Enhancing Nicotine Biodegradation: Acclimation of Activated Sludge and Formation of Aerobic Granules for Improved Performance

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This study investigates the adaptation of activated sludge for nicotine degradation by introducing glucose as a co-substrate. The acclimation process significantly improved nicotine biodegradability, leading to its complete degradation. In contrast, the non-acclimated sludge removed less than 10 % of nicotine over the same period. The acclimated system achieved effluent nicotine and COD concentrations of 0.95 mg L^{-1} and 31 mg L^{-1}, respectively, with removal efficiencies of 99.81 % and 93.80 %. 16S rRNA analysis identified *Bosea* sp. and *Paenarthrobacter* sp. as potential nicotine-degrading bacteria under long-term acclimation. This study also examines the impact of hydraulic circulation on the formation of aerobic granule. In a 6-hour cycle, granules with diameters of 2–3 mm and high strength had formed, whereas longer cycles (12 and 24 hours) led to incomplete granulation. Increasing the nicotine concentration up to 1000 mg L^{-1} tested the granules' degradation capability, achieving a COD removal efficiency of 91.02 %.

Keywords
acclimated activated sludge, aerobic activated sludge granule, tobacco biodegradation, nicotine biodegradation

Introduction

Nicotine [1-methyl-2-(3-pyridyl-pyrrolidine), $C_{10}H_{14}N_2$], a well-known alkaloid, poses chronic and serious ecological effects on most organisms[1]. Nicotine (NCT) is a water-soluble compound that can easily be transported into surface and groundwater[2]. Therefore, reducing NCT concentration in tobacco waste has become a significant focus of environmental remediation efforts. Various physicochemical technologies have been explored for "denicotinization" from aqueous media, including adsorption[3–6], photocatalysis[7,8], advanced oxidation processes[9], and extraction using organic solvents such as cyclohexane and 1-butanol[10]. However, biological methods have gained attention over the past 50 years due to their efficiency and cost-effectiveness in reducing nicotine levels in tobacco and detoxifying tobacco waste compared to other physical and chemical methods[11–13]. Consequently, exploring biological treatments using microbial processes is considered a promising alternative. Microbial communities play a crucial role in eliminating NCT by altering its content in final products. Several studies have focused on isolating and identifying specific nicotine-degrading microbial strains such as *Pseudomonas* sp.[14,15], *Arthrobacter* sp.[16,17], *Ochrobactrum intermedium*[18], *Rhodococcus* sp.[19,20], *Ensifer* sp. strain N7 [21], *Agrobacterium* sp. S33 [22], *Shinella* sp. HZN1 [23], *Bacterium s*p. strain J54 [24], among others. Additionally, the bioaugmentation of a specific type of NCT digester with activated sludge has been discussed[25,26]. Activated sludge, a collection of microbial communities, shows great potential for the biological removal of various pollutants. Previous research suggests that the acclimation process of microorganisms is an effective mechanism for protecting them against toxic substance shock. During acclimation, microorganisms in the activated sludge undergo morphological, physical, and biochemical changes that help them tolerate adverse environmental conditions and reduce biodegradation time[27–31]. On the other hand, compared to conventional activated sludge, aerobic granules – a type of biomass retention technology – offer advantages such as high settling ability, compact microbial structure, and greater resistance to toxicity and shock loadings[32–35].

The primary objective of this study was to investigate the long-term compatibility of microorganisms in activated sludge to NCT as a toxic pollutant, and to identify bacterial strains capable of degrading NCT and surviving in NCT- containing environments. In addition, the ultimate aim was to

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explore the possibility of cultivating aerobic granules using adapted activated sludge, determine the optimal cycle time for the process, and assess the ability of aerobic granules to treat synthetic wastewater (SWW) containing NCT.

Materials and methods

Seed sludge and materials

The raw activated sludge was obtained from an urban wastewater treatment plant (Anzali City, Iran), ensuring the presence of a diverse microbial population. Nicotine with a purity of 99 % was sourced from Sigma–Aldrich (Germany), and nutrient agar was obtained from Quelab (Canada). All other chemicals used in the experiments were of analytical grade.

