BIOLOGICAL NITROGEN REMOVAL FROM WASTEWATER BY DENITRIFICATION OF MIX-CULTURING FUNGI AND BACTERIA

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Abstract: Denitrification has been long thought to be a unique characteristic of prokaryotes, but in recent years, several filamentous fungi and yeasts were found to exhibit denitrifying activities. This paper deals with the examination of denitrification capabilities by mix-cultures of the fungus (*Fusarium oxysporum*) and the bacterium (*Pseudomonas stutzeri* TR2) in combination with a specific medium and using a synthetic wastewater of defined quality. The results revealed that *P. stutzeri* TR2 has strong and fast denitrifying capabilities under anaerobic conditions, and that co-denitrification of mix-cultures with *F. oxysporum* and *P. stutzeri* TR2 was more effective to remove nitrate under limited oxygen conditions. *P. stutzeri* TR2 was able to remove nitrate completely during cultivation for 12 hr in the specific medium and in mixed culture with *F. oxysporum*. A rapid N_2 evolution by mixed culture with *F. oxysporum* and *P. stutzeri* TR2 was observed in both mixed culture medium and synthetic wastewater. Using synthetic wastewater with a defined composition, about 87% of the nitrate was eliminated to form about 420μmol of N_2 from 1.0mmol of NO_3^- by co-denitrification of *F. oxysporum* and *P. stutzeri* TR2 after incubation for 6days. In co-cultures of *F. oxysporum* and *P. stutzeri* TR2, N_2O produced by *F. oxysporum* was rapidly consumed by *P. stutzeri* TR2. This indicated that mixed culture of *F. oxysporum* and *P. stutzeri* TR2 can be used to remove nitrate and nitrite from wastewater effectively.

Key words: Denitrification; Mix-culturing; Fungi; Bacteria

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Denitrification is a biological process in which nitrate and/or nitrite is reduced to gaseous nitrogen, dinitrogen (N_2) or nitrous oxide (N_2O) while carbon dioxide is the second gaseous product of the process. This is one of the main mechanisms of the global nitrogen cycle, and plays an important role as the reverse reaction of nitrogen fixation in maintaining global environmental homeostasis[1]. Denitrification has been long thought to be a unique characteristic of prokaryotes[2,3]. A number of bacteria (such as *Pseudomonas stutzeri*) perform this process in which nitrate or nitrite acts as the ultimate electron acceptor for anaerobic respiration. This full denitrification system is comprised of four reducing steps from NO_3^- to N_2 involving NO_2^-, NO and N_2O as the intermediates.

In recent years, several filamentous fungi and yeasts were found to exhibit denitrifying capabilities[4-6]. Denitrification in fungi differs from those of bacteria, and are incomplete in that they evolve nitrous oxide (N_2O) instead of dinitrogen (N_2) as the final denitrification product and thus seem to lack N_2O reductase[7]. In addition, fungal nitric oxide reductase (Nor) is the cytochrome P450 type (P450nor) employing NADH or NADPH as the direct electron donor, while bacterial Nor is the cytochrome cb type receiving electrons from the respiratory chain via cytochrome c[8,9]. The fungal denitrification system is located at the respiring organelle, the mitochondrion, and at least a portion of the denitrifying process is associated with the respiratory chain coupling to the syn-

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thesis of ATP[10]. Recently, the quantitative relationship between the O_2 supply and the denitrifying activity of *F. oxysporum* was investigated. It was shown that the minimal amount of O_2 is necessary for efficient denitrification by fungus although the fungal denitrification is completely repressed by excess O_2[11].

Biological denitrification is at present the most effective process to remove fixed nitrogen pollutants from aqueous ecosystems. Fungi may denitrify under somewhat oxic conditions and simultaneously perform denitrification and aerobic respiration[12] while bacteria can reduce nitrate to nitrogen under anaerobic condition. Both denitrifiers have important potential application in wastewater remediation and other environment contamination problems caused by entrophication, or by nitrate/nitrite. Here, we report a new model of denitrification by mix-culturing fungi and bacteria and characterizing their denitrification capabilities that produce N_2 gas by reducing NO_3^- under oxic conditions. This new way will be useful in the application to the nitrogen removal from wastewater.

