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Research Article

TEMPERATURE AND INITIAL BIOMASS CONCENTRATION RELATION TO NITRATE AND NITRITE UPTAKE BY IMMOBILISED AND FREE CELLS OF *PSEUDOMONAS AERUGINOSA*

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ABSTRACT
Although nitrogen is an essential nutrient to the existence of living organisms, its presence in excessive amounts in wastewater could have detrimental effects to humans and other living organisms. This study was aimed at assessing the effect of temperature and initial biomass of immobilised and free cells of *Pseudomonas aeruginosa* in the removal of nitrate and nitrite from nutrient media. The study was carried out in batches under shaking flask conditions. After inoculation with the test isolate, aliquots were withdrawn from the reactor every 24 h for the analysis of nitrate, nitrite and pH, using standard methods. The results revealed significant nitrate and nitrite removal at the different initial biomasses used for inoculation. This observation was irrespective of the immobilised or free cells. At the different incubation temperatures, significant nitrate and nitrite decreases in the media were observed at 25 °C and 35 °C. At 45 ^{o}C, nitrate or nitrite removal was either minute or no removal was observed. The study was able to reveal effects of initial biomass concentration and temperature affects nitrate and nitrite removal in presence of the immobilised and free cells of the test bacterial**.**

Keywords: *Nitrite, Nitrate, Temperature, Biomass, Nutrient Removal, Immobilised Cells*

INTRODUCTION
The enrichment of nutrients in water bodies are major problems leading to excess amounts in the two essential eutrophic nutrients known as nitrogen and phosphorus, whose availability in excess results in eutrophication with detrimental effects on human and aquatic life (Rocca *et al.,* 2007). In recent years, awareness on the importance to treat water pollution has been supported to remove these pollutants before its discharge for consumption and other industrial activities and agricultural uses such as irrigation. Physical, chemical and biological pollutants are removed by various physical, chemical and biological processes. Due to the high capital and operational prices, which can be related to the generation of secondary waste that present treatment issues, like volume of sludge made throughout chemical treatment, the method is not advocated in the recent years (Kumar *et al.,* 2010).
Approximately 25 % of all water body damages are the result of nutrient-related causes such as oxygen depletion, algal growth, nutrients, ammonia and harmful algal blooms (USEPA, 2007). In a bid to decrease the number of nutrient deficiencies, rigid effluent limits have been forced on several point source dischargers for nitrogen and phosphorus. In a bid to attain these new, lower effluent limits, amenities have begun to source beyond unconventional methods of treatment (Metcafe and Eddy, 2003).
To protect the environment from the harmful impacts of eutrophication, the stringent effluent limits placed on the discharger coupled with absorption with agricultural and industrial wastes similarly as changed wastes has been utilized for the removal of harmful nutrients from wastewater. However, the uptake of these nutrients by using microorganisms endowed with nutrient uptake or mineralization capacities has been a major breakthrough in area of wastewater treatment. Nitrate in aerobic systems by bacteria can be converted to nitrate and nitrite in a process called nitrification and in anaerobic systems can be reduced by other strains of bacteria to nitrous oxide or nitrogen gas by a process of denitrification (Anjali *et al.,* 2014).
Several methods such as assimilation, biodegradation and surface assimilation are the various means by which the microbes are able to detoxify water (Wu *et al.,* 2012). Although several traditional biological treatment procedures have the ability of eliminating voluminous segments of biodegradable organic

compounds residing in wastewater, several harmful compounds are rarely extracted due to their toxicity (Kumar *et al.,* 2010). In addition, they impose harmful effects on the composition and activities of microorganism populations in activated sludge flocs, thereby reducing the total performance of these amenities posing several problems for waste water engineers and scientists. Therefore, the process of cell immobilization overall term used in defining an extensive variety of the cell or the particle attachment or entrapment using algal polysaccharides such as agar, agarose, alginate, carrageenan and chitosan suitable for the treatment of waste by transforming the harmful compounds into nutrient, carbon dioxide and biomass by biodegradation through their intermediates (Peinado *et al.,* 2005; Kilonzo and Bergougnou, 2012).

Several research works have been carried out on waste water treatment using free cells however, with the recent knowledge of immobilised cells that have shown capacity for wastewater treatment. In comparison with conventional suspension system, the immobilized microorganism technique poses a variety of benefits, such as high biomass, high metabolic activity and solid resistance to toxic chemicals over free cells (Liu *et al.,* 2012). The utilization of immobilized microbial technology is specified as a promising tool for wastewater treatment in the past few years and in the future ahead of us as it is reasoned that immobilised free cells are enhanced options for nutrient uptake from wastewater due to their cost effectiveness, because they portray no significant loss of functionality despite continuous use (Devi and Sridhar, 2000). The aim of this study was to assess the effect of immobilised and free cells of *Pseudomonas aeruginosa* on nitrate and nitrite uptake in nutrient media.

MATERIALS AND METHODS

Media Composition

The media used for the study had the following composition dissolved in distilled water: sodium acetate (5 g/L), peptone (5 g/L), yeast extract (5 g/L), magnesium sulphate (0.5 g/L), potassium dihydrogen phosphate (0.5 g/L), sodium nitrate (0.5 g/L) and sodium nitrite (0.5 g/L). For preparation, each of the components was first weighed and dissolved separately in small quantity of distilled water before the different components were mixed together. Prior to use, the media was dispensed in 200 mL into 250 mL capacity conical flasks, cotton plugged and sterilized in an autoclave at 121 ℃ and 1.05 kg/cm^3 for 15 min.

Preparation of the Free and Immobilized Cells

The test bacteria used for the study was *Pseudomonas aeruginosa* ATCC 9027 P-1. The pure isolate was grown in sterile nutrient broth for 24 h before usage as free or immobilized cells. For immobilization, sodium alginate [KEM LIGHT LABORATORIES] with calcium chloride [SIGMA-ALDRICH], agarose [SIGMA-ALDRICH] and agar powder [LAB M] were used as respective substrates.

For preparations of the free cells, the 24 h old broth cultures of the isolate were first centrifuged at 5000 rpm for 30 min to separate the broth from the cells. After centrifugation, the supernatant was discarded while the cells were suspended in sterile normal saline (0.9 % NaCl solution). The suspended cells were vortexed and dispensed in sterile universal bottles and stored in a refrigerator until when needed. In order to now the estimate of cell population in the suspension, dilutions of the cell suspensions were made and a known volume plated in sterile nutrient agar using the pour plating technique and incubated at 37 ^{o}C ± 2 ^{o}C in an incubator for 24 h. At the end of the incubation, the number of colony forming units were counted and expressed as colony forming units per millitre of the cell suspension (CFU/mL).

For immobilization in sodium alginate solution, 20 mL of the broth culture of the isolate was mixed with 150 mL of sterile 5 % sodium alginate solution and allowed to stand on the work bench for 2 h. The mixture was then pitted dropwise into a sterile beaker that contained 2.5 % of sterile calcium chloride solution to produce the immobilized beads. The formed beads were allowed to harden for 3 h before washing off the calcium chloride-sodium alginate solution from the beads with sterile distilled water. The beads were placed in a capped bottle and incubated in a refrigerator until when needed. To ascertain the purity and viability of the beads, a few of the beads were removed and cultured in cultured in sterile nutrient agar using the pour plating technique. The number of cells in a bead was estimated.

The cell immobilization in the agar and the agarose powders were carried out by mixing the 20 mL of the broth cultures of the cells in 2.5 % and 5 % sterile agar and agarose solutions, respectively. Before mixing with the cells, the solutions were allowed to cool around 45 ^{o}C, after which the respective agar and agarose mixtures were poured in petri dishes and allowed to solidify. After solidifying, the beads were extracted from the plates using a sterile cork borer. The quantity of the cells in the immobilized beads were estimated as described earlier for the alginate immobilized cells.

