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RECENT DEVELOPMENT OF BIOAUGMENTATION METHODS FOR TOBACCO WASTEWATER TREATMENT

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ABSTRACT. In production of cigarettes a lot of tobacco waste, with nicotine, goes into the environment. Hence, there is a need for an economic and efficient method to diminish the discharge of hazardous materials from tobacco wastewaters. Bioaugmentation using specialized bacteria strains could improve the efficiency of tobacco wastewater treatment. In this review paper we present bioaugmentation methods for tobacco wastewater treatment that were published in last few years. Bioaugmentation systems have proven to be very effective in removal of nicotine and TOC; it was shown that Pseudomonas sp. HF-1 and TW bacteria strains can be successfully used in reactors. Recent studies showed that controlling pH in the reactors can improve reactor performance in removing nicotine and TOC from tobacco wastewater.

Keywords: *tobacco wastewater, nicotine, hazardous materials, bioaugmentation*

INTRODUCTION

In production of cigarettes a lot of tobacco waste, that includes nicotine, aminobiphenyl, naphthylamine and benzo(a)pyrene goes into the environment [1, 2]. A non-recyclable, powdery, nicotine-containing waste is formed during tobacco production, which has an average nicotine content of 18 mg/kg dry weight [3]. These wastes are classified as ‘‘toxic and hazardous wastes’’ under European Union Regulations when the nicotine content exceeds 500 mg/kg dry weight [4].

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For 1 t of cigarettes, more than 60 t tobacco wastewater is discharged [5]. Nicotine can dissolve in water and other organic solvents. It can cross blood-brain barrier and many other complicated biological membranes [6]. Because of this, nicotine is considered malignant pollutant among other things.

Analyses on the effluent of many sewage treatment plants (STPs) shows the presence of nicotine. Based on this it can be concluded that nicotine survives conventional treatment processes [7] and different methods for removal of nicotine from tobacco wastewaters are being developed. Nicotine adsorption by coconut fibers and saw dust is a good method for removal of nicotine from wastewaters [8], but it does not degrade nicotine. Teijon et al. [7] report that nicotine removal after conventional treatment processes based on flocculation–coagulation, lamellar clarification, filtration, and disinfection is about 79%, while after an additional treatment of chlorination the removal was about 97%. However, aqueous chlorination can react with natural organic matter and other organic compounds to form disinfection by-products (DBPs). These products have been shown to be more resistant to degradation and more toxic then the original compounds that they came from [9-11]. As can be seen denicotinization of tobacco wastes can be done with physio-chemical treatment, but compared to these methods, biological methods of nicotine degradation are more efficient and less costly [12].

Because of pollution problems and toxicity of nicotine, research on nicotine degradation by microorganisms (biodegradation) is very active filed of research. Large numbers of microorganisms, able to degrade nicotine were identified [13]. Biochemical pathways for degradation as well as genes responsible for nicotine degradation were described in reviews [13, 14]. Moreover, new microorganisms that degrade nicotine were found and described in the last few years [15-17].

NICOTINE DEGRADING BACTERIA

Many types of nicotine degrading bacteria have been isolated and identified [18-21]. Some of them have been used to degrade nicotine in liquid medium.

Wang et al. [21] demonstrated that *Sphingomonas* sp. TY had a greater ability to degrade nicotine than strain HF-1, with complete degradation of 1.0 g/l of nicotine within 18h. Strain TY could grow and degrade nicotine in a range of pH from 3-8, and temperature range 15-45°C. However, best results were obtained when initial pH was 6.0-7.0 and temperature 25-30°C [21].

There are a few strains of *Pseudomonas* sp. used in nicotine degrading studies. As shown by Wang et al. *Pseudomonas* sp. S16 was able to degrade 3.0 g/l nicotine within 10h. The optimal temperature for nicotine degradation

was 30°C, while optimal pH was 7.0 [22]. Another *Pseudomonas* sp. strain designated as CS3 was used for nicotine degradation in liquid medium [23]. Optimal conditions for nicotine degradation by strain CS3 were 30°C and pH 7.0. However, this strain showed high nicotine-degrading capabilities within pH range from 6.0 to 10.0. Strain CS3 can decompose 1.0 g/l nicotine within 24h, and could endure up to 4.0 g/l nicotine in liquid media [23]. Strain *Pseudomonas plecoglossicida* TND35 can degrade 0.5-5 g/l nicotine within 8 to 44h, with optimal conditions of 30°C and pH 7.0 [17].

