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Optimum growth requirements of nitrifying consortia developed from treated sewage

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The optimum growth requirements of two nitrifying consortia developed from treated sewage by enrichment technique were determined by a series of experiments. There was total inhibition of nitrification at above 2.75 g l^{-1} NH_4^+- N and 2.5g l^{-1} NO_2^- - N and the ammonia oxidizing consortium preferred a *p*H at 8.5 and the nitrite oxidizing consortium a *p*H of 7.5 as the optima for nitrification. Optimum temperatures were between 20° and 30°C for both the groups. As the rate of airflow was increased from 1 to 7 l/min, the build-up of NO_2^- -N increased 10-fold and the consumption of NO_2^- -N increased by a factor of 28.8 implying that the ammonia oxidizing consortium in a bioreactor required three times more aeration than that for nitrite oxidizers for expressing their full nitrifying potential. These data directly contribute for developing a fermentation process for the mass production of nitrifiers as well as for designing bioreactors for nitrifying sewage.

Keywords: Bioreactor, Nitrifying consortia, Optimum requirements

As sewage contains high concentrations of ammonia, toxic to aquatic life including photosynthetic bacteria[1], the latter should either be stripped off or oxidized to less toxic nitrates before being discharged in to surface waters. In nature, ammonia is oxidized to nitrates in a two step process by the aerobic chemolithotrophic bacteria (family Nitrobacteriaceae). Activated sludge systems and trickling filters are the only two available biological systems where nitrification of sewage can be achieved[2,3]. But in reality a series of drawbacks have been noticed in both activated sludge systems[3] and in trickling filters[4] suggesting the requirement of a terminal nitrification process for the treated sewage. This led to the conceptualization of nitrifying bioreactors in which large quantity of nitrifiers can be immobilized to carryout nitrification of sewage at a rapid rate.

In order to develop bioreactors for nitrifying sewage there were two prime requirements, such as (i) nitrifying consortia or a pure culture, which is designated as the software of the technology, and (ii) nitrifying reactor designated as the hardware. The process of developing nitrifying consortia involved stages such as (a) enrichment of nitrifiers, (b) determination of optimum growth requirements and (c) mass production of nitrifiers. The process of enrichment of nitrifiers including the selection of an appropriate growth medium could be achieved[5]. Ammonium and oxygen concentrations and *p*H are thought to be the environmental parameters most important to the nitrification rate and also are likely to determine the nitrifying community structure[6]. Determination of optimum growth requirements such as optimum substrate concentration, optimum *p*H, optimum temperature, optimum rate of airflow are essentially required for their mass production in fermentors. Further, these factors have to be determined for every culture or consortium because as per the existing literature, observations made by various workers on the above requirements on different cultures were contradictory and were within a range than specifically to a point[2,6]. Moreover, these works were carried out with either pure cultures in most of the cases or with consortia solely obtained from the temperate regimes and therefore were not applicable as such to be adopted for the ones under study. Therefore, the present study has been undertaken to determine the optimum growth requirements of nitrifying consortia developed from sewage by enrichment technique.

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Materials and Methods

Nitrifying consortia — The consortia of nitrifiers were mass cultured in 500 ml medium each prepared for both ammonia and nitrite oxidizers. The medium for ammonia oxidizers according to Lewis and Pramer[7]

contained g l^{-1} Na_2 HPO_4 13.5, KH_2 PO_4 0.7, $MgSO_4$. $7H_2O$ 0.1, $NaHCO_3$ 0.5, $(NH_4)_2$ SO_4 2.5, $FeCl_3$ $6H_2O$ 0.01444 and $CaCl_2.2H_2O$ 0.0184 and it was numbered as medium 1. The medium for nitrite oxidizers according to Rodina[8] contained (g l^{-1}) $NaNO_2$ 2, KH_2PO_4 1, NaCl 0.5, $MgSO_4.7H_2O$ 0.5, $MnSO_4$. $4H_2O$, trace and $Fe(SO_4)_3$ $9H_2O$, trace and numbered as medium 2. The nitrifying consortia developed by Ramachandran and Singh[5] were used as the inocula for producing sufficient biomass for this study. The media in 1 litre flasks setup over a magnetic stirrer, kept under obscurity were inoculated with the cultures to 1% (v/v). The flasks were incubated along with timely addition of the substrates when exhausted and manual adjustment of *p*H to 8 when it dropped to 5.5. On attaining stationary phase, about 50% the cultures was removed, centrifuged at 10000 *g* in a refrigerated centrifuge at an operating temperature of 4°C, the pellet was washed with the medium and re-suspended in fresh medium containing 100 μg ml^{-1} substrate and stored at 4°C. This was used as the inoculum for the present study where the inoculum size in all cases was regulated to be 3.0×10^{-5} gml^{-1} (dry weight) and in all cases the media were inoculated to a final concentration 1% (v/v).