Experimental Design

In 250 mL capacity conical flasks, 200 mL volume of media was prepared and sterilized. After sterilization, a known population of the respective free or immobilized cells were inoculated into each flask and incubated at the required temperatures for the study. Just immediately after inoculation and every 24 h for 120 h, aliquot samples (10 mL) were taken from each flask for the determination of nitrate and nitrite concentrations in the media and the pH of the media, using standard procedures (APHA, 2012).

Nitrate analysis was carried out using the salicylate method. To a 100 mL glass beaker, 2 mL aliquot of the sample was pipetted, after which 2 mL of 0.5 % sodium salicylate was added and evaporated to dryness on a hot plate. Two mL of concentrated H2SO4 was added to the beaker after cooling while tilting the beaker to wet the bottom and lower edge of the wall completely and allowed to stand for about 10 minutes. Thirty mL of distilled water was cautiously added which was followed by 8 mL 50 % (w/v) sodium hydroxide (NaOH) solution for colour development and made up to the mark by distilled water. The absorbance was measured after 10 minutes after addition of NaOH at a wavelength of 410nm using a 6705 uv/vis spectrophotometer. A reagent blank was also run with the samples to help the spectrophotometer to zero. A standard nitrate solution was prepared using different concentrations of sodium nitrate. All standards were treated like the samples as above. A calibration curve drawn was by plotting absorbance (y-axis) versus standard concentration (x-axis). Nitrate concentration was calculated as:

$$mg - nitrate/L = concentration\ from\ calibration\ curve\ X\ 20XD$$

Where D= dilution factor

For nitrite determination, 2 mL aliquot of the sample was pipetted into a 100 mL glass beaker, after which 1.0 mL of 5 % sulphanilamide solution using a micropipette, mixed and allowed to react for 3-5 min, after which 1.0 mL of 0.5 % N-(1-Naphthyl)-ethylenediamine dihydrochloride solution was added for colour development and mixed immediately.

The solution was shaken thoroughly and made up to the mark with distilled water. Prior to the measurement, the wavelength of the spectrophotometer was set at 543nm and then set to zero with distilled water and the reagents without the sample. The absorbance samples were then measured using a JENWAY 6705 uv/vis spectrophotometer. A standard nitrite solution was prepared using different concentrations of sodium nitrite. All standards were treated like the samples as above. A calibration curve drawn was by plotting absorbance (y-axis) versus standard concentration (x-axis). Nitrate concentration was calculated as:

$$mg - nitrite/L = concentration\ from\ calibration\ curve\ X\ 20\ X\ D$$

Where D= dilution factor

All reagents used for the study were of analytical grade. Also, all experimental setups were carried out in duplicate. Statistical analyses were carried out using the SPSS statistical software package. The test for the comparison of means was done using the one-way variance (ANOVA).

RESULTS AND DISCUSSION

Results

Nitrate and Nitrite Uptake Studies at the Different Temperatures

As shown in Figure 1, nitrate concentrations in the media in presence of the alginate immobilised cells were observed to show significant decreases after 72 h of incubation. These decreases were more evident at incubation temperatures of 35 ^{o}C and 45 ^{o}C. After the 120 h incubation period, nitrate levels in the

media showed a decrease from 338.77 mg/L to 22.38 mg/L, from 219.29mg/L to 30.86 mg/L and from 299.79 mg/L to 38.62 mg/L, at incubation temperatures of 25 ^{o}C, 35 ^{o}C and 45 ^{o}C (Figure 1). Although, the concentrations of nitrate in the media were observed to vary during the course of the period of incubation, these differences were not observed to differ significantly among the different temperature range ($p \leq 0.05$).

However, in the case of nitrite levels in the media, no decrease in concentration was observed at incubation temperature of 45 ^{o}C. This trend was irrespective of the sampling period. At incubation temperatures of 25 ^{o}C and 35 ^{o}C, remarkable decrease in nitrite concentration was observed after 120 h and 72 h, respectively. At the expiration of the period of incubation, nitrite levels showed reduction from 165.40 mg/L to 0.54 mg/L at 25 ^{o}C and from 193.80 mg/L to 2.05 mg/L at 35 ^{o}C (Figure 1). The concentration of nitrite at incubation temperatures of 25 ^{o}C and 35 ^{o}C were observed to be significantly lower than that at 45 ^{o}C ($p \leq 0.05$).

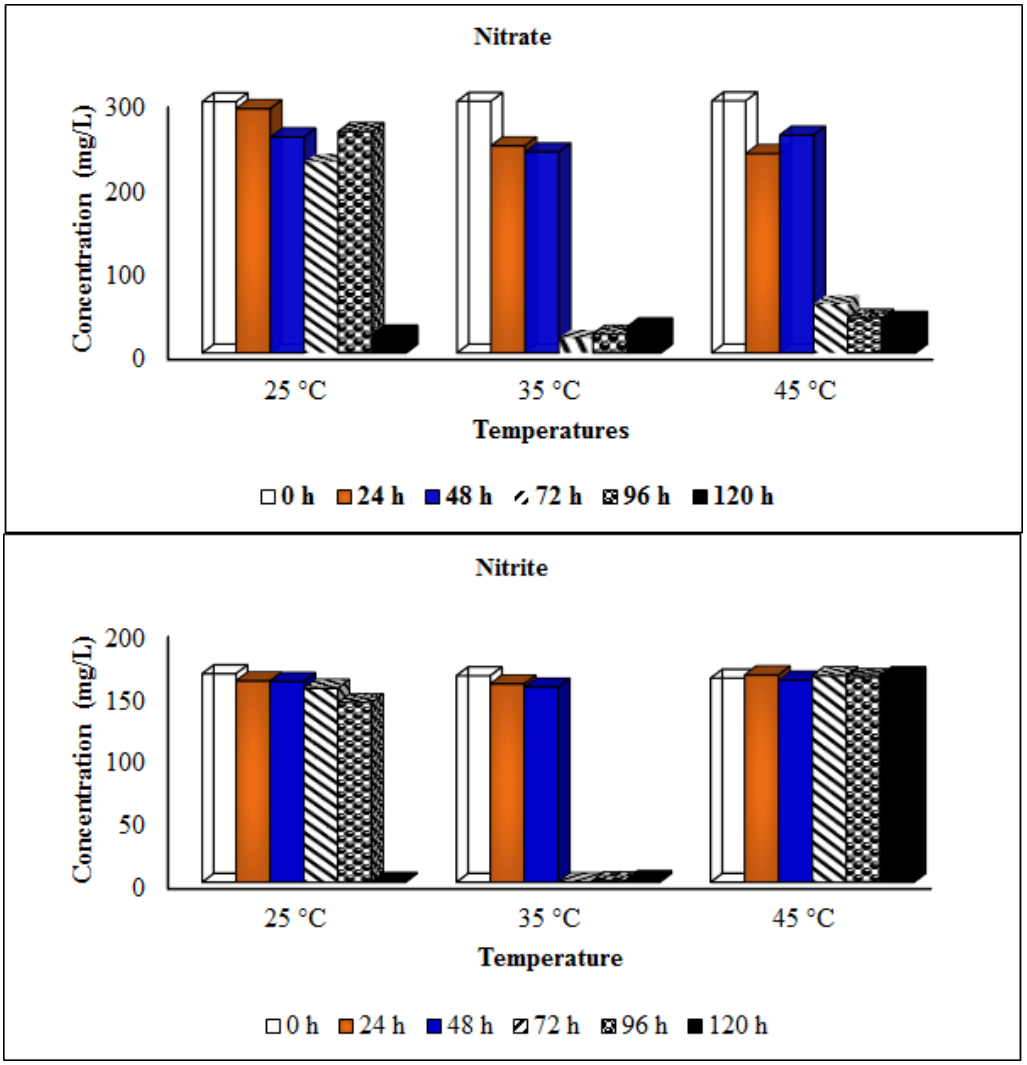

Figure 1: Nitrate and Nitrite Concentrations in the Media at the Different Incubation Temperatures in Presence of the Alginate-Immobilised Cells