Newly isolated *Ochrobactrum* sp. Strain SJY1 was tested in degrading nicotine [24]. The study showed that strain SJY1 could grow in a range of pH form 5.0 to 9.0, where optimal pH was 7.0. The optimal temperature was 30°C. Under optimal conditions strain SJY1 could almost completely degrade 4.0 g/l nicotine within 10h [24].

BIOAUGMENTATION

For the treatment of tobacco wastewaters bioaugmentation using specialized bacteria strains has proven to be one of the most effective, considering costs and sustainability as well [25-27].

The bacteria used in bioaugmentation have to be active, persistent and compatible, hence one has to find microorganisms that are suitable for bioaugmentation [28, 29]. To avoid unpredictable bioaugmentation results it is important to choose the right strain [30]. Two strains, that showed high nicotine degrading ability, were used for bioaugmentation of tobacco wastewaters; *Pseudomonas* sp. HF-1 [3, 31-33] and *Acinetobacter* sp. TW [21].

The principle behind bioaugmentation is colonization. This means that the nicotine degrading bacteria has to have the ability to coexist with native bacteria and keep its activity in the activated sludge system [34]. A sharp increase in autoinducers, small molecules that bacteria use to communicate, can indicate a change in behavior of bacterial community [35, 36]. Communication using autoinducers enable bacteria to acclimatize themselves to the environment. This way of bacterial communication is called the theory of quorum sensing [37-39].

When microorganism come in contact with toxic substances oxidative stress occurs [40]. Nicotine is one of the substances that can induce oxidative stress in microorganisms [41]. Bacteria capable of reducing the toxicity of nicotine, and thus reducing oxidative stress, can be beneficial to other bacteria in the active sludge.

Several studies on bioaugmentation of sludge were performed. In this review paper we present methods of bioaugmentation for tobacco wastewater treatment that were published in last few years.

Bioaugmentation with *Pseudomonas* sp. HF-1

Sequencing batch reactor (SBR) can be used for tobacco wastewater treatment [42]. However, using this method with conventional activated sludge culture in tobacco industry is not very efficient because of toxicity of nicotine and other substances in tobacco wastewater [43]. For example, in tobacco production corporation Liqun (Hangzhou, China) using sequencing batch reactor resulted in w20% nicotine degradation and w50% chemical oxygen demand (COD) removal [44]. These data show that conventional activated sludge is not optimal for treatment of tobacco wastewater. Hence, it was proposed to use bioaugmentation to increase efficiency of sequencing batch reactor [44], as bacterial strains specialized in nicotine degradation can reduce toxicity to the microbial community [45].

Comparison of Bioaugmented and Non-Bioaugmented reactors

In the study by Wang et al. *Pseudomonas* sp. HF-1, that possess high ability for nicotine degradation, was used for bioaugmentation in SBR [44]. The influence of nicotine degradation by bioaugmentation strain *Pseudomonas* sp. HF-1, on the structure and activity of microbial community, was monitored. In the study two reactors with activated sludge from the Sibao Sewage Treatment Plant (Hangzhou, China), as the indigenous population, were used. One of the reactors was bioaugmented by *Pseudomonas* sp. HF-1, while the other one, without *Pseudomonas* sp. HF-1 was a control. Wastewater that was used in this study was prepared by mixing tobacco waste (collected from Liqun Cigarette Co. Ltd., Hangzhou, China) and tap water in a ratio of 7:100 (g/mL). Nicotine and COD concentrations were observed in the study as target pollutant indexes.

Both systems were able of complete removal of nicotine when concentration of nicotine was 40 and 80 mg/L; however, removal of nicotine in bioaugmented system was faster. When concentrations of nicotine were from 130 to 250 mg/L non-bioaugmented system was able only partially to remove nicotine in 48h, while bioaugmented system removed nicotine completely in 12h.

An increase of nicotine concentration resulted in decrease of COD removal in non-bioaugmented system; increase from 40 to 250 mg/L corresponded to COD removal of 89.9% and 64.6%. An increase of nicotine concentration almost did not influence COD removal in bioaugmented system; COD removal was between 84.8% and 90.6%. These results indicated that bioaugmenteation by *Pseudomonas* sp. HF-1 improves removal of nicotine, but also removal of COD. Namely, nicotine as toxic substance inhibited growth and activity of indigenous sludge microorganism population. In bioaugmented system, *Pseudomonas* sp. HF-1 removed nicotine and enabled growth and activity of indigenous sludge microorganism contributing to increased COD removal.

