Characterization of biofilm of a rotating biological contactor treating synthetic wastewater

V. Singh and A. K. Mittal

ABSTRACT

A four-stage rotating biological contactor (RBC) was designed and operated to treat synthetic wastewater containing 1,000 mg/l chemical oxygen demand (COD) and 112 mg/l NH₄⁺-N. A mixed culture bacterial biofilm was developed consisting of a heterotrophic bacterium Paracoccus pantotrophus, nitrifiers and other heterotrophs. Applying the peculiar characteristics of P. pantotrophus of simultaneous heterotrophic nitrification and aerobic denitrification, high simultaneous removal of carbon and nitrogen could be achieved in the fully aerobic RBC. The microbial community structure of the RBC biofilm was categorized based on the nitrate reduction, biochemical reactions, gram staining and morphology. The presence of P. pantotrophus within the RBC biofilm was confirmed with an array of biochemical tests. Isolates from the four stages of RBC were grouped into complete denitrifiers, incomplete denitrifiers and non-denitrifiers. This categorization showed a higher relative abundance of P. pantotrophus in the first stage as compared with subsequent stages, in which other nitrifiers and heterotrophs were significantly present. High total nitrogen removal of upto 68% was in conformity with observations made using microbial categorization and biochemical tests. The high relative abundance of P. pantotrophus in the biofilm revealed that it could successfully compete with other heterotrophs and autotrophic nitrifiers in mixed bacterial biomass.

Key words | biofilm bacterial community, rotating biological contactor, wastewater treatment

INTRODUCTION

The rotating biological contactor (RBC) is an attached growth bioreactor that typically consists of a series of closely spaced discs that are mounted on a common horizontal shaft and are partially immersed in a tank through which wastewater flows. The shaft is continuously rotated by a mechanical motor and a biofilm is established on the entire surface area of the discs, which metabolizes the organic materials of the wastewater. As the attached biofilm comes out of the wastewater and gets exposed to atmospheric air, diffusion of oxygen into the biofilm takes place. When immersed in wastewater the organic matter and nutrients present in soluble form diffuse into the biofilm and the microorganisms consume the organic matter and oxygen for their cell growth and respiration.

In general RBC systems are intended to reduce organic carbon and ammonia by nitrification (Hoccheimer & Wheaton 1998). However, the system reported in this study was designed to perform simultaneous nitrification and denitrification (SND) by developing a mixed bacterial biofilm containing Paracoccus pantotrophus (Rainey et al. 1999), a heterotrophic nitrifier and aerobic denitrifier capable of removing both C and N under fully aerobic conditions. The bacterium has a respiratory metabolism and can use oxygen, nitrate, nitrite or a nitrogen oxide as the terminal electron acceptor making it capable of converting most of its oxidation products to gaseous nitrogen products (Robertson & Kuenen 1983, 1988). Conventionally in the process of removal of soluble organic matter, the organic matter serves as the electron donor and oxygen acts as the electron acceptor. When nitrification is the objective, NH₄⁺-N serves as the electron donor and oxygen again serves as the electron acceptor. And when denitrification is the objective NO₃⁻-N serves as the terminal electron acceptor and some form of organic matter generally serves as the donor. Unlike the
The conventional process the simultaneous use of oxygen and nitrate/nitrite co-respiration exist in aerobic denitrification (Schmidt et al. 2003). Electrons flow simultaneously to the denitrifying enzymes as well as to oxygen, thus the denitrification might occur in an aerobic environment (Robertson et al. 1988; Huang & Tseng 2001).

To optimize the removal of organic matter and nitrogen compounds from wastewater in a RBC, an adequate understanding of the nature and characteristics of the biofilm is essential. The aims of this study were therefore to characterize the RBC biofilm in order to confirm the presence and relative abundance of *P. pantotrophus* in the biofilm along the different stages of RBC as well as to determine whether it can compete with other microbes in the mixed bacterial biofilm or not. In addition to detailing the other bacteria contributing to denitrification under aerobic condition, the effect of substrate availability on the microbial structure of the biofilm is also studied.