In presence of the agar immobilized cells, nitrate concentration in the media at incubation temperature of 25 ^{o}C was observed to show significant decrease after 96 h of incubation. Significant reduction in concentration was however observed as from 72 h of incubation at incubation temperature of 35 ^{o}C while

there were no decreases in concentration throughout the sampling period at incubation temperature of 45 ^{o}C. At the expiration of the 120 h incubation period, nitrate levels in the media showed variation from 337.8 mg/L, 219.29 mg/L and 294.01 mg/L to 30.5 mg/L,12.5mg/L and 330.1 mg/L, at incubation temperatures of 25°C, 35°C and 45°C, respectively (Figure 2). In presence of the agar immobilized cells, the concentrations of nitrate in the media incubated at 25 ^{o}C and 35 ^{o}C were observed to be significantly lower than the one incubated at 45 ^{o}C ($p \leq 0.05$).

For nitrite levels in the media in presence of the agar immobilized cells, remarkable decreases in concentration were observed at incubation temperatures of 25 ^{o}C and 35 ^{o}C while at incubation temperature of 45 ^{o}C, no reduction in concentration was observed throughout the period of incubation. At the conclusion of the 120 h incubation period, nitrite levels in the media varied from 165.4 mg/L to 0.5 mg/L, from 193.8 mg/L to 0.78 mg/L and from 191.89 mg/L to 193.8 mg/L at incubation temperatures of 25 ^{o}C, 35 ^{o}C and 45 ^{o}C, respectively (Figure 2). Generally, nitrite concentrations in the media at incubation temperatures of 25 ^{o}C and 35 ^{o}C were shown to be significantly lower than that at 45 ($p \leq 0.05$).

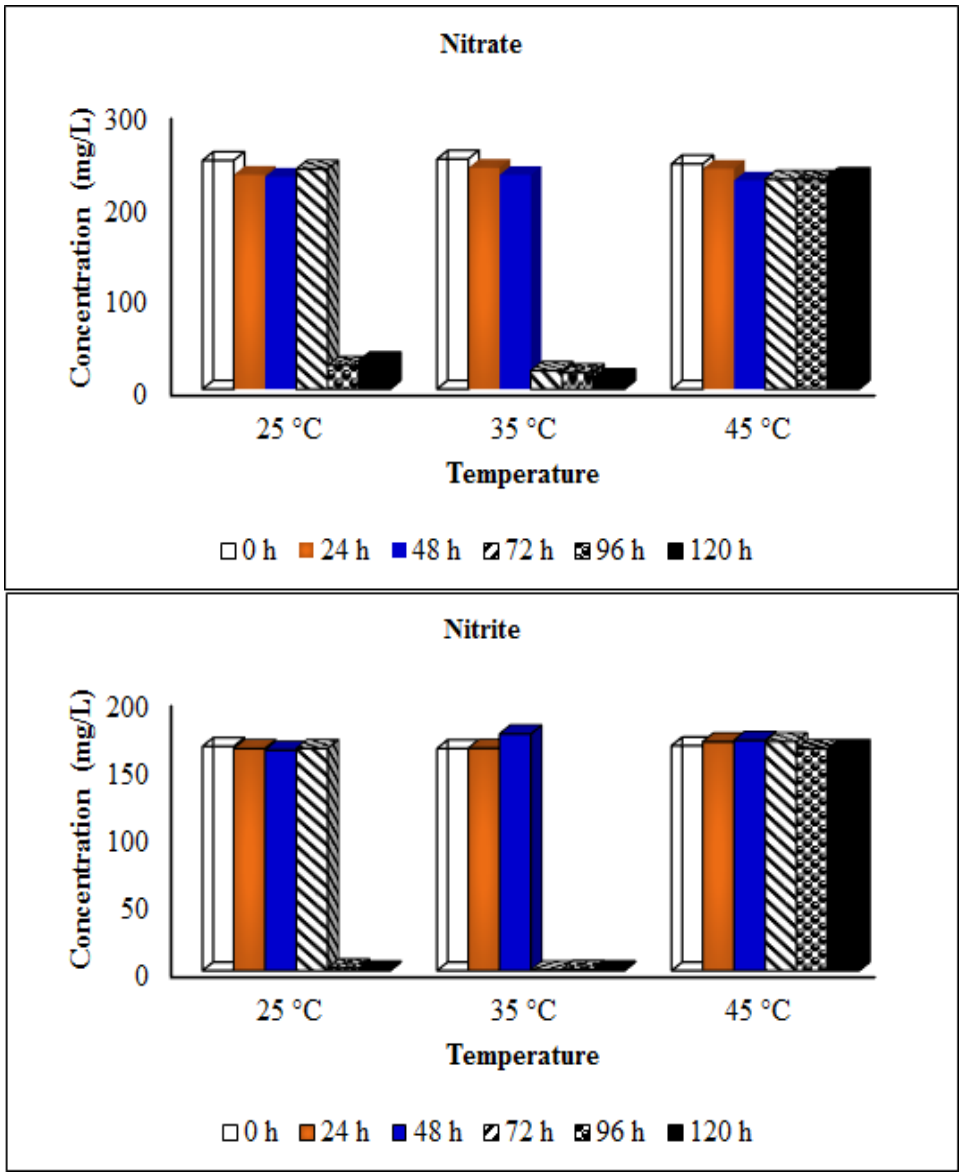

Figure 2: Nitrate and Nitrite Concentrations in the Media at the Different Incubation Temperatures in Presence of the Agar-Immobilized Cells

In presence of the agarose immobilized cells, nitrate concentrations in the media showed no remarkable decrease throughout the period of incubation at incubation temperature of 45°C. However, at incubation temperatures of 25 °C and 35 °C, remarkable decreases in nitrate concentration were observed as from 72 h incubation till the end of incubation. At the conclusion of the 120 h incubation period, nitrate levels in the media showed variations from 338.95 mg/L to 19.85mg/L, from 219.29 to 13.54mg/L and from 294.01 mg/L to 318.73 mg/L at incubation temperatures of 25 °C, 35 °C and 35 °C, respectively (Figure 3). The decreases in nitrate concentrations in the media at 25 °C, 35 °C were significantly lower than concentration at 45 °C ($p \leq 0.05$).

As for nitrite level in the media, remarkable decrease in concentration was observed from 96 h incubation at 25 °C in presence of the agarose immobilized cells. At 35 °C, nitrite levels in presence of the agarose immobilized showed reduction from 72 h incubation while no remarkable change in concentration was observed throughout the period of incubation at 45 °C. Nitrite levels in the media at the end of incubation were observed to change from 165.40 mg/L, 193.80 mg/L and 191.88 mg/L to 0.15 mg/L, 1.49 mg/L and 181.16 mg/L, at incubation temperatures of 25 °C, 35 °C and 45 °C, respectively (Figure 3). In the presence of the agarose immobilized cells, reduction nitrate concentration in the media at 25 °C and 35 °C was observed to be significantly higher than reduction at 45 °C ($p \leq 0.05$).

