



Temperature and pH Relation to Nutrient Uptake by Immobilized Cells of *Pseudomonas aeruginosa*

O. B. Akpor^{1*}, F. T. Otitolaye¹ and C. O. Adetunji¹

¹Department of Biological Sciences, Landmark University, PMB 1001, Omu-Aran, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Author OBA designed the study, interpreted the results, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author FTO performed the experiment, interpreted the results and contributed to the first draft of the manuscript and author COA interpreted the results and contributed to the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Nutrient enrichment of aquatic ecosystem can lead to increase in algae and aquatic plants and the loss of component species and ecosystem function. The aim of this study was to investigate the relationship of temperature and pH to nutrient uptake from nutrient media by immobilized cells of *Pseudomonas aeruginosa*. The study, which was carried out under shaking flask conditions used sodium alginate, agar-agar and agarose immobilized. The incubation temperatures used for the study were 25°C, 35°C and 45°C while the pH were 6, 8 and 10. Just before inoculation and every 24 h, for 120 h, aliquot samples were aseptically withdrawn from each flask for the estimation of phosphate and sulphate concentrations in the media, using standard procedures. The results revealed maximum decreases in phosphate and sulphate concentrations in the media in presence of the immobilized cells occurred at 25°C and 35°C, respectively. In presence of the alginate-immobilized cells, lowest phosphate decreases from the initial concentration of 71.1 mg/L to 50.1 mg/L, 50.6 mg/L and 46.8 mg/L, after 96 h incubation were observed at incubation temperatures of 25°C, 35°C and 45°C, respectively. There was however no significant between phosphate concentrations in the media at the different incubation temperatures in presence of the alginate-

*Corresponding author: E-mail: akpor.oghenerobor@lmu.edu.ng;

immobilized cells. For sulphate uptake, in presence of the alginate-immobilized cells, highest decrease was observed at incubation temperature of 35°C. At this temperature, remarkable decrease was observed from 24 h incubation till the end of incubation. Generally, the study showed optimum pH for sulphate removal by the cells to be 8. At pH 6 and 10, only slight decreases in sulphate levels were observed at the end of incubation in the presence of the immobilized cells. In presence of the alginate-immobilized cells, remarkable increases in sulphate levels were observed throughout the period of incubation. The study was able to give an insight into the phosphate and sulphate uptake efficiency of the immobilized cells of the test bacteria under the experimental conditions used for investigation.

Keywords: Phosphate; sulphate; immobilization; wastewater; nutrient removal.

1. INTRODUCTION

Although phosphorus is an important component of phospholipids, ATP and nucleic acid in cells, its presence in excess amount in receiving water bodies is a leading cause of eutrophication. Some of the effects of eutrophication are dissolved oxygen depletion, death of aquatic life and other hazards to human population and the environment [1,2]. The ecological and public health impacts caused by the presence of eutrophic nutrients in wastewater makes it crucial to lower their limits to comply with standards set by regulatory bodies before emission into receiving water bodies [3,4].

The most commonly used treatment methods used in the elimination of phosphorus and sulphate from polluted wastewater are chemical and biological [5]. Biological removal process is however advocated over chemical methods owing to its simplicity, economic and various other environmental benefits [6]. Over the few decades, biological nutrient removal processes have been employed for the removal of total phosphorus from wastewater by using different microorganisms under different environmental conditions [7]. Several methods including chemical precipitation, crystallization, biological treatment reverse osmosis and adsorption have been applied to promote the removal of dissolved sulfate [8,9,10].

Although other microorganisms such as fungi, protozoa and microalgae have been implicated in nutrient removal from wastewater, nutrient removal in wastewater treatment systems have been attributed mainly to bacteria [11]. Bacteria are known to be of great numerical importance in wastewater treatment systems and are responsible for the stabilization of wastes coming into any treatment system. A number of studies have revealed the involvement and efficiency of bacteria in nutrient removal from wastewater.

Examples of bacteria that have been reported as essential in biological nutrient removal include *Pseudomonas*, *Klebsiella*, *Acinetobacter*, *Escherichia coli*, *Bacillus* and *Enterobacter* [2,12].

Although the role and importance of free living bacteria in nutrient uptake from wastewater is well documented in literatures, in recent years, there has been increases interest in the use of immobilized microbial cells for wastewater treatment [13,14]. This is because immobilized cells are said to be advantageous over conventional suspension due to their high biomass and metabolic activity and strong resistance to toxic chemicals [15,16,17]. This study was therefore aimed at evaluating the relationship of temperature and pH in phosphate and sulphate removal from nutrient media by sodium alginate, agarose and aga-agar immobilized cells of *Pseudomonas aeruginosa*.

2. MATERIALS AND METHODS

2.1 Media Composition and Preparation

The media used for the study was composed of sodium acetate (5 g/L), peptone (5 g/L), yeast extract (5 g/L) potassium di-hydrogen phosphate (0.5 g/L) and magnesium sulphate (0.5 g/L). For preparation, each of the constituents were weighed and dissolved separately in small quantities of distilled water before mixing them in large flask and adding the required quantity of distilled.