1 Materials and Methods

1.1 Microorganisms and culture condition *Fusarium oxysporum* MT-811[4] was the fungal denitrifier used in this study. *Pseudomonas stutzeri* TR2 was selected as the bacteria denitrifier, which is a new strain of *P. stutzeri* and can rapidly reduce nitrate to form nitrogen gas without nitrite accumulation[13]. *P. stutzeri* TR2 was grown in LB medium for the seed culture, then incubated in 500ml Erlenmeyer flasks with two side arms containing 150ml of liquid medium that consisted of 0.085% sodium nitrate (10mM), 0.472% sodium succinate, 0.5% casamino acid, 0.79% disodium hydrogenphosphate, 0.15% potassium dihydrogenphosphate, 0.01% magnesium sulfate, and trace solution[2], on a rotary shaker (120 rpm) at 30℃. The top and side arms of the flask were sealed with butyl rubber stoppers after inoculation. *F. oxysporum* was cultivated in liquid medium as described by Shoun and Tanimoto[4]. The seed culture was aerobically grown in 50ml test tubes containing 10ml of medium consisting of 1% glycerol, 0.2% peptone, 5mM sodium nitrate and inorganic salts[2]. After 3days, 10ml of pre-cultured cells were transferred into 500ml Erlenmeyer flasks with two side arms containing the mix-culturing medium (150ml for each) or the artificial wastewater (100ml for each) for mix-cultivation. All experiments were run in triplicate.

The mix-culturing medium for *F. oxysporum* and *P. stutzeri* TR2 were composed of the following (g/L): 10mM sodium nitrate (^{15}N-$NaNO_3$ 0.85g), glycerol 5.0g, ammonium chloride 0.53g, sodium succinate 2.36g, casamino acid 2.5g, disodium hydrogenphosphate 4.0g, potassium dihydrogenphosphate 1.48g, magnesium sulfate 0.15g, and trace solution 1.5ml, pH 7.2. The synthetic wastewater of the following composition was used: ^{15}N-$NaNO_3$ 0.85g (10mM), polypepton 0.6g, bouillon extract 0.4g, urea 0.1g, NaCl 0.03g, KH_2PO_4 0.1g, KCl 0.014g, $MgSO_4 \cdot 7H_2O$ 0.02g, $CaCl_2 \cdot 2H_2O$ 0.0185g, and tap water 1000ml. ^{15}N-$NaNO_3$ purchased from Shoko Co.

1.2 Gas analysis The upper space gas of cultivation flasks was analyzed by gas chromatography (GC) with a Shimadzu gas chromatograph GC 12 A and gas chromatography-mass spectrometry (GC-MS) with a Shimadzu gas chromatography-mass spectrometer GCMS-9000C. Each sample gas was taken off through the double butyl rubber stopper in the arms with a syringe and applied to analyses. The amount of products during denitrification (N_2O, N_2, $^{30}N_2$, $^{29}N_2$, $^{28}N_2$) and O_2 remained in the flask were detected[4,14].

1.3 Determination of nitrate and nitrite The concentration of nitrate and nitrite in the culturing medium were determined by using brucine-sulfate and a-naphthyethylenediamine respectively[15], or by using ion chromatography with a 761 Compact IC (Metrohm Co).

2 Results

2.1 Denitrification by mix-culturing F. oxysporum and P. stutzeri TR2 in the specific medium

F. oxysporum was pre-cultured for 3days and *P. stutzeri* TR2 was pre-cultured overnight. After that, 10ml of *F. oxysporum* and 2ml of *P. stutzeri* TR2 were transferred into a 500ml Erlenmeyer flask containing 150ml of the mix-culturing medium for measuring denitrified products N_2 and N_2O. The mix-culturing *F. oxysporum* and *P. stutzeri* TR2 were in two ways. One was mix-culturing them directly, in which both of *F. oxysporum* and *P. stutzeri* TR2 were free. The other way was that *F. oxysporum* was packed in a tea bag first then mix-cultured