Optimum substrate concentration — The medium 1 and 2 were prepared with varying concentrations of the substrates (ranging from 0.1 to 5.0 gl^{-1} NH_4^+-N and NO_2^--N) added as $(NH_4)_2$ SO_4 and Na NO_2 respectively. Incubations were carried out at 28° ± 0.5°C in a humidity - controlled incubator under obscurity and the medium *p*H was initially adjusted to 8, monitored and regularly made up when got changed, using either 1*N* HCl or 10% aqueous sodium carbonate.

Optimum pH — The media 1 and 2 were prepared in varying *p*H ranging from 4 to 10 and in a uniform substrate concentration of 0.1 g l^{-1} of NH_4^+-N and NO_2^- –N. They were inoculated with the consortia of ammonia and nitrite oxidizers respectively to a final concentration of 1% (v/v). The flasks were incubated static under obscurity at 28° ± 0.5°C in a humidity-controlled incubator. Whenever the *p*H altered it was adjusted to the required level.

Optimum temperature — The media 1 and 2 having a uniform substrate concentration of 0.1 g l^{-1} and the *p*H adjusted to 8.5 for ammonia oxidizers and 7.5 for nitrite oxidizers were inoculated with ammonia and nitrite oxidizing consortia to a final concentration of 1% (v/v), incubated under obscurity at a wide range of temperature extending from 4° to 37°C (4, 20, 26, 30 and 37°C).

Rate of airflow — A series of 250 ml conical flasks containing 100 ml medium 1 having 330 μg l^{-1} NH_4^+ - N with *p*H adjusted to 8.5 and the same quantity of medium 2 having the same concentration of NO_2^- -N with *p*H adjusted to 7.5 were inoculated with ammonia and nitrite oxidizing consortia as described above to a final concentration of 1% (v/v). These flasks were set at different airflow rates ranging from 0 to 7 l/min drawn from an air compressor through a pipeline air filter and monitored by an airflow meter (Oxytech Equipments, India). Air spargers were used to increase the aeration efficiency and the flasks were incubated at room temperature (28° ± 10°C) in the dark. Prolonged incubation under this experimental set up was not practicable as the aeration led to higher rate of evaporation.

Measurement of growth of nitrifiers — Throughout the present study the amount of NO_2^- -N produced by ammonia oxidizing consortium and the amount of NO_2^--N consumed by nitrite oxidizing consortium were used as the measures of their growth as validate by Engel and Alexander[9]. The incubation was carried out for 7 days or the experiment was terminated when the substrate got exhausted in any one of the flasks in each series.

Results and Discussion

On testing with various substrate concentrations ranging from 0.1 to 5.0 g l^{-1}, the maximum allowable limits *per se* for the ammonium and nitrite oxidizing consortia were found to be 2.75g l^{-1} NH_4^+-N and 2.5g l^{-1} NO_2^--N respectively (Fig. 1a and b). Beyond this value progressive inhibition of ammonia oxidization and sharp decline in nitrite oxidation respectively took place.

In biological filter systems, for a given initial substrate concentration ranging from 0.035 to 1.96 g l^{-1} of NH_4^+-N, ammonia oxidation was in the first order reaction as described in Michaelis Menton Kinetics, implying that beyond 1.96g l^{-1} NH_4^+ - N, inhibition of nitrification could take place[10]. Even though similar comparable works could not be cited from literature with regard to nitrite oxidizers, the work of Boon and Laudelout[11] demonstrated that 1.4 g l^{-1} NO_2^--N caused 40% inhibition in the activity of pure culture of *Nitrobacter*. Studies of Bruns[12] and Suwa *et al.*[13] show that media containing low substrate concentrations (10 mg of NH_4^+ l^{-1}) can give larger MPN counts of ammonium oxidizers than the media containing higher ammonia concentrations[12,13]. The evidence of Suwa *et al.*[13] suggests that there is some correlation between ammonia oxidizer sensitivity or tolerance to ammonia and phylogeny.