Based on these results it was proposed that bioaugmentation with the nicotine-degrading *Pseudomonas* sp. strain HF-1 is a good and environmentally friendly alternative for tobacco wastewater treatment. Hence, this method has great potential for application on tobacco wastewater [44].

Biofilm Formation

The colonization of bioaugmented systems by the inoculated bacteria can be affected by many factors, such as competition from native bacteria [46]. The ability of bacteria to colonize a new environment can be improved by biofilms, multi-cellular communities formed by bacteria [47-49]. The process of biofilm formation has been reported to occur via quorum sensing, cell-cell communication among bacteria using auto-inducers [50, 51]. In order to develop better bacterial bioaugmentation system for tobacco wastewater treatment Wang et al. studied the roles and condition for release of acylated homoserine lactones (AHLs), the main auto-inducers that effect biofilm formation of *Pseudomonas* sp. HF-1.

Biofilm formation includes swarming by flagella and secretion of extracellular polymeric substances (EPS); these processes are induced by acylated homoserine lactones (AHLs). AHLs are released into and out of bacterial cells as the population of bacteria increases. Conditions influence the increases in bacteria population and affect the release and existence of auto-inducers [52].

The results on influence of temperature and pH on biofilm formation showed that during the start-up stage of the bioaugmentation, low temperature (20–25 ^{o}C) and acidic environment (pH value 6) were good for introduction of the strain culture. During biofilm formation, it was shown that temperature does not have significant influence, while alkaline environment (pH value 8) was beneficial for stable performance of the bioaugmentation

Biofilm formation can be also influenced by substances in the solution like nicotine and NaCl. Taking also concentrations of nicotine and NaCl, Wang et al. proposed conditions for biofilm formation [1]. Temperature of 25 °C, pH 5–6, concentrations of 3% inoculum, 1.5 g/L nicotine and 1% NaCl, was beneficial to the startup stage during bioaugmentation, since the amount of AHLs released was sufficient for quorum sensing of swarming and EPS formation for strain HF-1. Under conditions of pH 8 in the presence of 1.2–1.8 g/L of nicotine and 1% NaCl, the threshold for quorum sensing of biofilm formation was reached and the bioaugmentation system showed an efficient performance.

Influence of pH, inoculum amount and nicotine load on Reactor Performance

It is hypothesized that biofilm formation by *Pseudomonas* sp. HF-1 in a bioaugmented system was also regulated by quorum sensing [53]. Since it was found that pH had a significant effect on the release of auto-inducers by *Pseudomonas* sp. HF-1, it was investigated if regulation of pH could be used

to facilitate HF-1 colonization of activated sludge in bioaugmentation systems [1]. Reactor performance in removing nicotine and TOC is the most important evaluation in the experiment. Nicotine removal in pH controlled reactors remained at about 100% during whole experiment. In non-pH controlled reactors nicotine removal decreased after some time. Low pH (pH 5.5) in the beginning of the process induced the release of auto-inducers and increase in the swarming ability and EPS secretion for strain HF-1 biofilm formation. However, low pH hampered the growth of other bacteria in the activated sludge and caused low TOC removal in the beginning. Nevertheless, in the pH controlled reactor, in the later phases of process, when pH was 8.0, presents of strain HF-1 removed toxic nicotine, and enabled the growth of other bacteria and increased TOC removal. In non-pH controlled reactor, strain HF-1 did not make biofilm and disappeared in late phases of the process, which caused toxic nicotine to inhibit growth of other bacteria, resulting in decreased TOC removal.

Appropriate inoculum amount is crucial for successful setup of bioaumentation system. Small inoculum may not be enough to degrade the amount of nicotine present in the reactor, while too large inoculum could destroy the ecological balance in the reactor [54]. The amount of inoculum that is most suitable for the colonization of strain HF-1 was 1.10 mg/g (dry weight of strain HF-1/dry weight of activated sludge) [55].

The set-up of strain HF-1 bioaugmented system was not inhibited by nicotine load. The successful setup of bioaugmented reactor was done with nicotine concentrations from 250 to 1200 mg/L. However, it needs to be mentioned that TOC removal efficiency decreased when nicotine was above 1000 mg/L [55].