with *P. stutzeri* TR2. Denitrifying ability of *F. oxysporum* or *P. stutzeri* TR2 was detected in their specific medium. Fig. 1A showed time-dependent evolution of N_2 and N_2O during anaerobic incubation (oxygen-limited condition) of *F. oxysporum* and *P. stutzeri* TR2 with nitrate (^{15}N-$NaNO_3$). Both dinitrogen atoms in the N_2 molecule were shown by GC-MS to be derived from nitrate (by using ^{15}N-nitrate). The results showed that denitrifying ability of *P. stutzeri* TR2 was strong, and about 78% nitrate was reduced to form N_2 during 12hr (Tab. 1). In the case of mix-culturing, the reducing speed by *F. oxysporum* and *P. stutzeri* TR2 was faster than that of *P. stutzeri* TR2, even though *F. oxysporum* only reduce nitrate to form N_2O instead of N_2 (Tab. 1). The turnover rate of nitrate reduced by mix-culturing *F. oxysporum* (packed in tea bag) and *P. stutzeri* TR2 was very high and reached 88.1%. *F. oxysporum* seemed to lack N_2O reductase and evolved N_2O as the denitrification product. After incubated for 72hr, 467.5μmol of N_2O was produced by *F. oxysporum*. The amount of O_2 remained during the denitrification was determined by GC chromatography (Fig. 1B). When *F. oxysporum* and *P. stutzeri* TR2 were mix-cultured, O_2 was consumed fast, that seemed favorable to denitrification by *P. stutzeri* TR2.

Tab. 1 Removal of nitrate during denitrification by *F. oxysporum* (*Fus*) and *P. stutzeri* TR2 in flask cultures using different media (explanations see footnote)

Medium	Strain	NO_3^- consumed (μmol)	NO_2^- produced (μmol)	Time (h)	N_2 or N_2O produced (μmol)	N_2 or N_2O produced/ NO_3^- consumed (%)
GP	Fus	1500	15.00	72	467.50[a]	62.3
SC	TR2	1500	50.00	12	584.86	77.9
MC	TR2+Fus-p	1500	35.00	12	661.10	88.1
	TR2+Fus-f	1500	38.00	12	603.01	80.4

GP: contained glycerol and peptone and suitable for *F. oxysporum*, a: amount of N_2O, SC: contained succinate and casamino acid and suitable for *P. stutzeri* TR2, MC: mix-culturing medium for both *F. oxysporum* and *P. stutzeri* TR2 (as in method and materials), Fus-p: *F. oxysporum* was packed in tea bag, Fus-f: *F. oxysporum* was free. Each of them contained ^{15}N-$NaNO_3$ and each flask contained 150ml medium (initial amount of NO_3^- was 1500μmol). All experiments were run in triplicate