Experimental set-up and operating conditions for the acclimation process

The acclimation process was conducted using a 1.5-L fed-batch reactor (dimensions: 10×10×15 cm) with a working volume of 1 L. A phosphate buffer (pH 7.0) was used to wash the activated sludge, followed by aeration for 24 h to remove any residual organic compounds. The sequencing batch reactor (SBR) operated at room temperature (20 ± 1 ºC) with extended aeriation. An aeration pump with a capacity of 300 L h^{-1} was used to ensure proper mixing and aeration. Air was introduced through air stones placed at the bottom of the bioreactor, maintaining dissolved oxygen (DO) levels between 3–5 mg L^{-1}. The hydraulic retention time (HRT) was set at 24 h. At the end of each cycle, one hour was allocated for sedimentation, after which 50 % of the supernatant was drained and replaced with fresh synthetic NCT-substrate mixture. The basic composition of the medium in the bioreactor maintained a COD:N:P ratio of 100:5:1, with glucose (as co-substrate carbon source), NH_4Cl ($N–NH_4^+$) as the nitrogen source, and KH_2PO_4 ($P–PO_4^{3-}$) as the phosphorus source. The initial biomass concentration, MLSS (mixed liquor suspended solids), was 3±0.5 g L^{-1}. The ratio value of MLVSS/MLSS was about 87 %. During the acclimation process, the glucose concentration was gradually decreased by raising the concentration of NCT as carbon source. The COD concentration of the substrate was kept constant. Effluent NCT, COD and MLSS concentrations were measured regularly.

Comparative performances of fresh and acclimated sludges for NCT removal

Comparative trials were conducted simultaneously with acclimated and raw activated sludge to assess the benefits of acclimation sludge on NCT degradation. NCT was allocated as the sole carbon source to both SBRs (500 mg L^{-1} NCT). MLSS were maintained at 3±0.5 g L^{-1} in each SBR.

Identification of isolated NCT-degrading microorganisms

To identify the NCT-degrading microorganisms, 1 mL of the acclimated activated sludge was first diluted from 10^{-1} to 10^{-10} using autoclaved saline (0.8 % NaCl), and then distributed in nutrient agar plates containing 5 g L^{-1} peptic digest of animal tissue, 5 g L^{-1} sodium chloride, 1.5 g L^{-1} beef extract, 1.5 g L^{-1} yeast extract, and 15 g L^{-1} agar. The samples were then incubated for 24 h at 30 ºC. Nutrient agar streak plates were used to isolate the colonies, and two different isolated strains were selected for bacterial identification using 16S rRNA sequencing. Total DNA was extracted and purified using a modified Marmur method[36]. A PCR reaction was performed utilizing universal primers 27F and 1492R. The resulting PCR products were sequenced using the Applied Biosystems 3730/3730xl DNA Analyzer (Bioneer, Korea). The obtained sequences were assembled and edited using Chromas Pro version 1.5 software. The nucleotide sequences were then compared with existing sequences in the NCBI GenBank database (https://blast.ncbi.nlm.nih.gov).

Reactor setup and operation for aerobic granulation

Optimum cycle time

In order to determine the optimum cycle time for aerobic granulation, three 1000 mL graduated cylinders (42 cm in height and 7 cm in diameter, *H*/*D* ratio of 6) were used as column-type sequencing batch reactors (AGSBR1, AGSBR2, and AGSBR3), which were operated in parallel. The influent was added from the top of the reactor, and the effluent was discharged at a volumetric exchange ratio (VER) of 50 %. Each reactor was seeded with acclimated activated sludge taken from the previous step, at an initial biomass concentration of 3±0.5 g L^{-1} MLSS. The AGSBRs were supplemented with $CaCl_2$ and $MgSO_4$ at 40 mg L^{-1} of Ca^{2+} and Mg^{2+}, respectively. Detailed experimental conditions are provided in Table 1. The organic loading rate (OLR) in terms of COD was kept constant in the bioreactors. The settling time was gradually reduced from 10 to 1 minute over three operational phases, increasing aeration time to maintain the cycle time. The particle size distribution was determined using the wet sieve separation method as proposed by Laguna *et al.*[37] The bioreactors were maintained at ambient temperature (20 ± 1 ºC) and fed with a glucose-based SWW. Sludge volume index (SVI), MLSS, and COD parameters were measured regularly.

Table 1 – *Detailed experimental conditions for AGSBRs*

Bioreactor	Cycle (h)	Influent COD (mg L^{-1})	Feeding (min)	Aeration (min)	Settling (min)			Decanting (min)	Exchange ratio (%)
					Phase 1 (Day 1–7)	Phase 2 (Day 7–21)	Phase 3 (Day 21-onwards)		
AGSBR1	6	500	1	348–357	10	5	1	1	50
AGSBR2	12	1000	1	708–717	10	5	1	1	50
AGSBR3	24	2000	1	1428–1437	10	5	1	1	50

Aerobic granules' NCT removal ability

To investigate the effect of nicotine concentration on aerobic granules, the bioreactor was inoculated with 300 mL aerobic granules from the optimum feed cycle, and a feeding period with nicotine was performed on the granules. The hydraulic retention time (HRT) was 24 h. To prevent shock from changing the food source and the feeding cycle, glucose was gradually removed from the feed over one week, and nicotine was introduced into the system at a concentration of 1000 mg L^{-1} as the sole carbon source. This nicotine concentration, twice that used during the adaptation phase, was chosen to evaluate aerobic granules' resistance to shock. Effluent COD concentrations were analyzed frequently.