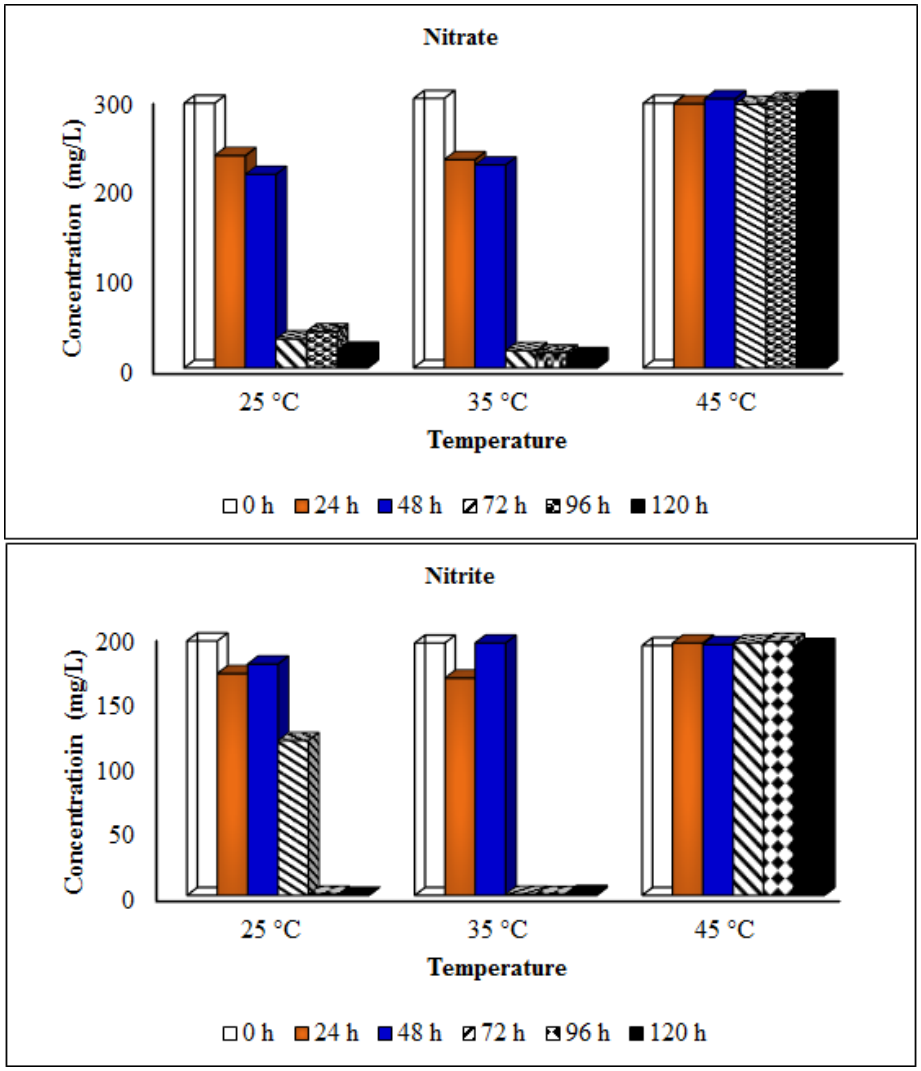

Figure 3: Nitrate and Nitrite Concentrations in the Media at the Different Incubation Temperatures in Presence of the Agarose-Immobilized Cells

As illustrated in Figure 4, in presence of the free cells, suspended in normal saline, remarkable decreases in nitrate concentration in the media were observed from 72 h incubation, at incubation temperatures of 25 °C and 35 °C. In presence of the free cells, no decrease in concentration was observed in media incubated at 45 °C. At the end of incubation, the concentration of nitrate in the media showed a decrease from 336.78 mg/L to 45.66 mg/L and from 219.65 mg/L to 11.55mg/L at incubation temperatures of 25°C and 35°C, respectively. At 45°C, an increase in nitrate level from 294.01 to 313.08 mg/L was observed. The concentration of nitrate in media incubated at 45 °C in presence of the isolate was observed to be significantly higher than at 25 °C and 35° ($p \leq 0.05$).

For nitrite concentration in the media, remarkable decrease in concentration was observed at 35 °C in presence of the free cells from 48 h incubation. This decrease was consistent till the end of the incubation period. At 35 ^{o}C, significant decrease in nitrite level was observed from 72 h incubation while at 45 ^{o}C, only minute decreases in concentration were observed. The concentrations of nitrite in presence of the free cells showed variations from 165.4 mg/L to 0.63 mg/L at 25 ^{o}C, from 193.8 mg/L to 0.62mg/L at 35 ^{o}C and from 191.89 mg/L to 182.82 mg/L at 45 ^{o}C (Figure 4). Generally, nitrite concentrations in the media incubated at 25 °C and 35 °C were observed to be significantly lower than that at 45°C ($p \leq 0.05$).

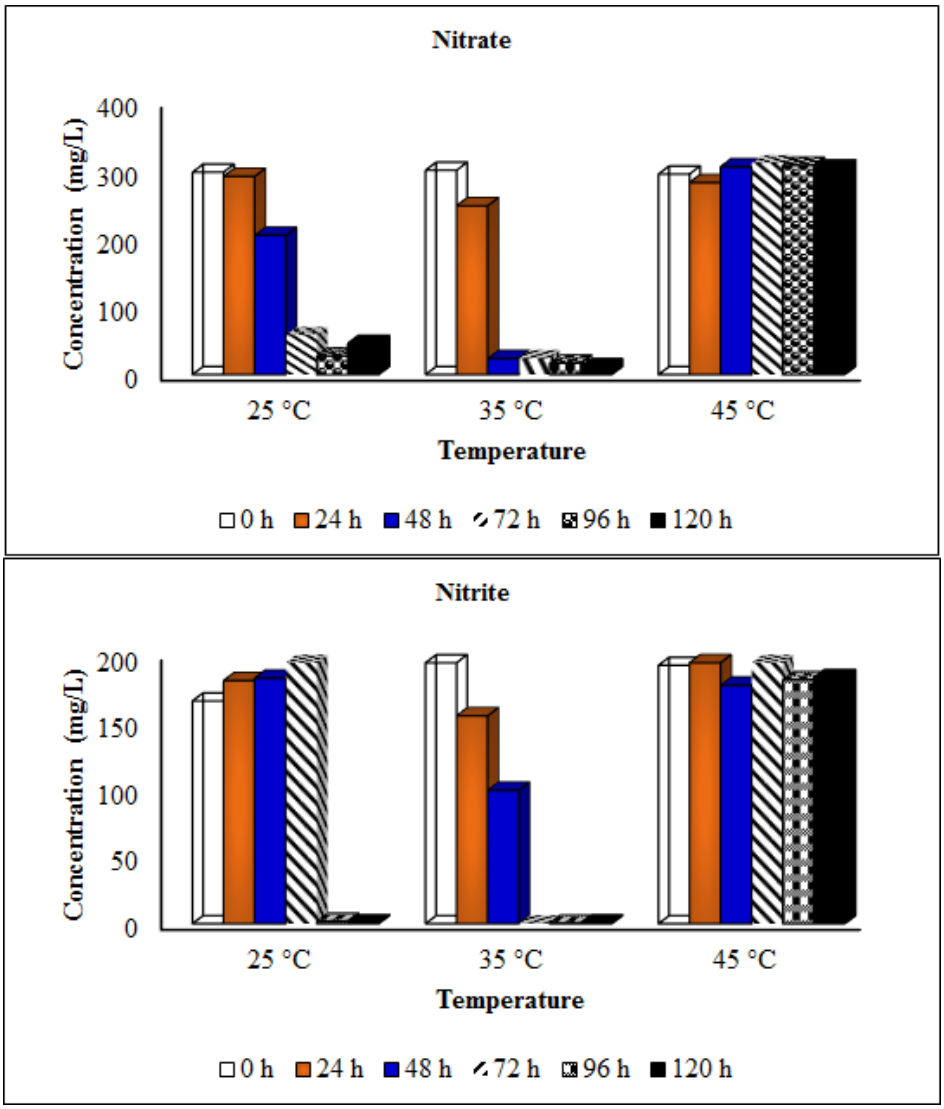

Figure 4: Nitrate and Nitrite Concentrations in the Media at the Different Incubation Temperatures in Presence of the Free Cells

As presented in Table 1, increases in pH of the media were observed at the different temperatures. This trend was irrespective of the cells used for inoculation. Generally, the highest pH increase was observed at incubation temperature of 35 ^{o}C.