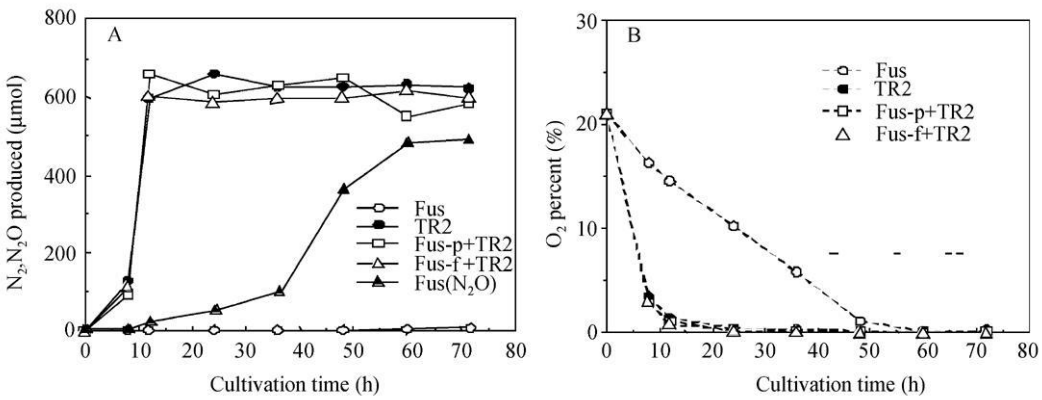

Fig. 1 Evolution of N_2 and N_2O by *F. oxysporum* and *P. stutzeri* TR2 from nitrate. *F. oxysporum* and *P. stutzeri* TR2 were cultured by the closed culture (500ml flasks) in the presence of ^{15}N-sodium nitrate (10mM, total 1.5mmol). A: total amounts per flask of N_2 and N_2O: N_2(○) and N_2O (▲) produced by *F. oxysporum*; N_2 produced by *P. stutzeri* TR2 (●), by mix-culturing *P. stutzeri* and *F. oxysporum* (△), or by mix-culturing *P. stutzeri* and *F. oxysporum* packed in tea bag (□). B: total amounts per flask of O_2: *P. stutzeri* TR2 (●); *F. oxysporum* (○); and mix-culture of *P. stutzeri* TR2 and *F. oxysporum* (△) or *F. oxysporum* packed in tea bag (□). All experiments were run in triplicate

2.2 Co-denitrification by *F. oxysporum* and *P. stutzeri* TR2 in synthetic wastewater

The denitrification capabilities of *F. oxysporum* or *P. stutzeri* TR2 and both of them in the synthetic wastewater were investigated (Fig. 2 and Tab. 2). Both *F. oxysporum* and *P. stutzeri* TR2 were cultivated in the same manner as described in Fig. 1 except that synthetic wastewater (100ml per flask contained 10mM of ^{15}N-sodi-

um nitrate, total 1.0mmol) was used instead of mix-culturing medium. A rapid N_2 evolution by mix-culturing *P. stutzeri* TR2 and *F. oxysporum* (in free) was observed as shown in Fig. 2A. After incubation for 6days, 87.1% of nitrate was reduced to form 420.44μmol of N_2 by co-denitrification of *F. oxysporum* and *P. stutzeri* TR2, while *P. stutzeri* TR2 seemed not to denitrify very well under shaking cultivation and only produced a little dinitrogen (about 16.72μmol of N_2). This indicated that mix-culturing *F. oxysporum* and *P. stutzeri* TR2 can be used to remove nitrate and/or nitrite from wastewater effectively. The oxygen derived from air and initially present in the flask was consumed rapidly during first 24 hr when *F. oxysporum* and *P. stutzeri* TR2 were mix-cultured (Fig. 2B). At the same time, the denitrifying speed was increased quickly, suggesting that the limited oxygen or anaerobic condition is benefited to the dissimilatery nitrate reduction. It was interesting that N_2O was produced (154.48μmol of N_2O in 72 hr) when the packed *F. oxysporum* and *P. stutzeri* TR2 were mix-cultured, while N_2O was not produced in the mix-culturing *F. oxysporum* and *P. stutzeri* TR2 directly.

Tab. 2 Removal of nitrate during denitrification by *F. oxysporum* and *P. stutzeri* TR2 in synthetic wastewater with different culturing manner

Culturing manner	NO_3^- consumed (μmol)	Time (d)	N_2 or N_2O produced (μmol)	N_2 or N_2O produced/NO_3^- consumed (%)
Fus	580.0	6.0	51.09[a]	17.5
TR2	360.0	6.0	16.72	9.2
TR2+Fus	965.0	6.0	420.44	87.1

Each flask contained 100ml of synthetic wastewater in which ^{15}N-$NaNO_3$ was used and initial amount of NO_3^- was 1000μmol. a: amount of N_2O. All experiments were run in triplicate as the same manner described in Tab. 1.