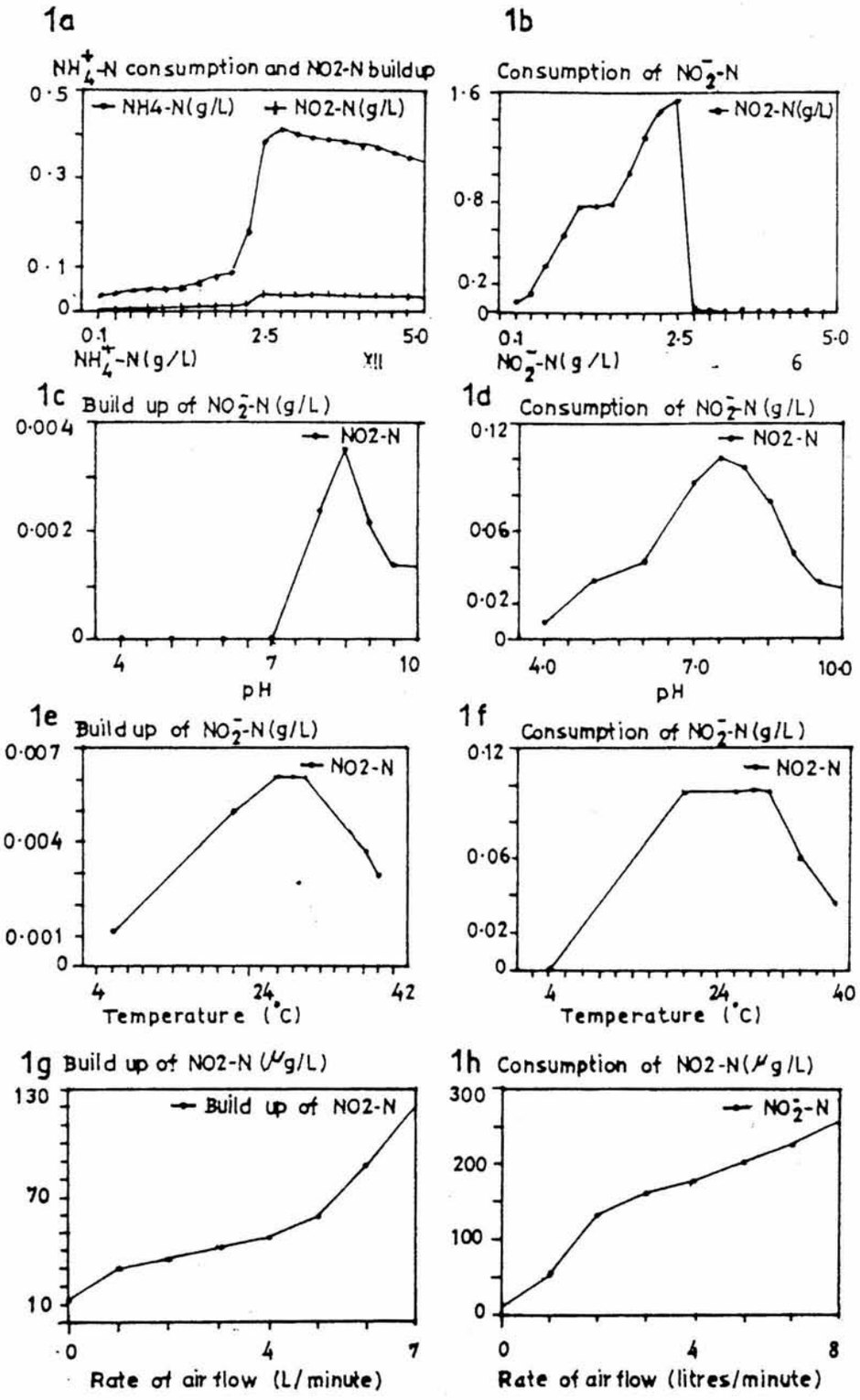

Fig. 1 — Optimum growth requirements of nitrifying consortia (a and b- Substrates; c and d-*p*H; e and f-Temperature; g and h-Rate of airflow).

Both *Nitrosomonas* sp. and *Nitrobacter* sp. were sensitive to their own substrates[14], and the degree of inhibition depended upon the ammonia-ammonium and nitrite-nitrous acid equilibria which were *p*H dependents[11,15,16]. In the light of these observations it could be ascertained that the consortia of ammonia and nitrite oxidizers developed here had better tolerance to higher concentration of NH_4^+-N and NO_2^--N, a requisite for ammonia removal from sewage where shock loading was often a reality.

The maximum nitrifying potential of ammonia oxidizing consortium as sharp peaks was exhibited at *p*H 8.5 and that of nitrite oxidizers at *p*H 7.5 (Fig. 1c and d), the *p*H optima falling slightly on the alkaline side.