Chemical and statistical analysis

NCT concentrations in all liquid samples were analyzed using high-performance liquid chromatography (HPLC) via a Perkin Elmer Flexar series (USA), (5 µm d, 250 × 4.6 mm L.D.), an Agilent C18 column, and a UV detector operating at a wavelength of 254 nm. NCT concentration was analyzed using 100 % methanol as the mobile phase at a flow rate of 1.0 mL min^{-1} with an injection volume of 20 µL at 35 °C. COD, SVI, MLVSS, and MLSS concentrations were determined following the procedure outlined in the Standard Method[38]. The internal microbial morphology of granules was examined using a scanning electron microscope (SEM, Philips XL30, Germany).

Results and discussion

Acclimation process

In the experimental procedure, the bioreactor was fed daily with a NCT-substrate mixture. As shown in Figs. 1 and 2, the medium was refreshed weekly, with the NCT concentration incrementally increased from 0 to 500 mg L^{-1} while the glucose concentration was simultaneously decreased from 500 to 0 mg L^{-1}. Changes in the amount of MLSS with varying influent concentrations of NCT are shown in Fig. 1. Initially, a significant increase in MLSS was observed due to the presence of glucose. However, at NCT concentrations of 200 and 375 mg L^{-1}, severe declines in MLSS occurred. This result, as reported by Liu *et al.*[39], can be attributed to the toxic effects of nicotine, which can impair the metabolic system of activated sludge and reduce microbial activity. The increase in nicotine concentration surpassed the tolerance threshold of some microorganisms, possibly leading to the removal of those unable to degrade nicotine, thereby causing the observed decrease in MLSS.

As the microbial community gradually adapted to NCT, the decline in MLSS stabilized and eventually began to increase at a concentration of 500 mg L^{-1}. Effluent COD and NCT concentration values, along with removal efficiency rates during the acclimation process, are illustrated in Fig. 2. At lower concentrations of NCT, the removal rates were notably high. However, at higher concentrations of NCT, the COD content of the bioreactor did not decrease significantly. As seen in Fig. 2(a, b), with the injection of 200 and 400 mg L^{-1} NCT, both COD and NCT removal efficiency dropped sharply. To mitigate shock and allow more time for microbial adaptation, the rate of increase in NCT concentration was temporarily slowed. Ultimately, the bioreactor achieved a more stable state, and with the elimination of glucose from the feed, 500 mg L^{-1} of NCT was provided as the sole carbon source. The removal rates of NCT and COD reached 99.81 % and 93.80 %, respectively.

Occasionally, during the degradation process, the color of the substrate changed, starting from light yellow and turning green, and eventually becoming deep brown at high concentrations of NCT. This spectrum of color change has been previously reported[23]. However, a blue pigment was sometimes observed in the substrate, which became more pronounced as the concentration of NCT increased. Previous studies have noted the production of blue pigment by microorganisms. For example, A. *nicotinovorans* strain metabolized nicotine to 2,3,6-trihydroxypyridine, which produced a blue pigment in the presence of oxygen and is known as blue nicotine[40]. Similarly, the strain *Nocardioides* sp. JS614,

from the *Actinomycetales* family follows a comparable pathway in the biodegradation of NCT[41]. However, the enzymes and genes related to nicotine catabolism in many bacterial strains have not yet been identified. The diversity of the microbial population in activated sludge and the different colors observed at the end of the degrading process suggest that various microbial strains present in the acclimated activated sludge contribute to nicotine degradation.