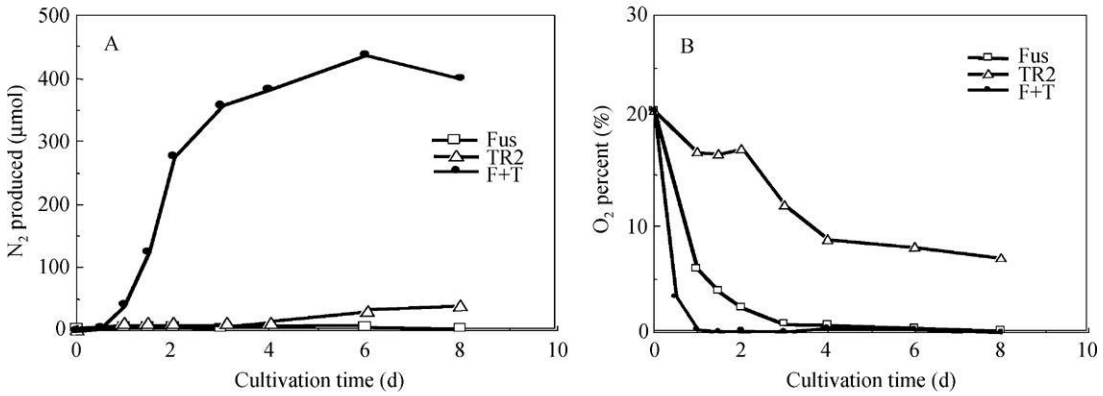

Fig. 2 N_2 evolution by *F. oxysporum* and *P. stutzeri* TR2 with the synthetic wastewater. *F. oxysporum* and *P. stutzeri* TR2 were cultured in the same manner as in Fig. 1 except that 100ml of the synthetic wastewater was used in the presence of ^{15}N-sodium nitrate (10mM, total 1.0mmol). All experiments were run in triplicate. A: total amounts per flask of N_2; B: total amounts per flask of O_2. Fus: *F. oxysporum* (□); TR2: *P. stutzeri* TR2 (△); F+T: mix-culturing *P. stutzeri* and *F. oxysporum* (in free●)

3 Discussion

Previous work proved that *F. oxysporum* is capable of reducing nitrate and nitrite to form N_2O during the denitrification[4]. In this study, we found that the denitrifying ability of *P. stutzeri* TR2, a new strain of *P. stutzeri*, was strong and fast under anaerobic condition, and that co-denitrification in mixed cultures *F. oxysporum* and *P. stutzeri* TR2 was more effective to remove nitrate under limited oxygen condition. Both mixed culture medium and synthetic wastewater were used to culture *F. oxysporum* and *P. stutzeri* TR2 to detect their denitrifying abilities. The results showed that about 87% nitrate was removed effectively by co-denitrification of *F. oxysporum* and *P. stutzeri* TR2 in 12 hr (with mix-culturing medium) or in 6days (with synthetic wastewater) respectively (Tab. 1 and 2). Although the turnover rate of nitrate by mix-culturing *P. stutzeri* TR2 and *F. oxysporum* (packed in tea bag) was a little lower (still more than 80%, data not shown) than that of them in free, it took

some advantages of recovering and reuse in their application. In co-cultures of *F. oxysporum* and *P. stutzeri* TR2, no N_2O was detected suggesting that *P. stutzeri* TR2 consumed N_2O quickly and reduced it to form N_2 without N_2O accumulation.

Oxygen is a critical factor in the denitrification by *F. oxysporum* and *P. stutzeri* TR2. A low amount of O_2 was required for fungal denitrification (cell growth and induction) although it was completely repressed by excess O_2[11]. Denitrification by bacteria is a typical anaerobic process[2]. When *P. stutzeri* TR2 was cultured in synthetic wastewater with 120 rpm shaking, there was no significant N_2 evolution, possibly since some oxygen was dissolved in the medium during agitation. When *F. oxysporum* and *P. stutzeri* TR2 were mix-cultured, oxygen was consumed quickly in first 24 hr because of cell growth. At the same time, N_2O produced by *F. oxysporum* was rapidly consumed by *P. stutzeri* TR2. This indicated that mix-culturing *F. oxysporum* and *P. stutzeri* TR2 can be used to remove nitrate and nitrite from wastewater effectively.

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