### MATERIALS AND METHODS

#### Schematics of the experimental setup

A four-stage RBC comprising four circular plates of acrylic sheets, 12 cm in outer diameter and 0.5 cm thickness was used. These discs were spaced at 2.5 cm and their outer surface was roughened so as to provide better adherence to the microbial film. The shaft was rotated at 2.5 rpm with a motor and reduction gear system. The RBC reactor vessel was fabricated out of stainless steel 2 mm thick. The total volume of the trough was 4.9 l. The direction of flow of wastewater in RBC was perpendicular to the discs. A schematic of the experimental set up is shown in Figure 1.

#### Composition of synthetic wastewater

Wastewater was prepared with glucose and acetate as carbon sources and ammonium chloride as a nitrogen source. The concentrations of carbon and nitrogen were maintained to simulate domestic wastewater (Stover & Kincannon 1975). Characteristics of the synthetic feed are presented in Table 1.

#### Development of the biofilm and the reactor operation

The bacterial strain *P. pantotrophus* (DSM 2944) was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) GmbH, Braunschweig, Germany. The isolate was cultivated in DSM medium 356 (DSMZ 1989). The biofilm development on acrylic plates was achieved using a high-strength medium twice as concentrated as the above composition and running the reactor in batch mode. A thin microbial film had

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>COD</td>
<td>1,000</td>
</tr>
<tr>
<td>NH₄⁺-N</td>
<td>112</td>
</tr>
<tr>
<td>C/N</td>
<td>3.32</td>
</tr>
<tr>
<td>pH</td>
<td>7.4</td>
</tr>
<tr>
<td>Alkalinity (as CaCO₃)</td>
<td>300</td>
</tr>
</tbody>
</table>

*aAll values in mg/l except pH.*

---

![Figure 1](https://iwaponline.com/wst/article-pdf/66/2/429/4429403/429.pdf)
formed on the plate in 1 week. After 20 days, continuous feeding was started. Hydraulic retention time (HRT) was kept at 2 days. Fresh liquid culture of *P. pantotrophus* was added thrice a week in the RBC trough during the biofilm development. Thereafter no addition of pure culture was made to the system. After substantial development of biofilm, the reactor was switched to the composition shown in Table 1. HRT was changed to 1 day.

**Sampling and analysis**

Effluent samples collected from four stages of RBC were analysed for various parameters according to APHA (1998). All parameters were monitored daily. Ammonia-nitrogen was measured by the kjeldahl distillation method, nitrate nitrogen by nitrate electrode (Thermo-Orion) and COD by the closed reflux method. Dissolved oxygen (DO) was measured using DO probe (Hach), pH by a pH meter and alkalinity by titrating against a standard H₂SO₄ solution.

**Biofilm sampling and isolation of microorganisms**

For determination of microbial characteristics biofilm samples were taken from the outermost discs of each stage and at different depths of the biofilm (average biofilm thickness, 5.33 mm) which can be broadly categorized as surface, middle and bottom layers of the biofilm. Primary isolation was effected by streaking microbial sample on the surface of a dry nutrient agar (HiMedia) plate. Such plates were then incubated at 32 °C for 36 h. Single colonies were removed from these plates and sub-cultured on new nutrient agar plates. This procedure was repeated to obtain pure cultures. For isolation of *P. pantotrophus*, cultures were streaked on DSM medium 356 (DSMZ 1989).

**Identification of dominant microorganisms**

Dominating microorganisms were identified by comparing their relative abundance, which was determined on the basis of denitrification capabilities of various isolates. The microbial isolates collected from all the four stages of RBC were subjected to nitrate reduction tests (Cappuccino & Sherman 1992) to evaluate the abundance of denitrifiers along the four stages of RBC. On the basis of their denitrification capability, the isolates were further categorized into three groups: completer denitrifiers (CDN), incomplete denitrifiers (IDN) and non-denitrifiers (NDN). Dominating microorganisms were then identified from the group of denitrifiers (CDN + IDN) by comparing their relative abundance within the group.