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Table 1: pH Profile of the Media at the Different Incubation Temperatures

Temperature	Initial	Final	% Change
Alginate-Immobilized Cells			
25 °C	6.56	8.75	33.4
35 °C	6.29	9.22	46.5
45 °C	6.53	8.69	33.1
Agarose-Immobilized Cells			
25 °C	6.59	9.06	37.5
35 °C	6.41	8.95	39.6
45 °C	6.54	8.66	32.4
Agar-Immobilized Cells			
25 °C	6.59	8.91	35.2
35 °C	6.50	8.96	37.9
45 °C	6.53	8.42	29.0
Free Cells			
25 °C	6.52	8.90	36.5
35 °C	6.61	9.04	36.8
45 °C	6.58	8.08	22.80
Uninoculated Control			
25 °C	6.58	6.58	0
35 °C	6.52	6.60	1.2
45 °C	6.52	6.53	0.15

Initial and final represent pH of the medium at 0 h and 120 h, respectively. All values are averages of triplicate analysis. All % values are increases a study on the treatment of nitrate contaminated water using an electrochemical method

Nitrate and Nitrite Uptake Studies at the Different Initial Biomass

As shown in Figure 5, nitrate concentrations in the media in presence of the different initial inoculum of the alginate immobilised cells were observed to show significant decreases after 48 h of incubation. This decrease in nitrate concentration was observed to be consistent till the end of incubation. After the 120 h incubation period, nitrate levels in the media showed a decrease from an initial concentration of 382.5 mg/L, to final levels of 4.69 mg/L,21.29mg/L, 24.55 mg/L and 20.04 mg/L, when 2, 4, 6 and 8 alginate beads were used for inoculation, respectively (Figure 5). Although, the concentrations of nitrate in the media were observed to differ throughout the period of incubation, these differences were not observed to differ significantly among the different inoculum concentrations ($p \leq 0.05$).

In the case of nitrite levels in the media a remarkable decrease in the concentration was observed after incubating for 48 hours. The decrease showed consistency till incubation was terminated. After the 120 h incubation period, nitrite levels exhibited a decrease from an initial concentration of 156.78 mg/L, to final levels of 0.18 mg/L,0.19 mg/L,0.48 mg/L and 0.32 mg/L when 2,4,6 and 8 alginate beads were used for inoculation respectively (Figure 5).

Although, the nitrite concentration in the media was perceived to vary throughout the period of incubation, these differences were not observed to vary considerably among the different inoculum concentrations ($p \leq 0.05$).

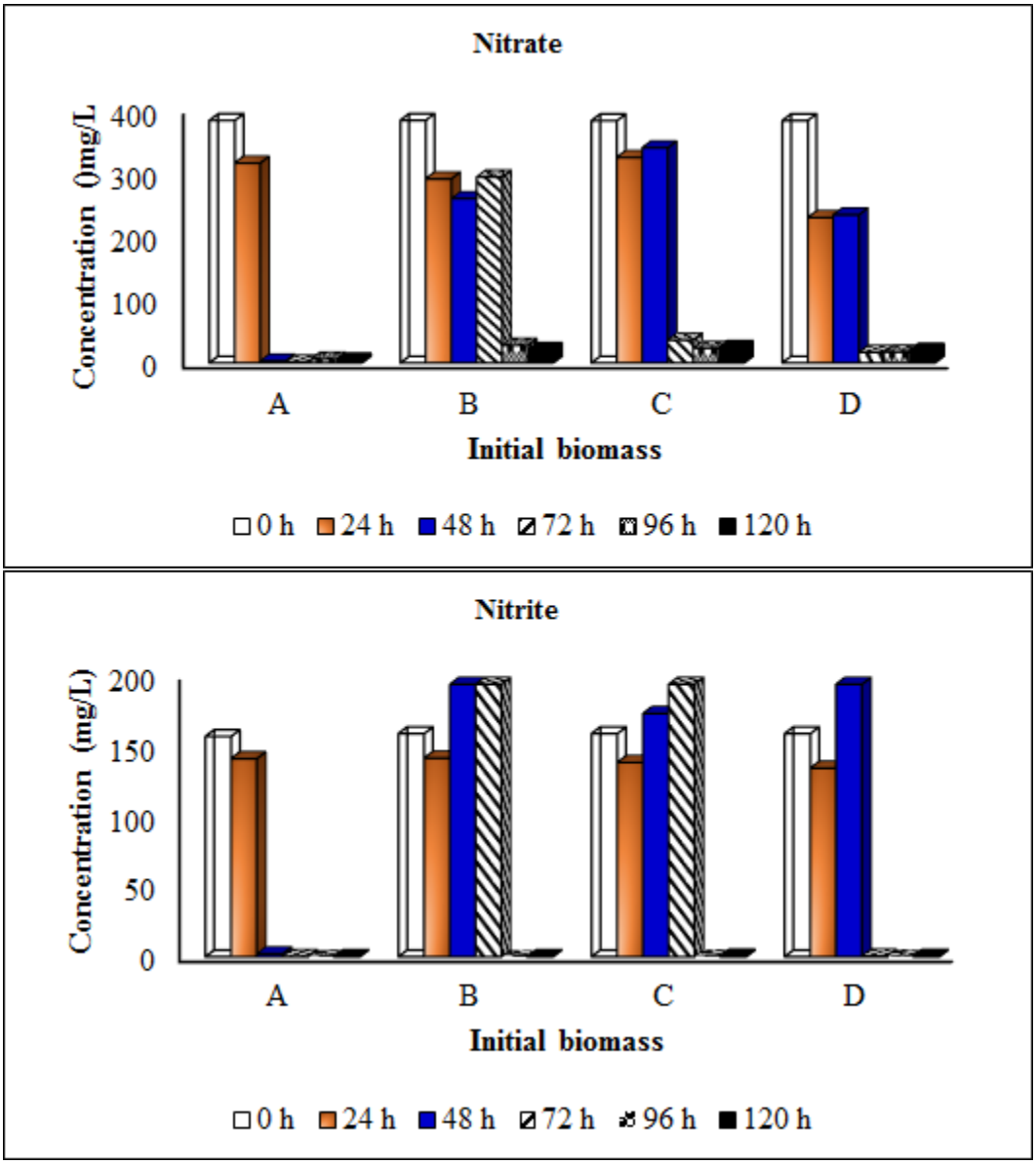

Figure 5: Nitrate and Nitrite Concentrations in the Media in Presence of the Different Initial Biomass of the Sodium Alginate-Immobilised Cells (A, B, C and D, Represent Alginate Immobilized Beads of 2, 4, 6 and 8, Respectively; an Alginate Immobilized Bead Contained Approximately 15 CFU/mL)

In presence of the agar immobilized cells, remarkable decrease in nitrate concentration was observed from 48 h of incubation, when 1 bead was used for inoculation. When 2, 4 and 6 beads were used as initial inoculum, remarkable decrease in nitrate concentration was observed from 72 h of incubation. At the end of the 120 h incubation period, nitrate concentration was observed to reduce from an initial level 310.50 mg/L to final levels of 4.7mg/L, 24.4mg/L,25.5mg/L,25.1mg/L when 1, 2, 4 and 6 agar beads were used as initial inoculum, respectively (Figure 6). Though the nitrate concentrations in the media were observed to differ between the different inoculum used for the study, these differences were not observed to be significant ($p \leq 0.05$).

