TIME DEPENDENT ACCUMULATION OF NICOTINE DERIVATIVES IN THE CULTURE MEDIUM OF *ARTHROBACTER NICOTINOVORANS* pAO1

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Abstract: Previous studies have shown that the metabolic intermediate 6-hidroxy-D-nicotine (6HNic) found in the *Arthrobacter nicotinovorans* pAO1+ nicotine catabolic pathway has the ability to bind nicotinic acetylcholine receptors and to sustain spatial memory in rats. These properties make 6HNic a valuable compound with some potential for medical applications, thereby a suitable, simple and efficient method for producing 6-hidroxy-D-nicotine is necessary. Here, we focus on identifying the best moment for harvesting *A. nicotinovorans* cells in order to directly convert nicotine to 6HNic with the best yield. The growth of *A. nicotinovorans* pAO1+ was monitored and the correlation between the growth phases and nicotine metabolism was established. After about 5 hours of lag, the strain entered the log phase and was fully grown after 10 hours. The nicotine concentration began to drop dramatically as the pAO1+ culture reached saturation and was depleted in 5 hours. As the nicotine concentration dropped, 6HNic began to accumulate, reaching the maximum levels after about 11 hours of growth. Two other products could be detected by HPLC, one which was identified as the nicotine-blue (NB) pigment and a second a still unknown end-product.

INTRODUCTION

Tobacco is reasonably cultivated worldwide, with a global production reaching 6.7 million tons per year. The main tobacco producer is China with 39.6% from the global production, followed by India (8.3%), Brazil (7.0%) and USA (4.6%). The tobacco processing industry produces annually about 3 million tons of waste (Gurusamy and Natarajan, 2013), with a mean nicotine content of about 18g / kg dry weight. EPA (The Environmental Protection Agency) and the UE regulations have designated these wastes as “toxic and hazardous” when the nicotine content is more than 0.05% (w/w). The waste containing nicotine is not only an unused source of aromatic compounds, but also a potential danger for the environment and human health.

Our previous studies have shown that 6-hidroxy-D-nicotine (6-HNic) has the ability to bind nicotinic acetylcholine receptors (nAChR) and thus, to sustain spatial memory in rats (Mihasan et al., 2013, Hritcu et al., 2011). These properties make 6HNic a valuable compound with high potential for medical applications, especially in the therapy of neurodegenerative disorders such as such as Alzheimer's disease (AD). Taking into account that the Alzheimer's Organization estimates the direct costs to American society of caring for those with Alzheimer's will total $214 billion for 2014 and a staggering $1.2 trillion by 2050 (Hebert et al., 2013), the ability to use an “toxic and hazardous” waste in order to produce active compounds with high impact on the medical sector is very appealing.

6-Hidroxy-D-nicotine is a metabolic intermediate found in the *Arthrobacter nicotinovorans* pAO1+ nicotine catabolic pathway. It is formed by a hydroxylation reaction catalyzed by the heterotrimeric enzyme nicotine-dehydrogenase. The three genes encoding the subunits of the active enzyme have been previously cloned (Sachelaru et al., 2006, Andrei and Mihasan, 2013) and expressed (Sachelaru et al., 2006), but the amount of purified enzyme is very low, making this approach unusable for production of 6HNic. Thereby, the current work makes the first efforts into developing a method of producing 6HNic by directly employing the wild type *A. nicotinovorans* pAO1 strain. Here, we focus on identifying the best moment for harvesting *A. nicotinovorans* cells in order to directly convert nicotine to 6HNic with the best yield.

MATERIAL AND METHODS

Chemicals. All chemicals were purchased from well-known suppliers and were of greatest purity available. 6-Hydroxy-nicotine was produce by chemical synthesis and is a kind gift from Prof. Dr. Roderich Brandsch - Institute of Biochemistry and Molecular Biology, Albert-Ludwigs University of Freiburg, Germany.

Strains and growth conditions. *Arthrobacter nicotinovorans* (strain ATCC 4991) harboring (pAO1+) or not harboring (pAO1-) the pAO1 megaplasmid were grown on citrate medium supplemented with 0,05% nicotine and 0,005% minerals solution (Eberwein et al., 1961) on a rotary shaker at 28^0C/190 rpm. The growth of the culture was followed at 660 nm. Samples from the growth medium were taken at specific time points and analyzed by UV-VIS spectroscopy and HPLC.