After preparation, the media was dispensed in 200 mL quantities in 250 mL capacity Erlenmeyer's flasks, cotton plugged with non-absorbent cotton wool and sterilized in an autoclave at 121°C for 15 min at 15 psi. To ensure that the sterilization was effective, during each batch study, the sterilized media were left on the work bench for 24 h to observe for

turbidity. Only flasks where no turbidity were observed were used for the study.

2.2 Preparation of Immobilized Cells

The bacteria isolate used for the study was *Pseudomonas aeruginosa* on (ATCC 9027). Prior to use, the purity of the isolate was ascertained by streaking a broth culture of the isolate on nutrient agar and incubated at $35 \pm 2^\circ\text{C}$ for 24 h for growth. Before using for immobilization, the pure culture of the isolate was cultured in sterile nutrient broth of 24 h, after which the cells were suspended from the broth by centrifugation at 5000 rpm for 30 min, using Anke TDL 5,000B Centrifuge. The supernatant was discarded while the pellets were suspended in sterile normal saline (0.85% NaCl).

For immobilization in sodium alginate, 20 mL of the free cells that were suspended in normal saline mixed with 150 mL of 5% sterile sodium alginate solution and homogenized on a vortex mixer. The bacteria-alginate mixture was pipetted drop wise into 1000 mL of crosslinking solution. The crosslinking solution was prepared by dissolving 25 g of CaCl_2 powder in 1000 ml of distilled water and then sterilized in an autoclave at 121°C for 15 min at 15 psi. Bead formation was achieved at room temperature as soon as the bacteria-alginate drops came in direct contact with the calcium chloride solution. The beads were allowed to fully harden for 1-2 hours. The beads were washed several times with sterile normal saline and then refrigerated in a sterile reagent bottle. To determine the viability of the immobilized cells, few of the beads were inoculated on nutrient agar plates and incubated at 37°C for 48 h for growth and estimation of the approximate number of cells.

For immobilization in the agarose and agar solutions, 20 mL of the suspended cells in normal saline were mixed with 150 mL of the 5% agarose solution or 3% of the agar solution at 45°C in a flask before dispensing in petri dishes to solidify. After solidifying, a sterile cork borer was used to bore holes on the gel produced to produce circular beads. The viability of the cells immobilized on the beads were determined at described earlier.

2.3 Phosphate and Sulphate Removal Studies

For the phosphate and sulphate removal studies, to a 250 mL capacity conical flask containing 200

mL of the media, a known inoculum of the respective immobilized cells was inoculated under aseptic conditions before incubating at in an orbital shaker (STUART: S1500) at a particular temperature. Just before inoculation and every 24 h, for 20 h, aliquot samples were aseptically withdrawn from each flask for the estimation of phosphate and sulphate concentrations in the media, using standard methods [18].

The study was carried out in batches at varying incubation temperatures (25°C , 35°C and 45°C) and pH (6, 8 and 10). For each batch experiment, uninoculated control setups were run concurrently with inoculated setup.

Statistical analysis was carried out using the PAST (Paleontological Statistics Software Package for Education and Data Analysis) software. The test for the comparison of means was done using the one-way analysis of variance (ANOVA) at a probability level of 0.05.

3. RESULTS AND DISCUSSION

3.1 Effect of Temperature

At the different incubation temperatures in presence of the alginate-immobilized cells, lowest phosphate decreases were observed after 96 h incubation, decreasing from the initial value of 71.1 mg/L to 50.1 mg/L at 25°C , 50.6 mg/L at 35°C and 46.8 mg/L at 45°C . At the end of the 120 h period of incubation, final phosphate values were observed to be 51.30 mg/L, 55.00 mg/L and 57.70 mg/L, at incubation temperatures of 25°C , 35°C and 45°C , respectively (Fig. 1). There was no significant between phosphate concentrations in the media at the different incubation temperatures ($p \leq 0.05$). For sulphate levels, in presence of the alginate-immobilized cells, the highest decrease was observed at incubation temperature of 35°C . At this temperature, remarkable decrease was observed after 24 h till the end of incubation. After the end of the incubation period, sulphate levels was observed to decrease from the initial level of 1017 mg/L to 577 mg/L at 35°C . At 25°C and 45°C , increases in sulphate concentration were observed at the expiration of incubation, although some decrease was observed at 96 h and 72 h, respectively (Fig. 1). A significant decrease in sulphate concentration was observed at 35°C , when compared with the other incubation temperatures ($p \leq 0.05$).