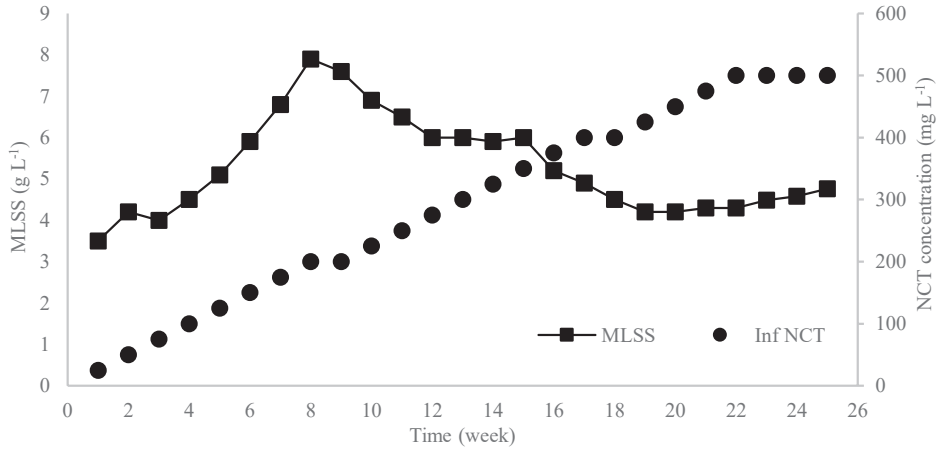

Fig. 1 – *Variations in MLSS at different influent NCT concentrations*

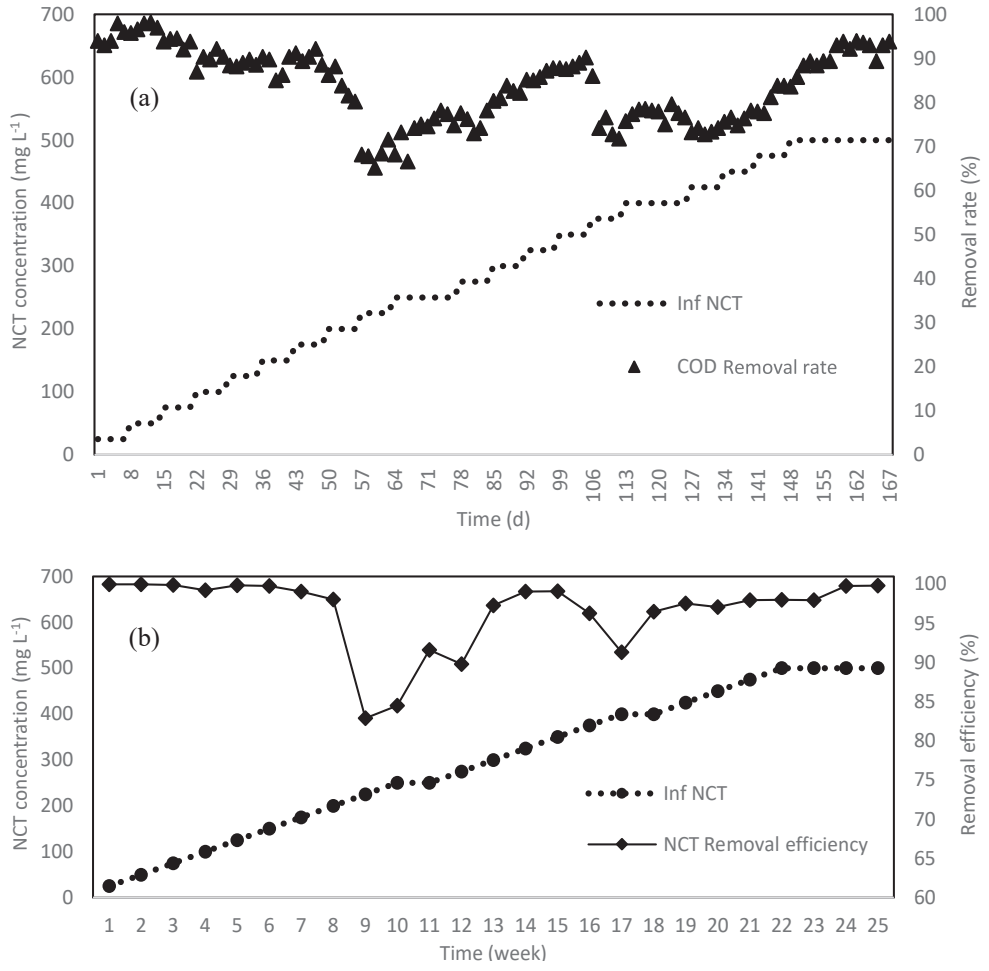

Fig. 2 – *Removal rate changes: a) COD removal rate and b) NCT removal rate at different influent NCT concentrations*

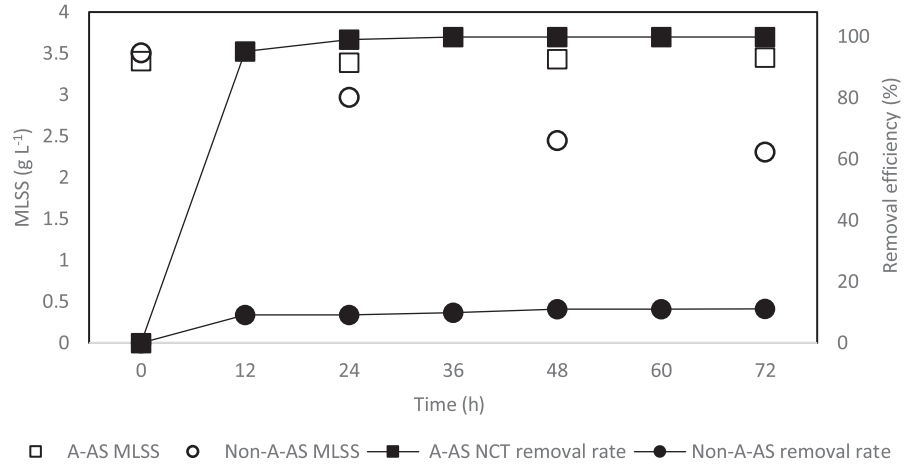

Fig. 3 – *Comparative performances of non-acclimated activated sludge (Non-A-AS), and acclimated activated sludge (A-AS) for NCT removal*