Bioaugmentation with *Acinetobacter* sp. TW

Evaluation of whether the strain *Acinetobacter* sp. TW can colonize activated sludge, was done in a synthetic tobacco wastewater system [56]. Synthetic wastewater system was used to study the effects of native sludge bacteria on the colonization of TW strain, since actual tobacco wastewater contains many unknown toxic materials that can affect the experiment [57]. The activated sludge used in this study was obtained from the Qige wastewater treatment plant (Hangzhou, China).

Polymerase chain reaction-denaturing gradient gel electrophoresis (PCRDGGE), quorum sensing autoinducer detection, and toxicological indicators monitoring were used to give a view on ecological relationships involved in bioaugmentation.

Three reactors divided into two groups were used in this study. The first group was non-bioaugmented system (non-BA system) and the other group included two parallel bioaugmented systems (BA system-1 and BA system-2). All three reactors were started with 3200 ± 50 mg/L of initial COD and 1.0 ± 0.1 g/L of nicotine. The only difference between them was that the first group did not contain TW strain. BA system-1 and BA system-2 were inoculated with TW strain 6 times,

every 2 days. The inoculation ended on the 12th day. The performance was followed for 28 days.

To see whether the colonization of strain TW on the active sludge was successful a sample was taken and analyzed in the end of the experiment. For the detection of TW in the activated sludge a reverse transcriptase PCR (RT-PCR) was used because it can monitor gene expression in viable cells, as dead cells are not detected [58]. Young has reported that gene *hsp* is one of the most important genes involved in nicotine degradation in strain TW, as well as in *Pseudomonas* sp. strain HF-1 [44, 59]. The sequences for *hsp* was found in BA system -1 and BA system - 2, which indicates that strain TW was present. No such sequences were seen in the original activated sludge and non-BA system, indicating absence of strain TW. This proves that strain TW has successfully colonized the activated sludge in the two BA-systems

The experiment was divided into 2 stages [56]. First stage was the bioaugmentation stage that lasted until day 12 when the inoculation ended. The second stage started from day 13, when a stable synthetic wastewater influx was maintained.

In the first stage non-BA system had nicotine removal of only 10%, while in BA systems it was up to 95%. The second stage lasts the remaining 16 days. Although no more inoculations were carried out, the BA systems maintained nicotine removal at above 95%, while non-BA system had merely 0-10%. Considering that there were no other differences between the non-BA system and BA systems, except strain TW, the 98% removal of nicotine in BA systems can be almost totally contributed to nicotine degradation by strain TW, which also contributes to the conclusion that strain TW has colonized the active sludge.

The efficiency of the reactors was also evaluated by monitoring COD removal. On non-BA system the removal of COD was maintained above 60%, while in two BA systems it was from 80% to 90% during the whole process. This indicates that colonization of strain TW improves the efficiency of COD removal.

BA systems have proven to be very effective in removal of nicotine and COD, which goes to 95% and 80%-90% respectively. Nicotine toxicity has made it easier for TW strain to colonize the activated sludge, because it had caused oxidative stress in the native bacteria. Further degradation of nicotine protected the native bacteria from its toxic effects and increased the biodiversity of the active sludge. With the increase in biodiversity the overall efficiency of BA systems was also increased, including efficient COD removal. Microbial community in bioreactors is popularly tested by PCR-DGGE technique [60, 61]. After colonization of the active sludge by strain TW there was a change in the structure of the bacterial colony and the BA systems reached a new ecological balance. On the last day of the experiment the activated sludge in BA reactor-1 and BA reactor-2 contained more types of bacteria when compared to the original sludge. More complex reactors ecosystem means that it is more stable and resilient [62].

As was described above many bacterial strains can be used to degrade nicotine in liquid media. Except strains *Pseudomonas* sp. HF-1 and *Acinetobacter* sp. TW, others have not been used in bioaugmentation of tobacco wastewater. The future research should concentrate on studying the use of these bacterial strains in bioagmentated treatment of tobacco wastewater.

CONCLUSION

BA systems have proven to be very effective in removal of nicotine and COD; it was shown that *Pseudomonas* sp. HF-1 and *Acinetobacter* sp. TW bacteria strains can be successfully used in reactors. Recent studies showed that controlling pH in the reactors can improve reactor performance in removing nicotine and TOC from tobacco wastewater.

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