J Ind Microbiol Biotechnol (2007) 34:567–570
DOI 10.1007/s10295-007-0212-x

SHORT COMMUNICATION

Biodegradation of nicotine from tobacco waste extract by *Ochrobactrum intermedium* DN2

Y. J. Yuan · Z. X. Lu · L. J. Huang · Y. Li · F. X. Lu · X. M. Bie · Y. Q. Teng · Q. Lin

Received: 8 June 2006 / Accepted: 4 February 2007 / Published online: 28 February 2007

Abstract *Ochrobactrum intermedium* DN2 was used to degrade nicotine in tobacco waste extracts. The optimal temperature and pH of nicotine degradation by strain DN2 was 30–37 °C and 7.0, respectively. Under these optimal conditions, the average degradation rate of nicotine in a 30L fed-batch culture was 140.5 mg l^{-1} h^{-1}. The results of this study indicate that strain DN2 may be useful for reducing the nicotine content of reconstituted tobacco.

Keywords Biodegradation · Nicotine · Reconstituted tobacco · *Ochrobactrum intermedium*

Introduction

Large quantities of waste containing high concentrations of nicotine are generated during the tobacco-manufacturing process and all activities that use tobacco [8]. Because nicotine is harmful to human health and the environment [4], the United States Environmental Protection Agency (EPA) has designated it as a Toxics Release Inventory chemical since 1994 [12]. This waste also has been classified as "toxic and hazardous" by European Union Regulations when its nicotine content exceeds 0.05% (w/w) [8].

Y. J. Yuan · Z. X. Lu (✉) · L. J. Huang · Y. Li · F. X. Lu · X. M. Bie · Y. Q. Teng · Q. Lin
College of Food science and Technology,
Nanjing Agricultural University,
1 Weigang, Nanjing, People's Republic of China
e-mail: fmb@njau.edu.cn

Nicotine is not readily degradable and is very toxic to most types of microorganisms. However, several microorganisms that are able to degrade nicotine have been isolated from the environment. These microbes incude *Arthrobacter nicotinovorans* [5], *Pseudomonas* sp. [2, 9, 13, 14], *Arthrobacter nicotianae* [3], *Enterobacter cloacae* [10], *and Cellulomonas* sp. [2], *Alcaligenes paradoxus* and *Arthrobacter globiformils* [11], which play an important role in the manufacturing process of tobacco by altering the content of nicotine in the final product and in treating nicotine pollution [1].

Reconstituted tobacco, or filler, because of its advantageous economic impact on the manufacturing cost of cigars and cigarettes, has been widely used by the tobacco industry [7]. Reconstituted tobacco is created from inexpensive materials such as tobacco plant stems, leaf scraps, dry tobacco dust, adhesives, reinforcing fibers, mineral ash modifiers, and humectants. The tobacco industry has been using reconstituted tobacco, which is cheaper than tobacco leaf, as a cigarette ingredient since the 1940s and 1950s. In addition, a high "filler" content can reduce the density of the cigarette and slightly reduce tar delivery [http://www.ash.org.uk/html/factsheests/html/fact12.html]. However, a high content of nicotine is present in the tobacco waste extract (TWE), which is used for manufacturing reconstituted tobacco, and all manufactures are attempting to reduce this.

Little information on the methods presently used to reduce nicotine content in TWT is available as the technology of the reconstituted tobacco industry is mostly protected by patents.

In previous study, we isolated and identified a novel nicotine-degrading strain, *Ochrobactrum intermedium* DN2, and optimized its medium for treating tobacco

wastes [16, 17]. The objective of this study was to examine parameters affecting nicotine removal in TWE by strain DN2. To our knowledge, this is the first report of microbially mediated nicotine biodegradation in TWE used for manufacturing reconstituted tobacco.

Materials and methods

Microorganism and preparation of inoculum

Ochrobactrum intermedium DN2, isolated from soil and identified by Yuan et al. [17], was used in this study. The organism was maintained at 4 °C by periodic transfer on nutrient agar slants containing (pH 7.2, l^{-1}): 11.34 g tryptone, 3 g beef extract, 5.0 g NaCl, 3.71 g $MgSO_4 \cdot 7H_2O$ and 20 g agar.