Comparative performance of fresh and acclimated sludge for NCT removal

To investigate the influence of adaptation on the biodegradation of NCT, experiments were conducted with an initial NCT concentration of 500 mg L^{-1} as the sole carbon source. The removal profiles of NCT and COD for both SBRs over 72 h are illustrated in Fig. 3. As shown, fresh sludge biodegraded 9.20 % NCT in 24 h, whereas the system with acclimated microorganisms achieved 99.02 % biodegradation in the same period, reaching 99.86 % after 48 h. However, beyond 48 h, biodegradation ceased in both bioreactors, and the removal rate of the non-adapted bioreactor only slightly improved to 11.02 %, likely due to the applied shock. These results indicate that the adaptation of activated sludge with NCT significantly enhances its performance, reducing the biodegradation time. Additionally, the acclimated activated sludge maintained the MLSS at a level similar to the initial value, without a decrease, while fresh sludge exhibited a reduction of approximately 1.2 g L^{-1} in MLSS over a 72-h period. Liu *et al.*[39] also compared the efficiency of glyphosate removal by adapted sludge and fresh sludge, observing similar results to this study.

In conclusion, the acclimation process significantly improved the pollutant removal efficiency, and after adaptation, enabled microorganisms to effectively degrade wastewater containing NCT.

Isolation and identification of NCT-degrading strains

The acclimated activated sludge contained a total of 10^{7-8} CFU mL^{-1} colonies per plate. Among the bacterial species capable of degrading nicotine, two strains were identified. The PCR products were sequenced, and the sequences of related species were compared with existing sequences in the NCBI GenBank database. The 16S rRNA analysis revealed that one of the candidate strains (1290 nucleotides in length) was phylogenetically closest to *Paenarthrobacter nitroguajacolicus* G2-1(T) (99.4 % 16S rRNA similarity) and *Paenarthrobacter aurescens* NBCR 12136(T) (99.3 % 16S rRNA similarity). The other isolated strain (1360 nucleotides in length) showed sequence similarity to *Bosea vestrisii* 34635T and *Bosea eneae* 34614T (99.7 and 99.6 % 16S rRNA similarity, respectively). Therefore, these two strains were classified within the families *Bosea* sp. and *Paenarthrobacter* sp., both rod-shaped and aerobic, with *Bosea* sp. being gram-negative[42] and *Paenarthrobacter* sp. being gram-positive[43]. The GenBank accession numbers for the 16S rRNA gene sequences of *B. vestrisii* 34635T, *B. eneae* 34614T, *P. nitroguajacolicus* G2-1(T), and *P. aurescens* NBCR 12136(T) are AF288306, AF288300, AJ512504, and BJMD01000050, respectively.

Granulation discussion

Properties of granules cultivated with different cycle times (optimum cycle time)

Seed sludge underwent changes in all AGSBRs over time. In AGSBR1, granulation began after the 4th day (16th hydraulic cycle), and larger, round granules formed within a week. In contrast, AGSBR2 and AGSBR3 only developed fragile granules at their 14th and 7th hydraulic cycles, respectively. As described in Table 1, the granulation process was conducted in three operational phases. At the start of each phase, the reduction in settling time led to sudden drops in MLSS due to biomass washout. However, as granulation commenced, MLSS levels increased. Notably, the early onset of primary granulation in AGSBR1 resulted in less severe washout

compared to the other reactors, thus minimizing biomass loss. Eventually, large granules (2–4 mm), dark brown in color, had formed in AGSBR1, and most of the sludge transformed into small yellow granules by the end of phase 2. In phase 3, these small granules grew larger and more mature, becoming uniformly sized, dense, round-shaped and yellow in color. By the end of phase 3, AGSBR2 and AGSBR3 contained mostly floc sludge, with irregularly shaped granules that lacked desirable settling ability compared to AGSBR1. AGSBR3 predominantly formed large, fragile granules. The sludge volume index (SVI) was used to assess the density and settling ability of the sludge. A complete granulated system typically achieves an SVI_{30}/SVI_5 ratio of 1.0 or a fraction less but close to 1.0 [33,34,44]. As seen in Fig. 4, after 55 days of cultivation, AGSBR1 reached a SVI_{30}/SVI_5 ratio of 1.0, indicating complete granulation. By the end of phase 3, AGSBR2 and AGSBR3 had SVI_{30}/SVI_5 ratios of 0.81 and 0.78, respectively. This suggested that the granules in AGSBR2 and AGSBR3, despite their large size, did not exhibit optimal settling ability. Aerobic granulation with a 6-h cycle was significantly faster compared to 12-h and 24-h cycles. As seen in Fig. 5, the granules from each bioreactor displayed varying colors and appearances, likely due to differences in feast and famine periods. Liu *et al.* also observed that darker granules form under conditions of prolonged starvation[45].

