus, strain TY was not suitable for tobacco wastewater treatment when its antioxidant abilities were considered. In most reports, a single indicator is used for evaluation of the suitability of a treatment method. However, the results of the present study indicate that at least three antioxidant indicators should be used to select a suitable bacterium for tobacco wastewater treatment. This was primarily because damage to one antioxidant defense line could be implemented by another to a certain extent.

Table-2: Correlation between pollutant treatment abilities and antioxidant abilities.

Variable X	Variable Y	Strain TW (%)		Strain TY (%)	
		r^a	p-value	r^a	p-value
Nicotine degradation	SOD	0.053	0.893	-0.665	0.051
	CAT	0.771	0.015 ^a	0.917	0.001 ^{**}
	GSH	-0.284	0.459	0.537	0.136
COD removal	SOD	-0.187	0.723	-0.870	0.024 ^a
	CAT	0.755	0.083	0.989	0.000 ^{**}
	GSH	-0.709	0.115	-0.941	0.005 ^{**}

^ar: Pearson correlation coefficient

Experimental

Strains

Acinetobacter sp. TW and *Sphingomonas* sp. TY were obtained from tobacco waste produced by the Liquin Environment Protecting Paper Co., Ltd. (Hangzhou, China) [19]. Both bacteria are capable of using nicotine as the sole source of carbon, nitrogen and energy at nicotine levels as high as 1 000 mg/L.

Treatment

Nicotine-degrading strains TW and TY were separately incubated in Luria-Bertani (LB) medium, nicotine inorganic salt medium (N), LB medium amended with nicotine (LB+N) and tobacco wastewater medium (TWW).

LB medium was composed of 10 g tryptone, 5 g yeast extract and 10 g sodium chloride per liter of distilled water at pH 7.0. N medium was composed of 0.2 g K₂HPO₄, 0.8 g KH₂PO₄, 0.2 g MgSO₄, 0.1 g CaSO₄·H₂O, 0.0033 g NaMoO₄, and 0.005 g FeSO₄·H₂O per liter of distilled water, with 1 000 mg/L nicotine. LB+N medium were composed of the LB medium and 1 000 mg/L nicotine. TWW medium was prepared by combining tobacco waste and H₂O at a ratio of 7:100 (g/mL), and then amending this mixture of nicotine to 1 000 mg/L. The TWW medium was designed to mimic the oxidative stress that organisms are exposed to in tobacco wastewater. Sources of carbon, nitrogen, energy and oxidative stress in the four culture media are shown in Table 3.

Table-3: Sources of carbon, nitrogen, energy and oxidative stress in the culture media.

Medium/ Source	Carbon, Nitrogen and Energy	Oxidative Stress
LB	tryptone and yeast extract	- ^a
N	nicotine	nicotine
LB+N	tryptone, yeast extract and nicotine	nicotine
TWW	nicotine and various materials	nicotine and other pollutants

^a:- no existence of oxidative stress in LB medium.

All tests were performed in 250 mL flasks with 70 mL of medium on a reciprocal shaker at 30°C and 140 rpm.

Toxicity Assay

Antioxidant indicators including CAT, SOD and GSH were measured by spectrophotometry using Kits A007, A001 and A006, respectively. The total protein content was determined using a modified

Bradford method with Kit A 045-2 as described by Wang and Jia [20]. The specific antioxidant enzymes activities (CAT and SOD) were expressed as units per mg of cellular protein, and GSH was reported as mg GSH per g of cellular protein. All test kits were purchased from the Nanjing Jiancheng Bioengineering Institute (Jiangsu, China).

Degradation Assay

Nicotine content was analyzed by high performance liquid chromatography using the method described by Wang *et al.* [20]. Chemical oxygen demand (COD) was detected in accordance with the Standard Methods for the Examination of Water and Wastewater [21].

Data Analysis

The indicator activities were determined based on the Value_{media}/Value_{LB}. All data were expressed as the mean ± standard error (SE). Statistical analyses consisted of the paired-samples *t*-test, one-way ANOVA and Pearson correlation coefficient and were determined using SPSS (version 16.0). Differences were considered significant at *p* < 0.05.

Conclusion

The following conclusions were drawn based on the results of this study:

- (1) The antioxidant ability of strain TW was higher than that of strain TY, while the pollution treatment ability of strain TW was stronger than that of strain TY, both toward nicotine and other components in tobacco wastewater. Thus, a stronger antioxidant ability was associated with a higher potential for tobacco wastewater treatment.
- (2) A series of antioxidant indicators in all three media should be employed for suitable selection of nicotine-degrading bacteria for tobacco wastewater treatment.

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