The strain DN2 was transferred from a stock slant to 250-ml Erlenmeyer flask containing 100 ml of medium and incubated at 30 °C with shaking at 120 rev min^{-1}. Thirty-six hours incubated cultures were used as inoculum solution. The medium was sterilized in autoclave for 20 min at 121 °C.

The tobacco waste extract for manufacturing reconstituted tobacco

TWE was supplied by Hangzhou Liqun Environment Protecting Paper Co., Ltd (China). The contents of solid, nicotine and reducing sugar in TWE were 54.04% (w/v, dry weight), 2.07% (w/v) and 12.08% (w/v), respectively. Diluted TWE (DTWE), which was supplemented with (l^{-1}) 0.025 g $FeSO_4$, 0.25 g $MgSO_4 \cdot 7H_2O$, 2.0 g KH_2PO_4 and 1.0 g yeast extract, directly used without being sterilized in the study. The amount of TWE used was 60 ml in 1 l DTWE in pH and temperature studies, while amounts used in the fed-batch experiment are listed in Table 1.

Effects of temperature and pH on nicotine degradation

Experiments were performed in 250-ml Erlenmeyer flasks containing 100 ml of DTWE and the initial content of nicotine was about 1,228 ± 26.32 mg l^{-1}. In temperature studies, cultures (pH 7.0) were incubated at 25, 30, 37 and 40 °C, respectively. In pH studies (30 °C), the initial pH of culture media was adjusted to 5.0, 6.0, 7.0, 8.0 and 9.0. NH_3–H_2O (1:3, v/v) solution was used to adjust pH of culture media. Fifteen percentage (v/v) inocula were used in all experiments. All cultures were agitated at 120 rev min^{-1}.

Fed-batch degradation of nicotine in 30 l bioreactor

Nicotine degradation was studied in three fed batches culture, which was carried out in a 30-l reactor. The experiment design for each batch is presented in Table 1. The pH of culture medium in the reactor was initially adjusted to 7.0 and readjusted to 7.0 at each feeding event (43 h and 61 h,) using NH_3-H_2O. The reactor was agitated at 120 rev min^{-1} at 30 °C and aerated at 0.25 v/v/m.

Analytical method

Nicotine content was analyzed by high performance liquid chromatography (Agilent 1100 series, America) according to the method of Yang et al. [15]. Cell growth was measured as the dry cell weight. Samples were centrifuged at 3,000*g* for 10 min, and the cell pellets were washed twice with distilled water, dried to constant weight at 80 °C, and then weighed. Reducing sugar was determined using the dinitrosalicylic acid (DNS) method [6]. In pH and temperature studies, all tests were conducted in triplicate and the results are reported as mean values. In the reactor test, duplicate samples were analyzed and the mean values are reported.

Results

Effect of temperature and initial pH on nicotine degradation

Figure 1 shows effect of incubation temperature on nicotine degradation. Nicotine degradation by strain DN2 was best between 30 and 37 °C. At 30 °C, nicotine was degraded from 1,241 to 69 mg l^{-1} within 24 h, while at 37 °C, nicotine was degraded from 1,248 to 89 mg l^{-1} within same time.

Table 1 Experiment design for fed-batch nicotine degradation

Batch	TWE (l)	Water (l)	Inoculum solution (l)	Removal of culture (l)	Total volume (l)
The 1st fed-batch (0–42 h)	3.0	16.2	3.0	0	22.20
The 2nd fed-batch (43–61 h)	4.2	0	0	6.17	20.23
The 3rd fed-batch (61–85 h)	5.3	0	0	5.57	19.96

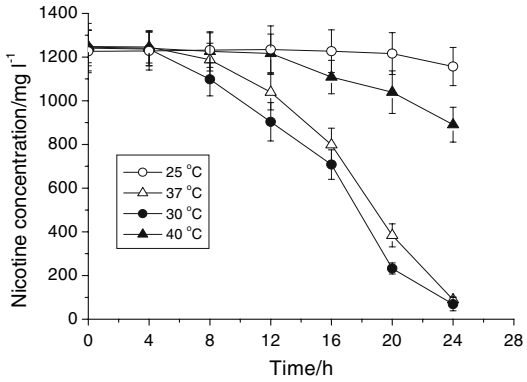

Fig. 1 The effect of temperature on degradation of nicotine by *O. intermedium* DN2. All data represent the mean of three replications. *Error bars* indicate standard error

Figure 2 shows the effect of the initial pH value on nicotine degradation. The optimum pH was 7.0, when nicotine was degraded from 1,211 to 80 mg l^{-1} within 24 h.