Absorption spectra's were recorded on a Beckman Coulter DU 730 Life Science spectrophotometer using citrate medium as blank. **HPLC analysis** was performed on a Bischoff system equipped with 2 pumps, a DAAD detector and a Machery-Nagel Nucleodur RP C18 ec column (150x4.6 mm, particle size 3µm). 20 µL of sample was injected and isocratic elution at room temperature was used for separation. The mobile phase was a mixture of 1mM H_2SO_4 and methanol at various ratios.

RESULTS AND DISCUSSIONS

Separation on nicotine from 6HNic. Despite the fact that the same reverse-phase principle is applied to all the described methods for separation of nicotine intermediates in complex mixtures, different authors employ various conditions in terms of mobile phase concentration (Table 1).

Table 1. Separation conditions employed for resolving nicotine derivatives.

Column	Mobile phase	Ratio	Flow rate/ temperature	Detection	Reference
Kromasil KR100-5C18, 150mm×4.6mm; particle size 5µm	Methanol:1 mM H_2SO_4	15:85	0,5 ml/ min / 30°C	210 nm	(Wang et al., 2005)
Kromasil KR100-5C18, 150mm×4.6mm; particle size 5µm	Methanol:1 mM H_2SO_4	25:75	0,5 ml/ min / 30°C	207 nm	(Tang et al., 2008)
Kromasil KR100-5C18, 150mm×4.6mm; particle size 5µm	Water:Methanol	75:25	0,5 ml/ min / 30°C	210 nm	(Tang et al., 2009)
Grace Alltima C18, 4.6×250 mm; particle size 5µm	Methanol:Water	10:90	0,5 ml/ min / 30°C	263 nm; 232 nm; 307 nm	(Ma et al., 2014)
Eclipse XDB-C18, 250×64.6 mm; particle size 5µm	Methanol:1 mM H_2SO_4	5:95	0,5 ml/ min / 30°C	-	(Liu et al., 2014)

In order to identify the best separating condition the complex mixture and system used in our experiment, pure nicotine and 6-hydroxy-nicotine were injected in the HPLC system and eluted at different methanol concentrations. The mobile phase mixture that gave the best results in terms of differences between retention time for nicotine and 6-hydroxy-nicotine was found to be 1mN H_2SO_4 : methanol 75:25 (Figure 1, A).

Growth of *Arthrobacter nicotinovorans* strains on nicotine containing medium. As 6HNic is a metabolic intermediate, one can clearly expect that its half-life is rather low as it is further processed by the nicotine-degradation machinery (Brandsch, 2005).

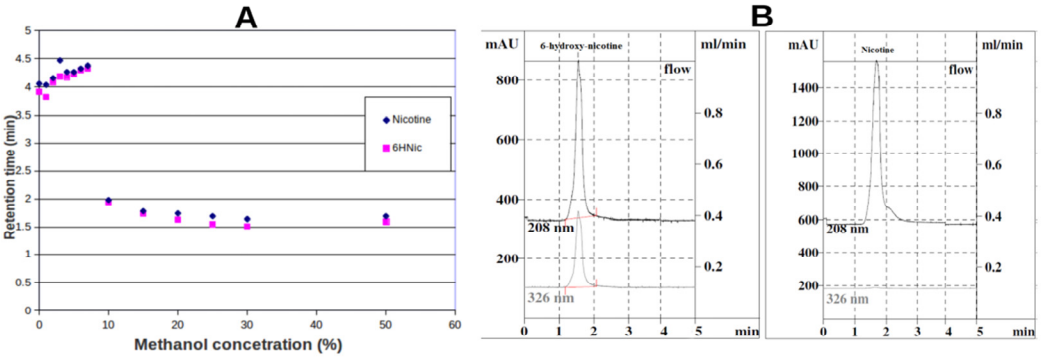
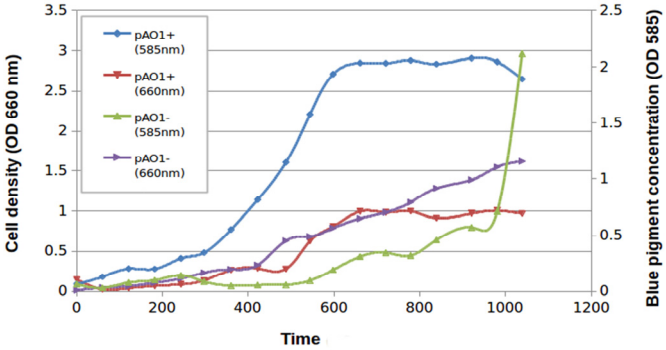


Figure 1. **A.** Dependence of nicotine and 6-hydroxy-nicotine retention times on the methanol concentration. Largest differences in retention times were obtained at a methanol concentration of 25%. **B.** Typical HPLC run for separation of nicotine and 6-hydroxy-nicotine using 1mN H_2SO_4 : methanol 75:25 as mobile phase.