**Screening for denitrification**

To assess the nitrate reduction capacities of all the bacteria isolated, monocultures were screened in triplicate for nitrate reduction using the nitrate broth comprising of 2 g/l potassium nitrate (KNO₃), 1 g/l meat extract, 2 g/l yeast extract and 5 g/l each of peptone and ammonium chloride (NH₄Cl). Microbial culture tubes containing 4 ml nitrate broth were incubated at 32 °C for 5–7 days. Nitrate reduction by each of the isolates was determined using alpha-naphthylamine and sulphamic acid colour reagent as prescribed by APHA (1998). The colourimetric biochemical reduction test was used to screen for nitrate and nitrite reduction (Cappuccino & Sherman 1992).

After nitrate reduction screening, dominant bacterial isolates were subjected to microbial characterization tests using biochemical test arrays API 20 E (BioMérieux, Marcy l’Etoile, France) and HiMedia test kit number KB008 and KB011. Morphological and physiological

---

### Table 2 | COD and nitrogen removal efficiencies along successive stages of RBC

<table>
<thead>
<tr>
<th>RBC Stage</th>
<th>COD (mg/l)±</th>
<th>NH₄-N (mg/l)±</th>
<th>Total N (mg/l)±</th>
<th>NO₃-N build-up (mg/l)±</th>
<th>NO₂-N build-up (mg/l)±</th>
<th>DO (mg/l)±</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>86.94 ± 3.76</td>
<td>59.82 ± 12.85</td>
<td>54.91 ± 15.74</td>
<td>1.77 ± 1.59</td>
<td>2.64 ± 1.82</td>
<td>0.46 ± 0.55</td>
</tr>
<tr>
<td>2</td>
<td>89.89 ± 2.65</td>
<td>65.87 ± 14.25</td>
<td>60.88 ± 15.32</td>
<td>2.16 ± 1.68</td>
<td>3.41 ± 1.88</td>
<td>0.95 ± 1.38</td>
</tr>
<tr>
<td>3</td>
<td>90.99 ± 2.42</td>
<td>76.52 ± 14.36</td>
<td>68.82 ± 14.16</td>
<td>4.28 ± 1.85</td>
<td>4.42 ± 1.33</td>
<td>1.56 ± 1.40</td>
</tr>
<tr>
<td>4</td>
<td>91.88 ± 2.16</td>
<td>84.77 ± 15.23</td>
<td>74.58 ± 12.65</td>
<td>5.57 ± 1.89</td>
<td>5.01 ± 1.52</td>
<td>1.81 ± 1.44</td>
</tr>
</tbody>
</table>

*Values represent average and standard deviation during steady state days.*
characterization of selected isolates was also carried out as per APHA (1998).

RESULTS AND DISCUSSION

Performance and nutrient removal characteristics of RBC

As described above, the RBC was designed and operated to remove both carbon and nitrogen from synthetic wastewater (Table 2). Most of the carbon was consumed in the first stage. The COD removal efficiency in the first stage remained at 86.94 ± 3.76% for the corresponding loading rate of 1,000 mg/l COD. The NH4+-N removal efficiency in this stage reached 59.82 ± 12.85% for nitrogen loading of 112 mg/l. The ratio of COD removed to NH4+-N removed was 11.5 in this stage. A similar ratio (13) was obtained by Gupta & Gupta (1999) while treating synthetic wastewater in a three-stage RBC. Very low nitrate build up of 2.64 ± 1.82 mg/l along with total nitrogen removal rate of 54.91 ± 15.74% in the first stage indicates a high SND rate. There was further removal of COD (5–10%) and NH4+-N (22–28%) in subsequent stages with an overall COD removal efficiency of 91.88 ± 2.16% and NH4+-N removal efficiency of 84.77 ± 15.23% as observed in the final effluent. Nitrate build up of 5.01 ± 1.52 mg/l and an overall nitrogen removal of 74.58 ± 12.65% in the final effluent indicates the aerobic denitrification along with significant nitrification in the system.