Fed-batch degradation of nicotine in 30 l reactor

Nicotine degradation was studied in a "fill and draw" fed batch culture which was carried out in a 30l biorector. Figure 3 shows that after the initial 24 h period, nicotine content decreased from 2,100 to 28 mg l^{-1} with a degradation rate of 115.1 mg l^{-1} h^{-1} within the first 18 h. After the first-fill and draw 4,300 mg l^{-1} of nicotine was degraded to 35 mg l^{-1} within 22 h at a degradation rate of 193.8 mg l^{-1} h^{-1}. After the second-fill and draw, 5,500 mg l^{-1} of nicotine was degraded to 400 mg l^{-1} within 22 h at a rate of 231.8 mg l^{-1} h^{-1}. At the same time, however, 62.35% of reducing sugar was also utilized. Cell growth increased from 0.027 to 1.89 mg ml^{-1}

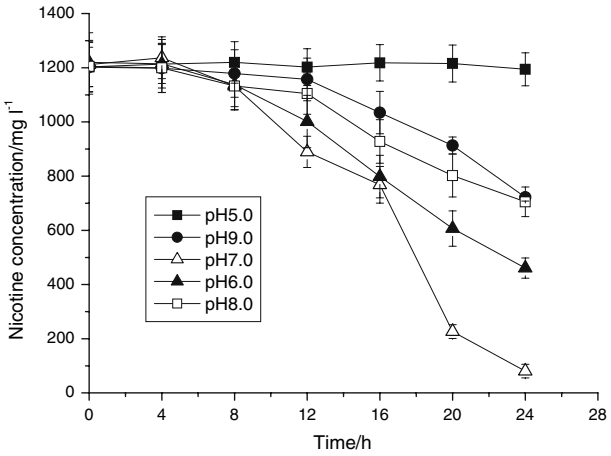

Fig. 2 The effect of initial pH values on degradation of nicotine by *O. intermedium* DN2. All data represent the mean of three replications. *Error bars* indicate standard error

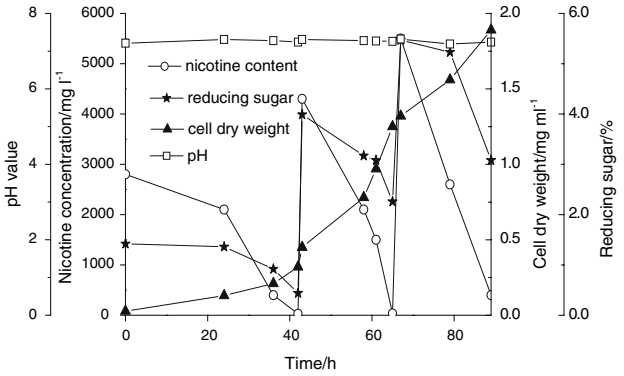

Fig. 3 Fed-batch degradation of nicotine by *O. intermedium* DN2 in 30 l reactor. All data represent the mean values of analysis on two samples at the same time. All errors < 10%

with nicotine degradation and reducing sugar utilization, while pH value lay between 7.19 and 7.32.

Discussions

The optimal pH and temperature of nicotine degradation by strain DN2 in TWE were 7.0 and 30–37 °C, respectively, which were consistent with growth and nicotine degradation by strain DN2 [17]. In the earlier study, it was found that pH in buffered or unbuffered medium by phosphate solution increased markedly during nicotine transformation by strain DN2 (data not shown). The pH of the medium changed only slightly, which is advantageous for growth and nicotine degradation by strain DN2.