Still, primary reports on nicotine metabolism in this strain indicate that 6HNic is present in the growth medium in small amounts (Hochstein and Rittenberg, 1959). In order to identify the best moment for harvesting *A. nicotinovorans* pAO1+ cells and obtain largest amounts of 6HNic a strict correlation between the age of the culture and the level of nicotine consumption is required. Thereby, the pAO1+ and pAO1- strains growth on liquid citrate medium was measured at 660 nm and was at first correlated with an primary indicator of nicotine depletion in the medium: the accumulation of the final end-product (Nicotine-blue pigment, NB), followed at 585 nm. After about 5 hours of lag, both the pAO+ and pAO1- strains entered the log phase and were fully grown after 10 hours. As the lag phase ended, the characteristic blue-pigment appeared in the medium of the pAO1+ strain (Figure 2, A).

6-Hydroxy-nicotine is excreted in the *Arthrobacter nicotinovorans* growth medium. When grown on citrate medium supplemented with nicotine, the *Arthrobacter nicotinovor*ans pAO1 strain is able to use nicotine as a carbon and nitrogen source (Baitsch et al., 2001; Igloi and Brandsch, 2003). The HPLC analysis of the growth medium has allowed to strictly correlate the main growth phases of the bacterial culture with the first steps of the nicotine catabolic pathway. As shown in figure 2, B, the nicotine concentration began to drop dramatically as the pAO1+ culture reached saturation and was depleted within 5 hours. As the nicotine concentration dropped, 6HNic started to be exported into the medium and began to accumulate, reaching the maximum levels after about 11 hours of growth. Two other products could be detected by HPLC, one which was identified as the nicotine-blue pigment and a second a still unknown aromatic ring containing end-product.

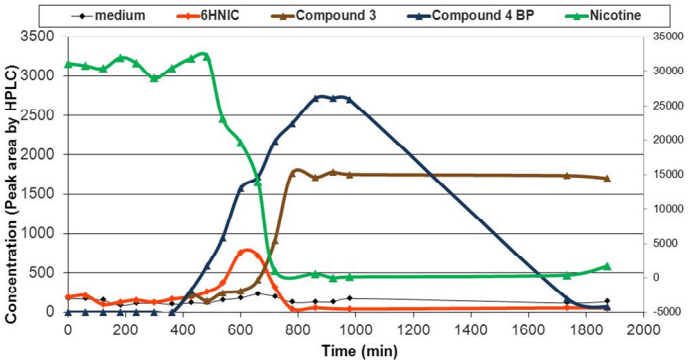
A



B

Figure 2. Dynamics of *Arthrobacter nicotinovorans* cultures on citrate medium supplemted with nicotine **A.** Growth curve and the time-dependent accumulation of nicotine-blue pigment accumulation **B.** Dynamics of nicotine and nicotine metabolites concentration during growth.

As it can be inferred from Figure 2, B, the levels of 6HNic dropped quite rapidly when the nicotine levels were low. This fact has two major practical implications. On one hand, it proves that there is a bottleneck (Liu et al., 2002) in the catabolism of nicotine due probably to different catalytic efficiencies of the involved enzymes. It seems that nicotine-dehydrogenase is able to process its substrate much faster then the fallowing enzyme 6-hidroxy-L-nicotine-oxidase



(6HLNO). This leads to the accumulation of 6HNic in the medium, but as nicotine is depleted, 6HLNO is able to catch up and starts using the excreted 6HNic. On the other hand, the small interval of lag between these two enzyme gives a one hour time frame on which the bacteria could be harvest and for the isolation of 6HNic. Still, as the accumulation levels of this compound are quite low, the yield of the process would be low also.

CONCLUSION

The direct utilization of A. nicotinovorans pAO1+ cells for transformation of nicotine into the biotechnological valuable product 6-hydroxy-nicotine is feasible, although with a rather low yield. The best moment for cells harvesting is after 11 hours of growth on citrate medium supplemented with 0,05% nicotine.

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