Additionally, Fig. 6 shows the growth of filamentous bacteria on the outer surface of the granules formed in AGSBR3 and, to a lesser extent, in AGSBR2, while AGSBR1's granules were completely smooth and round. According to Li *et al.*[46], filamentous bacterial growth on the granule surface can impair sedimentation ability of the granule, explaining the lower settling abilities observed in AGSBR2 and AGSBR3.

To gain a comprehensive understanding of the granules cultivated in AGSBR1, at the end of the granulation process, SEM images (Figs. 7a-d) were taken at varying magnifications. The first image (Fig. 7a) provided qualitative insights into the compactness and surface characteristics of the granules. The second image (Fig. 7b) was taken for a closer look at the specific surface areas of the granules. Further magnification (Fig. 7c-d), revealed that, while filamentous bacteria were present, the surface predominantly featured coccoid bacteria.

Diameter distribution of aerobic granules

The diameter of aerobic granules as an important characteristic that indicates growth and maturity. The diameter distributions of aerobic granules

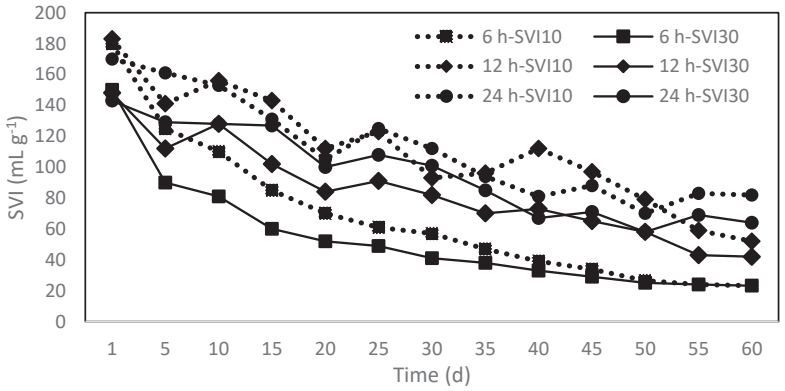

Fig. 4 – *SVI profile in the AGSBRs*

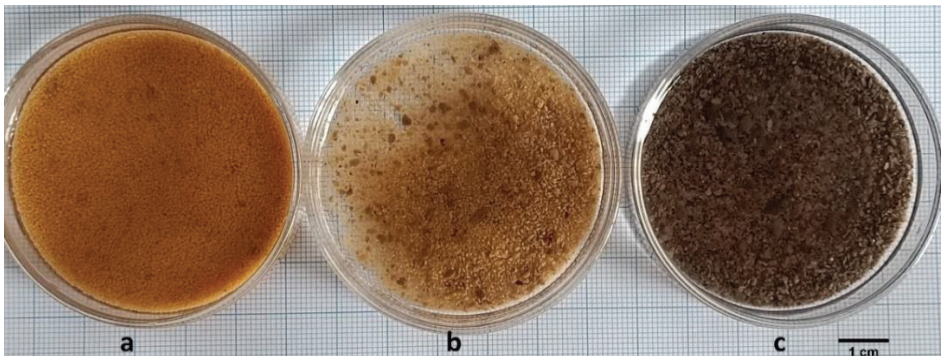

Fig. 5 – *Photographs of aerobic granules on day 60 a) 6-h cycle, b) 12-h cycle, and c) 24-h cycle*

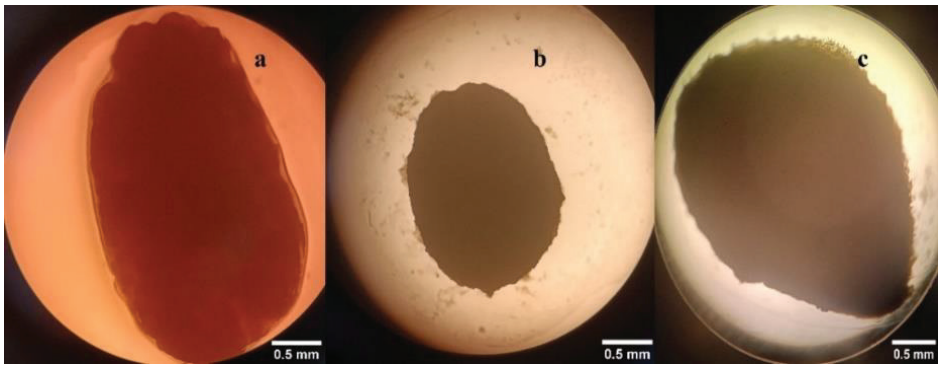

Fig. 6 – *Image analysis photographs of granules in AGSBRs: a single granule of a) 6-h cycle, b) 12-h cycle, c) 24-h cycle*