In an RBC, as the wastewater flows through the system, each subsequent stage receives an influent with an organic concentration lower than the previous stage. Conventionally, because of low growth rate of nitrifiers, the first stage tends to be an organic removal device, unless the wastewater organic carbon is very low (Cortez et al. 2008). However, P. pantotrophus being a heterotrophic nitrifier with a much higher growth rate than those of autotrophs is at competitive advantage (Robertson et al. 1988). Thus, a substantial 54.91 ± 15.74% total N removal along with 86.94 ± 3.76% COD removal in the first stage of RBC can be attributed to the heterotrophic nitrification and aerobic denitrification performed by P. pantotrophus.

In this study the second, third and fourth stages were able to remove a substantial part of remaining organics and nitrogen emanating from the first stage, resulting in an overall higher COD and N removal efficiency than those previously reported for RBC systems (Gupta & Gupta 1999, 2001; Lee et al. 2008). This situation can be attributed to batch development of biofilm which resulted in the presence of P. pantotrophus in all the stages. The overall COD removal efficiency (91.88 ± 2.16%) was comparable with the values obtained by Chen et al. (2006) while simultaneously removing C and N in a RBC.

Relationship between biofilm growth and nutrient removal characteristics

Various observations of RBC biofilm are presented in Figure 2. The external appearance of biofilm was clearly distinctive among the four stages of RBC. As described above, the start-up of the RBC unit was initiated by inoculating the synthetic wastewater with culture of P. pantotrophus while the reactor was operated in batch mode to provide the initial colonization and accumulation of microorganisms. A thin biofilm in the form of light brown coloration of the discs appeared in 3 days. Over a period of 1 week a uniform thin growth of biofilm on the entire disc surface was observed and a fast growth of the biofilm was observed after that. Appearance of biofilm after 1 week can be seen in Figure 2(a). After 20 days, substantial biofilm was developed and reactor was switched to continuous mode. Soon after, the reactor was run in continuous mode, a difference in the appearance of biofilm of the four stages started to appear. Figure 2(b) shows the appearance of biofilm in continuous mode operation of RBC. Biofilm in all four stages appeared compact with maximum thickness in the first stage and became thinner in subsequent stages. Biofilm in the first stage was off-white in color while it grew yellowish brown in later stages.

Figure 2(c) shows luxuriant growth of biofilm in the first stage of RBC. It was also observed that COD removal efficiency decreased after the biofilm got sloughed off (Figure 2(c)). In the first stage it decreased to less than 85% COD removal from an initial level of above 90% COD removal. However, with high substrate availability, the thickness of biofilm again increased as can be observed in Figure 2(d). Before getting thicker as shown in Figure 2(c) and (d), some white zones started to appear in the biofilm on the disc surfaces as indicated by arrow in Figure 2(e). A possible explanation of the bulk growth of biofilm in the first stage may be due to the possible presence of filamentous bacteria as indicated by the white zones. Filamentous bacteria proliferate on the biofilm surface due to their ability to grow under aerobic conditions. However, their affinity to low oxygen and nitrate concentrations may be the reason of their possible development in the first stage of RBC (Saikaly & Ayoub 2005; Ramothokang et al. 2006; You & Ouyang 2007).
Biofilm characteristics and microbial communities

The main objective of biofilm characterization was to determine the presence and relative abundance of *P. pantotrophus* in the RBC biofilm as the process was developed to perform both simultaneous C and N removal under fully aerobic conditions by employing the typical heterotrophic nitrification and aerobic denitrification capabilities of the bacteria. Another aim was to determine the presence of other denitrifiers contributing to the overall N removal efficiency of the RBC. Microbial cultures (21–25) were isolated from each stage of the RBC. A total of 92 isolates were collected from four stages. These were subjected to a nitrate reduction test. Based on the results of the nitrate reduction screening the isolated bacteria were characterized into specific groups.