One mechanism by which *p*H affects the rate of nitrification, as proposed by Anthonisen[15] is that both free ammonia (NH_3) (FA) and free nitrous acid (HNO_2) (FNA) inhibit nitrifying organism, by being able to penetrate the cells and altering the *p*H equilibria of the cytoplasm. This makes the species more inhibitory than NH_4^+ and NO_2^-, especially when the intra-cellular *p*H of a nitrifying organism is lower than the *p*H of the extra-cellular environment. In the optimum *p*H of the consortia recorded here, the quantity of FA and FNA may be comparatively lesser and this situation support maximum degree of nitrification. Studies on influence of *p*H over nitrifying biofilm activity in submerged biofilters show that, within a *p*H range of 5-9 a *p*H increase of one unit produce a 13% increase in nitrification efficiency [17].

The optimum temperature for nitrification was within the range 20°-30°C with no growth below 4°C or above 37°C (Fig.1e and f).

Nitrification in general follow the Vant Hoff Arrhenius law up to 30°C[18] suggesting that the process is better in warmer season or climate as has been observed in the present study. Meanwhile, Painter[11] even reported for *Nitrobacter* sp. an optimum temperature of growth as high as 42°C.

Ammonia and nitrite oxidizing consortia exhibited 10-fold increase in the build up of nitrite and 28.8-fold increase in the consumption of nitrite respectively as the rate of airflow was increased from 1 to 7 l / min (Fig. 1g and h).

It has been accepted that 3.43 mg O_2 was required for conversion of 1mg NH_4^+-N and 1.14 mg O_2 for conversion of 1mg NO_2^--N[19]; in other words ammonia oxidation to nitrite demanded three times more oxygen than that required for nitrite oxidation. In addition, the ammonia oxidizing population had greater specific affinity for O_2 than the nitrite oxidizing bacteria[20]. Obviously, given the same airflow rate as per the present study, nitrite-oxidizing activity always would be greater than ammonia oxidation.

Studies showed that *Nitrosomonas oceanus* lost 25% of its nitrifying capacity when the O_2 concentration was reduced from 100 to about 10% of air saturation[21]. Steady state culture of *Nitrosomonas* and *Nitrobacter* gave complete conversion of ammonia to nitrate at dissolved oxygen concentration at about 100 $\mu mol.l^{-1}$. Below this, the concentration of nitrate went down and nitrite started accumulating which indicated that the nitrite oxidizers were in trouble. At dissolved oxygen concentration below 50 μ mol.l^{-1}, ammonia also began to accumulate and the culture was washed out. However, pure O_2 at higher concentration was inhibitory, as the free radical formation inhibited oxygenase[22]. Therefore, it is recommended to use air with increased flow rate to cope up with the Nitrogenous Biological Oxygen Demand (NBOD). The above observation also implies that the ammonia oxidizing consortium in a bioreactor requires three times more aeration than the one required for a nitrite oxidizing consortium for the expression of their full potential.

However, in the present work the rate of airflow varied from 0-7 l/min passed through 100-ml medium taken in 250-ml conical flask. In this experimental facility the lowest quantity of airflow at the rate of 1 l/min was sufficient to saturate the medium with oxygen. In this situation when the volume of air passed through the medium was increased to 7 l/min it was not contributing to the dissolution of oxygen rather it was increasing the extent of turbulence and agitation of the medium. Therefore, the heightened activity of the consortium obtained during high airflow rate cannot be correlated with the dissolved oxygen content rather to the extent of turbulence. It is postulated that the enhanced turbulence may have increased the mass transfer resulting in high nitrification.

Conclusion

The ammonia and nitrite oxidizing consortia developed here are unique systems, which oxidize ammonia and nitrite and have not been developed elsewhere. For the mass production of nitrifying consortia in a fermentor and also to obtain highest level of activity in bioreactors, the optimum growth requirements such as substrate concentration, *p*H,

temperature and rate of airflow were determined. Accordingly, there is total inhibition of nitrification at above 2.75 g l^{-1} NH_4^+ -N and 2.5 g l^{-1} No_2^- -N in the test media 1 and 2. Ammonia oxidizing consortium preferred a *p*H of 8.5 and nitrite oxidizing consortium a *p*H of 7.5 as optima. Optimum temperature for nitrification was between a range of 20°C and 30°C for both ammonia and nitrite oxidizing consortia. As the rate of airflow increased from 1 to 7 l/ min, the build up of NO_2^- -N in medium 1 showed a 10-fold increase while consumption of NO_2^- -N in medium 2 increased by a factor of 28.8. As the increased rate of airflow does not lead to more dissolved oxygen content, the reason for higher nitrification has been correlated with the higher mass transfer, which might have happened during higher turbulence. These informations are vital for designing a fermentation process for mass production of nitrifiers and for redesigning bioreactors for nitrifying sewage. The consortium developed is maintained by the Environmental Microbiology Laboratory, School of Environmental Studies, Cochin University of Science and Technology, Cochin.

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