For nitrite concentration in the media at the different concentrations of the agar immobilized cells, decrease in concentration was observed with time. This trend was irrespective of the initial inoculum

used. As was observed for nitrate, remarkable decrease in nitrite concentration was observed after 48 h incubation when 1 agar-immobilize bead for used for inoculation. When 2, 4 and 6 beads were used as respective inoculum, remarkable decrease in nitrite concentration was observed from 72 h incubation. At the end of the 120 h incubation time, nitrite levels showed a decrease from 158.58 mg/L to 0.18 mg/L, 0.60 mg/L, 0.73 mg/L and 0.43 mg/L, when 1, 2, 4 and 6 agar-immobilized beads were used as initial inoculum, respectively (Figure 6). Although the nitrite concentrations in the media differed throughout the period of incubation, these differences were not observed to vary considerably among the different initial inoculum concentrations ($p \leq 0.05$).

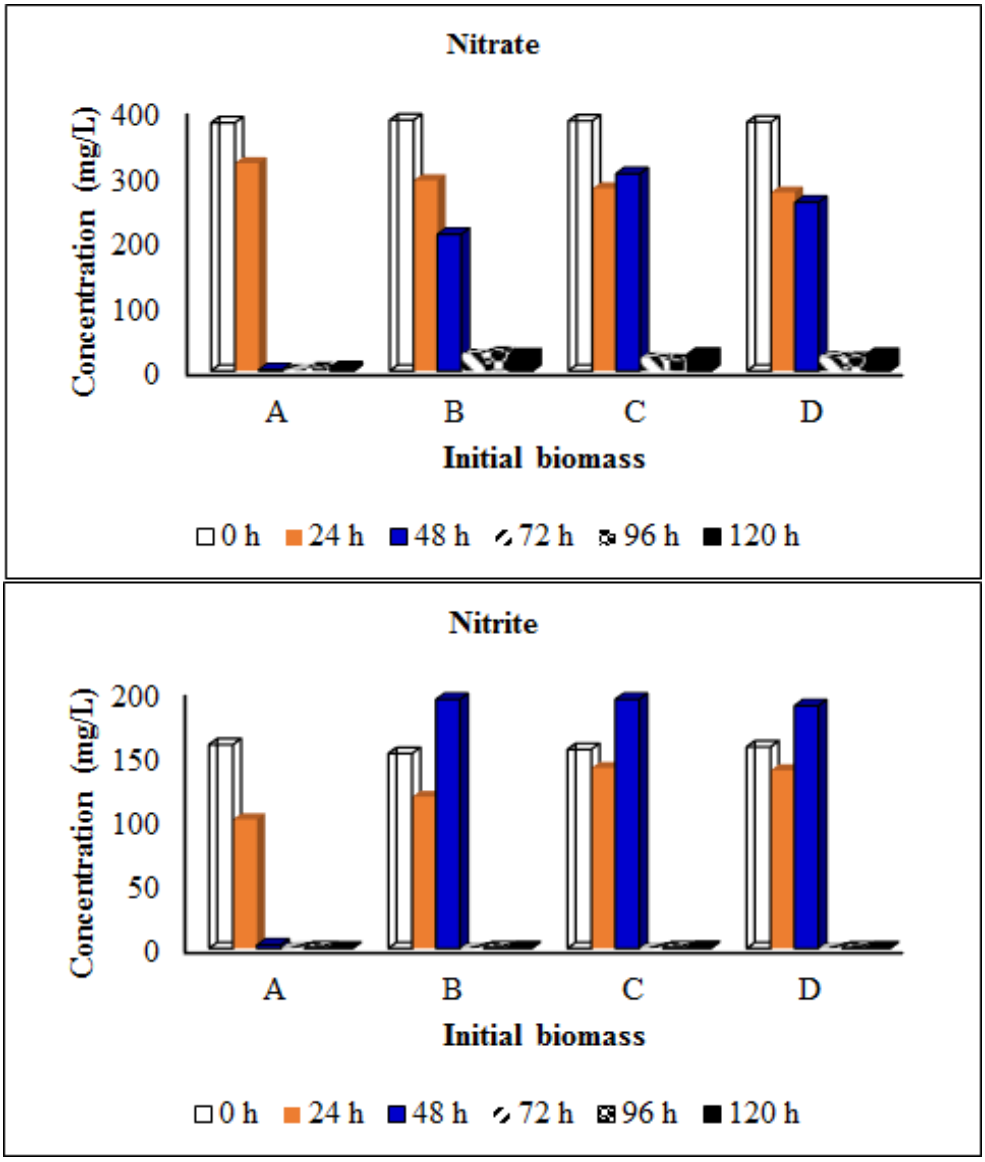

Figure 6: Nitrate and Nitrite Concentrations in the Media in Presence of the Different Initial Biomass of the Agar-Immobilised Cells; (A, B, C and D, Represent Agar Immobilized Beads of 1, 2, 4 and 6; an Agar Immobilized Bead Contained Approximately 68 CFU/mL)

When different initial inoculum sizes of the agarose immobilized cells were used for inoculation, remarkable reduction in the concentration of nitrate in the media was observed after 48 h of incubation. This reduction in concentration was consistent till the end of incubation. At the end of the 120 h

incubation period, nitrate levels in the media depicted a decrease from the initial concentration of 380.5 mg/L to final levels of 4.7 mg/L,18.6mg/L,16.7mg/L,16.2mg/L, when 1, 2, 4 and 6 agarose lumps were used, respectively (Figure 7). Though the nitrate concentrations in the media were observed to differ during the course of incubation, these variations were not observed to differ considerably among the different inoculum concentrations ($p \leq 0.05$).

In the case of nitrite levels in the media, significant reduction in concentration was observed after 48 h of incubation. The reduction in nitrite concentration was consistent with time throughout the duration of incubation. After the 120 h incubation period, nitrite levels showed decreases from 157.8 mg/L at 0 h to final levels of 0.4 mg/L,1.0 mg/L,0.7 mg/L and 1.1 mg/L when 1, 2, 4 and 6 agarose lumps were used for inoculation respectively (Figure 7). Although the nitrite concentrations in the media were observed to differ throughout the period of incubation, these differences were not observed to contrast considerably amid the different inoculum concentrations ($p \leq 0.05$).