Figure 2  Appearance of various growth stages of RBC biofilm during the course of reactor operation. (a) After 1 week of start-up in batch mode; (b) after 1 month of start-up in continuous mode; (c) sloughing of biofilm upper layer in first stage; (d) re-growth of sloughed biofilm in first stage; (e) white zones. 1, first stage; 2, second stage; 3, third stage; 4, fourth stage; white arrows, direction of wastewater flow.
delineating their capacity for nitrate reduction under aerobic conditions. These groups were classified as complete denitrifiers, IDN and NDN, depending upon their nitrate reduction capacity.

**Complete denitrifiers (CDN)**

Bacteria that were able to reduce nitrate completely into gaseous nitrogen without any build-up of nitrite were grouped as complete nitriﬁers. This characteristic of these bacteria can be attributed to the presence of nitrate as well as nitrite reductase enzymes (Cappuccino & Sherman 1992; Robertson & Kuenen 1992). Drysdale et al. (1999) termed these bacteria as true denitrifiers due to their capability of efﬁciently reducing nitrate and nitrite simultaneously with no apparent build up of nitrite as an intermediate. A total of 22.82% of the isolates was made up of complete denitrifiers.

**Incomplete denitrifiers (IDN)**

A total of 17.39% isolates was found to be IDN. These bacteria were only capable of reducing nitrates to nitrites. Thus nitrite would be the end product. Robertson & Kuenen (1992) attributed it to the lack of key nitrite reductase enzymes which enable true denitrifiers to reduce nitrites.

**Non-denitrifiers (NDN)**

It was found that 58.69% isolates was NDN. These isolates lack both nitrate and nitrite reductase enzymes. So these bacteria cannot carry out the denitrification (Drysdale et al. 1999).

Stage 1 (Table 3) showed the highest density of denitrifying bacteria with 47.62% as complete denitrifiers and 19.04% as IDN. A gradual decrease in the abundance of complete denitrifiers can be observed at successive stages, leading to a minimum of 8.7% in the fourth stage, whereas the quantity of non-denitrifiers continues to increase from 33.33% in first stage to 78.26% in the final stage. These results correspond with the observed variation in COD and N removal within stages of RBC. It can be considered that *P. pantotrophus* which is a heterotroph and other denitrifiers receive maximum influent carbon in the first stage supporting their high growth resulting in a high total nitrogen removal (54.91 ± 15.74%) along with carbon consumption of more than 90% (Table 2). The successive stages receive less carbon resulting in gradual decrease in denitrifiers’ population. On the other hand, after first stage, 19% of total N was removed while final stage contributed to only 3–4% of total N removal. These results are in agreement with a decreasing denitrifiers’ population which varied from 66.66% at the first stage to 22.13% in the final stage. In the case of initial high nitrate concentrations, Drysdale et al. (1999) has attributed nitrite build-up to the predominant presence of IDN. In the present study concentrations of nitrates in the first stage were very low. Besides, ratio of IDN to CDN was also low. Therefore, there was no significant build-up of nitrite in initial stages. It is evident from Table 3 that the IDN to CDN ratio continuously changed from 0.39 at first stage to 1.54 at final stage. This corresponds to the accumulation of nitrite along the successive stages due to the inability of IDN to reduce nitrite. Gupta & Gupta (2001) have reported a gradual build-up of nitrite nitrogen while treating domestic wastewater in a lab-scale RBC. In this study, bulky growth of biofilm was observed in the first stage of RBC (Figure 2) which can be attributed to the possible growth of filamentous bacteria. However, a study by Drysdale et al. (1999) indicated that filamentous bacteria can reduce nitrate at a rate similar or higher than