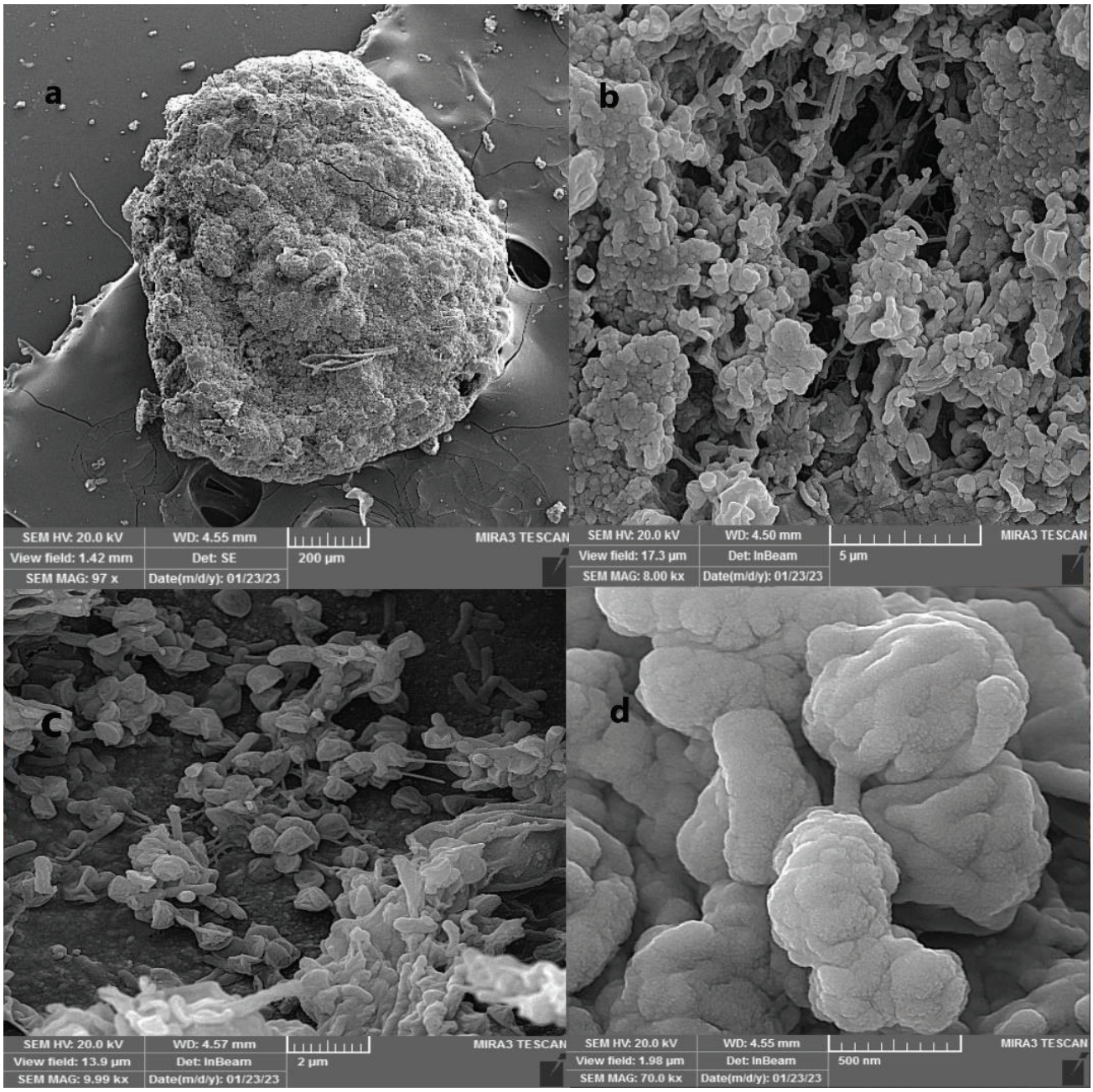

Fig. 7 – *SEM images of a granule cultivated in AGSBR1 at different magnifications (a) 97 x, b) 8.0 kx, c) 9.9 kx, d)70 kx mag.)*