"Fill and draw" fed batch culture showed an average rate of nicotine degradation of 140.5 mg l^{-1} h^{-1}, which is much higher than the 3.30–82.38 mg l^{-1} h^{-1} reported in other studies [2, 9, 13]. In a previous study, using an optimized medium containing 0.094% yeast extract, 0.101% glucose and 0.080%, Tween 80 improved nicotine degradation by strain DN2 and produced a degradation rate of 116.6 mg l^{-1} h^{-1} [17]. Here, we did not use the optimized medium as TWE contains in itself high content of reducing sugar and use of Tween 80 would not be allowed in the reconstituted tobacco process. However, nicotine degradation by strain DN2 depended on the presence of yeast extract [16], which could not be replaced by vitamin B_{12} (data not shown). Hence, in this study, yeast extract, at the optimal level of 0.1% [16], was added to TWE. To our knowledge, no report is available in the literature regarding reducing nicotine content in TWE by a biological method in manufacturing reconstituted tobacco. The results of this study indicate that the process of nicotine degradation by fed-batch culture using strain DN2 may prove to be useful and effective.

Acknowledgments The authors would like to thank Director YingBo Xu and Engineer HaiSheng He (The Technology Center of BengBu Cigarette Co. Ltd, Province AnHui of P.R. China), and Engineer QinAn Zheng (Hangzhou Liqun Environment Protecting Paper Co., Ltd, Province ZheJinag of P.R. China) for their warmhearted help and valuable discussions in nicotine degradation.

References

1. Brandsch R (2006) Microbiology and biochemistry of nicotine degradation. Appl Microbiol Biotechnol 69:493–498
2. Civilini M, Domenis C, Sebastianutto N, de Bertoldi M (1997) Nicotine decontamination agro-industrial and its degradation by micro-organisms. Waste Manag Res 15:349–358
3. Hochstein LI, Rittenberg SC (1959) The bacterial oxidation of nicotine: I. Nicotine oxidation by cell-free preparations. J Biol Chem 234:151–156
4. Holmstedt B (1988) Toxicity of nicotine and related compounds. In: Rand MJ, Thurau K (eds) The pharmacology of nicotine, ICSU Symposium Series 9. McLean, VA: IRL Press, pp 61–68
5. Kodama Y, Yamamoto H, Amano N, Amachi T (1992) Reclassification of two strains of *Arthrobacter oxydans* and proposal of *Arthrobacter nicotinovorans* sp. Nov. Int J Syst Bacteriol 42:234–239
6. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–427
7. National center for tobacco-free kids. Agriculture briefing paper 03: lowering leaf content, boosting profits. http://www.tobaccofreekids.org/campaign/global/pdf/ag3.pdf
8. Novotny TE, Zhao F (1999) Consumption and production waste: another externality of tobacco use. Tob Control 8:75–80
9. Ruan AD, Ming H, Peng XH, Huang Z (2005) Isolation and characterization of *Pseudomonas* sp. Strain HF-1, capable of degrading nicotine. Res Microbiol 156:700–706
10. Ruiz GV (1983) Degradacion de la nicotina por bacterias, 2: *Enterobacter cloacae* como degradador de nicotina. An INIA Ser Agric 22:85–98
11. Uchida S, Maeda S, Masubuchi K, Shinohara T, Kisaki T (1976) Isolation of nicotine-degrading bacteria and degradation of nicotine in shredded tobacco and tobacco extract. Nippon Sembai Kosha Chou Kenkyusho Kenkyu Hokoku 18:197–201
12. United States Environmental Protection Agency (1996) Emergency planning and community right-to-know section 313: list of toxic chemicals. (EPA745/B-96/002) United States Environmental Protection Agency, Washington, DC
13. Wang SN, Xu P, Tang HZ, Meng J, Liu XL, Huang J, Chen H, Du Y, Blankespoor HD (2004) Biodegradation and detoxification of nicotine in tobacco solid waste by a *Pseudomonas* sp. Biotechnol Lett 26:1493–1496
14. Ward E (1957) Microbial degradation of the tobacco alkaloids, and some related compounds. Arch Biochem Biophys 72:145–162
15. Yang J, Zhang DY, He ZF (1999) Determination of nicotine in tobacco by high performance liquid chromatography. Tob Sci Technol 1:27–28
16. Yuan YJ, Lu ZX, Huang LJ, Bie XM, Lü FX, Li Y (2006) Optimization of a medium for enhancing nicotine biodegradation by *Ochrobactrum intermedium* DN2. J Appl Microbiol 101:691–697
17. Yuan YJ, Lu ZX, Wu N, Huang LJ, Lü FX, Bie XM (2005) Isolation and preliminary characterization of a novel nicotine-degrading bacterium, *Ochrobactrum intermedium* DN2. Int Biodeter Biodegr 56:45–50