**Table 3 | Population and distribution of microbial communities along successive stages of RBC**

<table>
<thead>
<tr>
<th>RBC Stage</th>
<th>CDN (%)</th>
<th>IDN (%)</th>
<th>NDN (%)</th>
<th>Total denitrifiers (%)</th>
<th>Relative abundance of <em>P. pantotrophus</em> (%)</th>
<th>IDN/CDN</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>47.62</td>
<td>19.04</td>
<td>33.33</td>
<td>66.66</td>
<td>52</td>
<td>0.39</td>
</tr>
<tr>
<td>2</td>
<td>26.09</td>
<td>21.73</td>
<td>52.17</td>
<td>47.82</td>
<td>32</td>
<td>0.83</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>16</td>
<td>72</td>
<td>28</td>
<td>10</td>
<td>1.33</td>
</tr>
<tr>
<td>4</td>
<td>8.7</td>
<td>13.43</td>
<td>78.26</td>
<td>22.13</td>
<td>9</td>
<td>1.54</td>
</tr>
</tbody>
</table>

*Relative abundance of *P. pantotrophus* within the denitrifying microbial community.
heterotrophic bacteria. This may be an explanation of high total N removal in the first stage in spite of the possible growth of filamentous bacteria.

Morphological and biochemical characterization

Of the total 92 isolates, 43 isolates with denitrifying capability (CDN + IDN) were subjected to biochemical and morphological characterization. Out of these 43 isolates, 25 isolates were found to be *P. pantotrophus* while 18 isolates did not match completely the characteristics of *P. pantotrophus* (Table 4). On this basis, 58.14% of the total isolates subjected to biochemical and morphological characterization were *P. pantotrophus* (Table 3). Gram-negative cocci constituted the majority of bacteria and high similarities were found among many of these isolates with *P. pantotrophus*. Higher numbers of similar isolates were observed in the first stage compared with successive stages. Also, the majority of these are denitrifiers. It is possible that these isolates are mutant strains of *P. pantotrophus*. Although, the isolates other than *P. pantotrophus* could not be identified to their genus level, a fair relative abundance of *P. pantotrophus* could be confirmed in the RBC.

CONCLUSIONS

Analysis of the biofilm is important for understanding the treatment process within an RBC. Evaluation of microbial community structure is of utmost importance, especially...
when both nitrification and denitrification processes are combined. There have only been a few studies on the microbial community related to the treatment processes occurring within an RBC (Egli et al. 2003; Pynaert et al. 2003; Lee et al. 2008). However, these studies were based on non-culture-based methods. This study is the first one that examines denitrifying bacterial populations within an RBC using a culture-dependent method. The methodology proposed in the present study clearly evaluated the relative abundance of *P. pantotrophus* in different stages of RBC. Despite the limited sampling, the study clearly revealed the distribution pattern of denitrifiers along the successive stages of RBC.

The findings of this research validate that *P. pantotrophus* can efficiently compete with other heterotrophs. The COD and NH$_4^+$-N removal efficiencies at different stages and, a high overall C and N removal efficiency shows the capability of *P. pantotrophus* for simultaneous C and N removal in a mixed microbial biomass. The presence of denitrifiers as indicated by biochemical characterization agreed with the high total N removal efficiency of the RBC. Current findings also contribute towards the present understanding of the behaviour and applicability of *P. pantotrophus* for the treatment of wastewater in a mixed bacterial biofilm. Although current investigation focussed on determining the capability of aerobic denitrifiers for simultaneous removal of carbon and nitrogen, the findings also substantiates the ability of a mixed group of microbes to remove nutrients.

**REFERENCES**


Robertson, L. A. & Kuenen, J. G. 1992 *Nitrogen removal from water and waste*. In: *Microbial Control of Pollution* (J. C. Fry,


First received 25 December 2011; accepted in revised form 15 March 2012