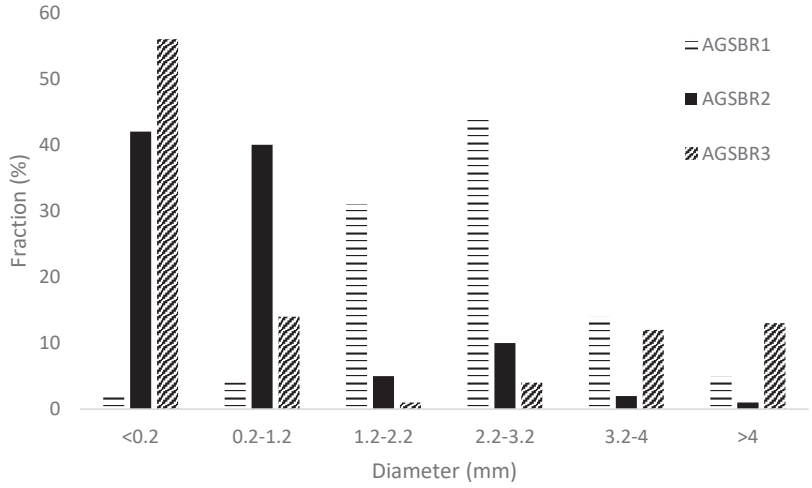

Fig. 8 – *Diameter distribution of granules*

cultivated in all bioreactors are presented in Fig. 8 (a, b and c). In AGSBR2 and AGSBR3, the granules predominantly ranged from 0.2 to 1.2 mm, and above 3.2 mm, respectively, although these sizes represented a small percentage of the total biomass. According to previous studies, the minimum size for biomass to be considered granules is 0.2 mm; biomass smaller than this size was considered unconverted[34,47]. The presence of both small and large aerobic granules in AGSBR2 and AGSBR3 indicates ongoing granular growth even after 60 days of establishment. While some granules in these two bioreactors were large, they were generally loose, fragile, and unstable. A significant portion consisted of small granules, which were likely to increase in size over time. In contrast, the majority of the granules formed in AGSBR1 measured between 1.2–3.2 mm. The amount of unconverted sludge or small granules in this bioreactor comprised a low percentage of its total biomass concentration. A significant percentage of the granules formed in AGSBR1 were in a more uniform size range compared to the granules in the other two AGSBRs, likely due to the optimal operating conditions maintained in AGSBR1.

NCT removal ability of aerobic granules

The granules developed in AGSBR1 were used for this section. A 24-h HRT was considered for NCT removal. Initially, to prevent shock, a concentration equal to one-quarter of the adapted NCT levels, along with glucose, was introduced into the system for a brief period. Gradually, glucose was removed from the feed, and NCT was supplied to the system as the sole carbon source. Due to the increase in NCT concentration and changes in HRT, which reduced the organic loading rate from 2 kg COD m^{-3} d^{-1} to 0.5 kg COD m^{-3} d^{-1} compared to the granulation stage, the granules' size decreased to some extent. However, the granular state of the sludge was well-preserved, with no noticeable changes in SVI. The bioreactor achieved removal rates of 99.83 % for NCT and 94.90 % for COD with a feed of 500 mg L^{-1} NCT over 24 h. Subsequently, the nicotine concentration was increased by 100 mg L^{-1} daily, reaching a concentration of 1000 mg L^{-1} (twice the adapted concentration) to assess the strength and shock tolerance of the contaminant-loaded granules. The system's performance, evaluated in terms of COD removal efficiency, initially decreased as the nicotine concentration increased, but reached an acceptable COD removal efficiency level after two weeks. Despite the nicotine concentration being double the acclimatized concentration, the aerobic granules reduced COD by 91.02 % without requiring a prolonged acclimatization period. With the adaptation of activated sludge to nicotine, the nicotine-degrading microorganisms, including the identified strains, persisted and proliferated in the sludge. After granule formation and doubling of the nicotine concentration, the granules efficiently removed nicotine without additional adaptation. This suggested that, during the granulation process, nicotine-degrading strains became more tolerant to nicotine toxicity. This finding underscores the high potential of aerobic granules for removing toxic substances and their substantial resistance to shock. Liu *et al.* compared the effect of phenol toxicity on aerobic granules and activated sludge by examining microbial activity levels with increasing phenol concentrations[48]. They observed that at a phenol concentration where 50 % of microbial activity was inhibited, the granular structure protected microbial cells against phenol toxicity, especially during longer exposure. They also attributed the increased tolerance of the granules to toxicity to diffusional resistance, noting that granules with a larger diameter can protect more microorganisms within their inner portion from the toxic substance[48].

Conclusions

This study explored the adaptation of activated sludge to NCT using a strategy that involved the addition of glucose as a co-substrate and gradually increasing the influent NCT concentration, culminating in the granulation of the acclimated activated sludge. The key findings are as follows:

– The adaptation process significantly increased the biodegradability of NCT in activated sludge.
– Through 16S rRNA analysis, *Bosea* sp. and *Paenarthrobacter* sp. were identified as potential NCT-degrading bacteria under long-term acclimation conditions, for future use.
– In a hydraulic circulation time of 6 h, the granules achieved an average diameter of 2–3 mm with a well-defined round outer shape, and exhibited high strength. In contrast, in cycles of 12 and 24 h, a significant portion of the sludge did not convert into granules. Granulation of the sludge significantly affected its settling ability.
– The tolerance of microorganisms to toxic substances shock was notably increased in the granulated state compared to the activated sludge. The granules removed nicotine at a concentration of 1000 mg L^{-1} (double the concentration used in the adaptation stage) with an efficiency of 91.02 %.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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