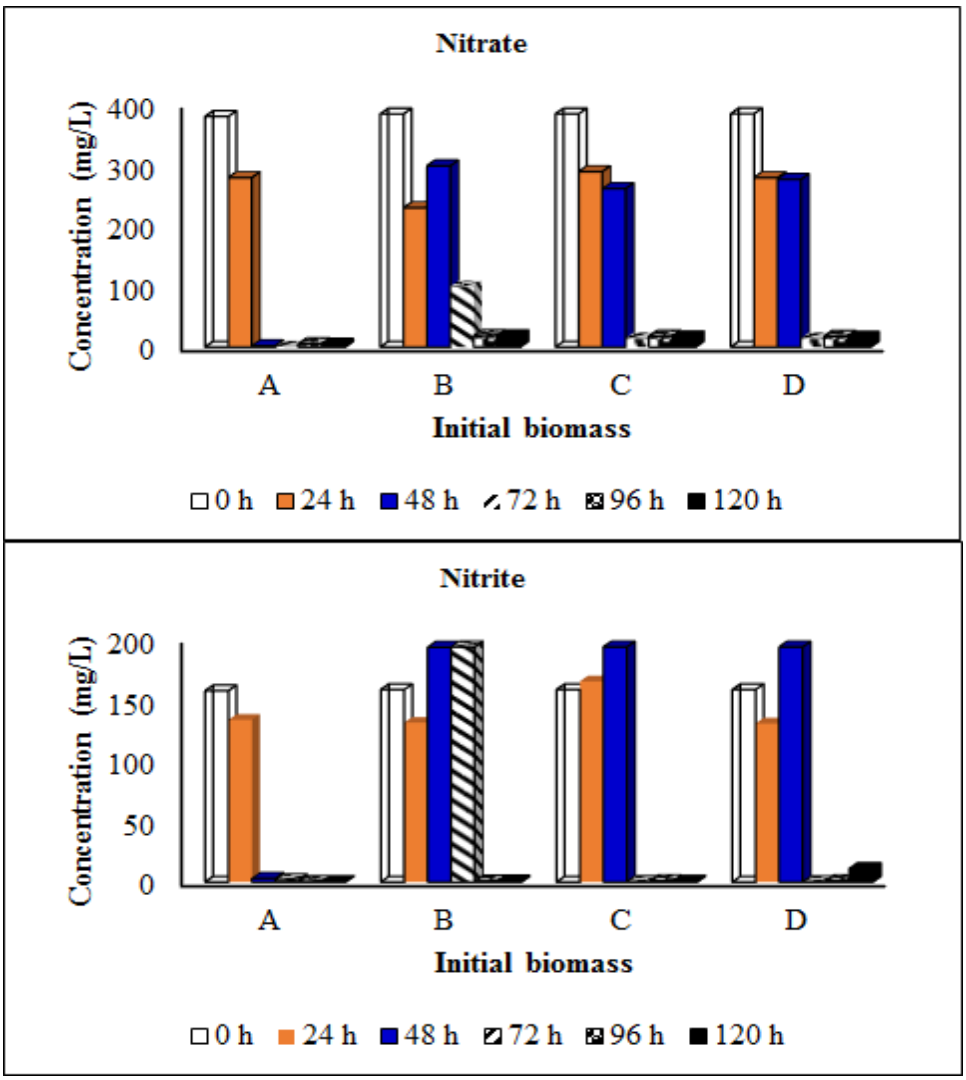

Figure 7: Nitrate and Nitrite Concentrations in the Media in Presence of the Different Initial Biomass of the Agarose-Immobilised Cells (A, B, C and D, Represent Agarose Immobilized Beads of 1, 2, 4 and 6; an Agarose Immobilized Bead Contained Approximately 58 CFU/mL)

As indicated in Figure 8, nitrate concentrations in the media in presence of the different initial inoculum of the free cells, were observed to display substantial decreases after 24 h incubation. This decrease was

consistent with time and was irrespective of the different initial inoculum used for investigation. At the expiration of the 120 h incubation period, nitrate levels in the media revealed a decrease from the initial concentration of 340.5 mg/L to final levels of 4.3 mg/L, 21.3 mg/L, 18.8 mg/L, 22.4 mg/L when 1.32 x 10^3 CFU/mL, 2.64 x 10^3 CFU/mL, 3.96 x 10^3 CFU/mL and 5.28 x 10^3 CFU/mL were used as initial inoculums, (Figure 8). Despite the differences in nitrate levels observed at the different inoculum concentrations, these were not observed to be significantly different ($p \leq 0.05$).

For nitrite, remarkable decrease in the concentration was observed after 48 h incubation. This trend was irrespective of the initial inoculum used for investigation. After the 120 h incubation period, nitrite levels were observed to decrease from the initial concentration of 158.7 mg/L, to final concentrations of 0.33 mg/L,0.3 mg/L,0.2 mg/L and 0.9 mg/L, when 4.3 mg/L, 21.3 mg/L, 18.8 mg/L, 22.4 mg/L when 1.32 x 10^3 CFU/mL, 2.64 x 10^3 CFU/mL, 3.96 x 10^3 CFU/mL and 5.28 x 10^3 CFU/mL of the free cells were used inoculation, respectively (Figure 8). As was observed for nitrate, although the nitrite concentration in the media differed at the different initial inoculum sizes used for inoculation, these differences were not observed to be significant ($p \leq 0.05$).

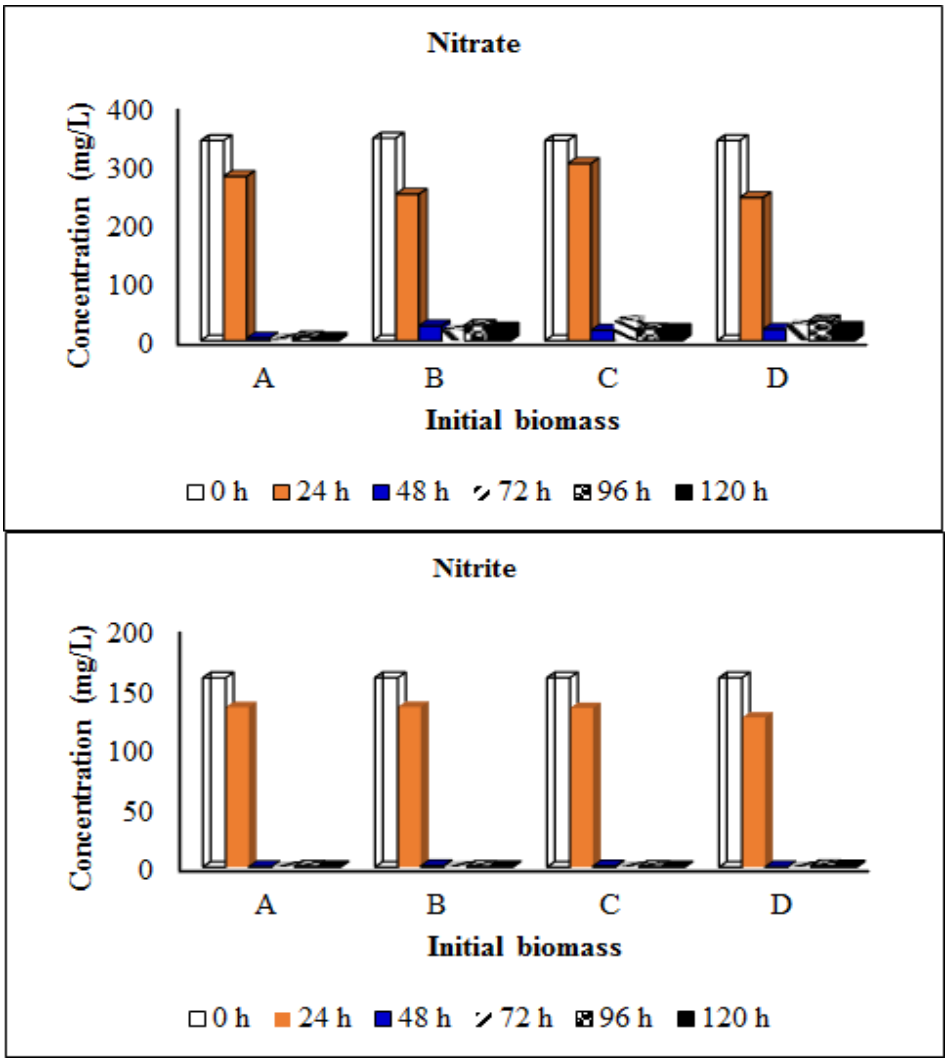

Figure 8: Nitrate and Nitrite Concentrations in the Media in Presence of the Different Initial Biomass of the Free Cells (A, B, C and D Represent 4.3 mg/L, 21.3 mg/L, 18.8 mg/L, 22.4 mg/L when 1.32 x 10^3 CFU/mL, 2.64 x 10^3 CFU/mL, 3.96 x 10^3 CFU/mL and 5.28 x 10^3 CFU/mL, Respectively)

As presented in Table 2, in the presence of the immobilized and free cells of the *Pseudomonas aeruginosa*, pH of the media at the different initial inoculum were observed to show increases with time. At the expiration of incubation, highest pH increases were observed at 45 %, 40 %, 43.10 % and 34.80 % when 12 alginate immobilized beads, 6 agarose immobilized beads, 4 agar immobilized beads and 2 ml of free cells were used as initial inoculum (Table 2).

Table 2: pH Profile of the Media at the Different Initial Biomass Concentration

Initial Biomass	Initial	Final	% Change
Alginate-Immobilised Cells			
2 beads	6.93	8.78	27.0
4 beads	6.24	8.81	41.2
8 beads	6.22	8.60	38.3
12 beads	6.24	9.05	45.0
Agarose Immobilised Cells			
1 bead	6.92	8.16	17.9
2 beads	6.25	8.60	37.6
4 beads	6.26	8.67	38.5
6 beads	6.28	8.76	40.0
Agar Immobilised Cells			
1 bead	6.92	8.74	26.3
2 beads	6.26	8.88	41.9
4 beads	6.19	8.86	43.1
6 beads	6.16	8.52	38.3
Free Cells			
1.32×10^3 CFU/mL	6.92	8.20	18.5
2.64×10^3 CFU/mL	6.49	8.75	34.8
3.96×10^3 CFU/mL	7.03	8.85	25.9
5.28×10^3 CFU/mL	7.07	8.80	24.5
Uninoculated Control			
	7.00	6.95	-0.7

Initial and final represent pH of the medium at 0 h and 120 h, respectively. All values are averages of triplicate analysis. All % values were increases, except negative value that represent decrease. An immobilized bead of alginate, agarose and agar contained approximately 15 CFU/mL, 58 CFU/mL and 68 CFU/mL, respectively.

Discussion

In the present study, optimum temperature for nitrate and nitrite uptake in presence of the cells was observed to range from 25°C to 35°C. In presence of the immobilized and free cells, no decrease in nitrate and nitrite concentration was observed at 45°C. Wastewater temperature is considered as an important factor that determines the nutrient uptake abilities of bacteria. Although, all the temperatures explored during the period of this research work were within the mesophilic range, according to reports, biological treatment processes are hastened in warm temperatures and slow in cool temperatures but treatment processes can stop in extremely cold temperatures (Metcalf and Eddy, 2003).

Despite the fact that temperature is indicated to be a vital factor that affects the growth and metabolic processes of microorganisms, there are conflicting reports on its influence on nutrient removal in biological systems (Mino, 2000; Seviour *et al.*, 2003). The discrepancies in temperature findings among different investigators is suggested to be due to the differences in substrates, variation in analytical procedures, differences in system configuration and use of varying operations settings, thus, making the comparison of results a difficult task (Larsdotte, 1994). Temperature is known to affect reaction kinetics and performance of biological nutrient removal systems (Erdal *et al.*, 2003). When temperature is low,

microbial activities are restricted. In extremely high temperatures, microbial death can occur (David *et al.*, 1997). Although, growth of the immobilized and free cells was observed at the different temperatures, nitrate and nitrite removal were observed to occur only at 25 ^{o}C and 35 ^{o}C. No nitrate or nitrite removal was observed at 45 ^{o}C, apart from in the presence of the alginate-immobilized cells when nitrate removal was observed from 72 h of incubation. Some investigators have indicated that the ideal temperature for nutrient removal is within the range of 28 ^{o}C to 33 ^{o}C. Despite the fact that different organisms have varying temperature ranges for growth, it is indicated that optimal growth temperature do not necessarily translate to optimum for nutrient uptake (Mamais and Jenkins, 1992; Sabalowsky, 1999; Saito *et al.*, 2004).

In a study on carbon, plant, and temperature control of nitrate removal from wetland mesocosms, at a temperature of between 11-12° C water temperature over a 7-day time span, nitrate concentrations in the overlying water decreased approximately to about 50 % while at 27 ^{o}C water temperature, concentrations were observed to zero for all treatments within 48 h (David *et al.*, 1997). Li *et al.*, (2010) have reported that high temperature is a favourable condition for nitrate reduction in In the present study, significant decreases in nitrate concentrations in the media were observed in presence of the different initial inoculums concentrations of the cells. This observation was irrespective of the free or immobilized cells used.

When investigating the effect of initial inoculum concentration on the efficiency of the non-nitrogen-fixing cyanobacterium *Phormidum uncinatum*, the results revealed that under standard assay conditions, nitrate uptake increased with increase in biomass used (Jone and Juan, 1993). In the present study, nitrate uptake was evident from 48 h of incubation. It is reported that rapid nitrate uptake is often observed in nitrate-deprived cells over a time scale of minutes. In determining if nitrate-deprived or nitrate-sufficient cells were more appropriate for rapid, consistent, and complete removal of nitrate from groundwater, nitrate uptake and cell growth were monitored in parallel in both types of cells by Hu *et al.*, (2000). They indicated in the report that in presence of the nitrate-deprived culture, rapid nitrate uptake was evident during the first 2 h of incubation, after which there was a decline in uptake for the next 30 h before uptake resumed when cell growth resumed. For the nitrogen-sufficient culture, lower initial nitrate uptake rate was initially observed during the lag phase, after which the rate of growth and uptake was increased exponentially. However, the latter showed a much shorter lag phase in both nitrate uptake and growth than the former. After the lag phase had been overcome, the rate of nitrate uptake by cells was independent of whether cells were nitrate deprived or nitrate sufficient (Jone and Juan, 1993). Nitrogen starvation in had also been reported to enhance significant increase in nitrate removal by free-living and immobilized cells (Jone and Juan, 1993). When investigating bioremediation of wastewater using microalgae, Chalivendra (2014) reported significant increases in removal percentages of nitrate, phosphate and heavy metal with increase in initial algae concentration. Similar observation has been reported by Rossi *et al.*, (2015), when investigating nitrate removal from wastewater through biological denitrification with OGA 24 in a batch reactor

The present study revealed decrease in nitrite concentration without respect to increase in inoculum size, although remarkable decreases were observed from 48 h of incubation. According to Payne (1976), temporary accumulation of nitrite was observed when *P. aeruginosa* was cultured under denitrifying conditions. This accumulation was reported to be due to consecutive induction. In this study, simultaneous nitrate and nitrite uptake from the medium was observed and this was irrespective of the initial inoculum concentration used for inoculation. Earlier investigators have reported that nitrite uptake can equal or exceed nitrate uptake in similar studies (Collos, 1998).

Conclusion

This study which was aimed at investigating the relationship of temperature and initial biomass concentration with nitrate and nitrite removal ability from nutrient media in presence of the immobilised and free cells of *Pseudomonas aeruginosa* revealed that uptake of the nutrients by the cells had no direct relationship with the concentration of the initial biomass of the cells used. This observation was irrespective of the immobilised and free cells.

The optimum temperature for nitrate and nitrite removal was observed to range from 25°C to 35°C. At 45°C, only negligible or no removal was observed. This observation was consistent in presence of the respective immobilised or free cells.

Despite the fact that the results of this study cannot be said to be exhaustive, it has still provided valuable information on the role of temperature and initial biomass on nitrate and nitrite removal ability of the test cells under the experimental conditions used for investigation.

Further studies on the effects of serial adaptation of the immobilized cells on removal of these nutrients could be the subject of future studies.

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