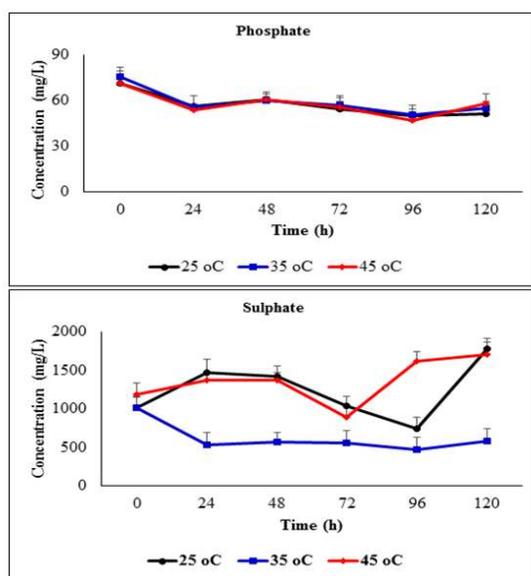


Fig. 1. Phosphate and sulphate levels in the media in presence of the alginate-immobilized cells at the different temperatures

As shown in Fig. 2, in the presence of the agarose-immobilized cells, a decrease in phosphate concentration in the media were observed at the different incubation temperatures, rather it was only a minute decrease at 45°C. At incubation temperature of 35°C, decrease in phosphate concentration was observed from 24 h of incubation and was consistent with time till the expiration of the incubation period. At the expiration end of the 120h incubation period, phosphate concentration in the media was observed to decrease from the initial value of 71.5 mg/L to 43.79 mg/L, 46.80 mg/L and 58.60 mg/L at incubation temperatures of 25°C, 35°C and 45°C, respectively (Fig. 2). The decrease in phosphate concentration in the media was observed to be significant higher at 35°C than at either 25°C or 45°C ($p \leq 0.05$). For sulphate concentrations, although increases were observed with time, at incubation temperatures of 25°C, 35°C and 45°C, remarkable decreases in concentration were observed after 120 h and 96 h, and 72 h, respectively. After 120 h, sulphate levels in the media at 25°C showed a decrease from the initial 1010 mg/L to 538 mg/L. At incubation temperature of 35°C, sulphate levels decreased from 1017 mg/L to 799 mg/L after 96 h incubation. At 72 h incubation, sulphate levels were observed to decrease from 1191 mg/L to 886 mg/L at 45°C (Fig. 2). No significant

difference was observed between the sulphate decreases at the different incubation temperatures ($p \leq 0.05$).

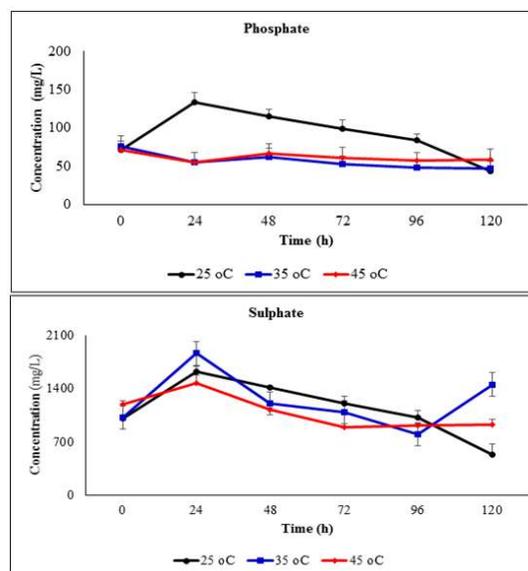


Fig. 2. Phosphate and sulphate levels in the media in presence of the agarose-immobilized cells at the different temperatures

As depicted in Fig. 3, in the presence of the agar-immobilized cells, phosphate levels in the media were observed to show decreases throughout the period of sampling. This trend was evident at incubation temperatures of 35°C and 45°C. The lowest reduction in phosphate concentration was observed after 96 h, at 35°C and 45°C and 72 h at 25°C. At the end of the 120 h incubation period, final phosphate concentrations were observed to vary from initial levels of 71.10 mg/L, 75.50 mg/L and 71.20 mg/L to 121.52 mg/L, 53.50 mg/L and 45.50 mg/L, at 25°C, 35°C, and 45°C respectively (Fig. 3). Phosphate decreases at incubation temperatures of 35°C and 45°C were observed to be significantly higher than the decrease at incubation temperature of 25°C ($p \leq 0.05$). For sulphate levels in presence of agar-immobilized cells, concentrations in the media show significant decreases after 72 h incubation at 25°C and 45°C. Although significant decreases in concentration were also observed at the end of the 120 h incubation at 35°C and 45°C, a spike in concentration was observed at 120 h, at 25°C. Sulphate concentrations after the 120 h incubation were observed to decrease from the initial concentration of 1017 mg/L to final concentrations of 1482 mg/L, 727 mg/L and

930 mg/L at 25°C, 35°C and 45°C respectively (Fig. 3). The variations in sulphate concentration at the different incubation temperatures were not observed to vary significantly ($p \leq 0.05$).

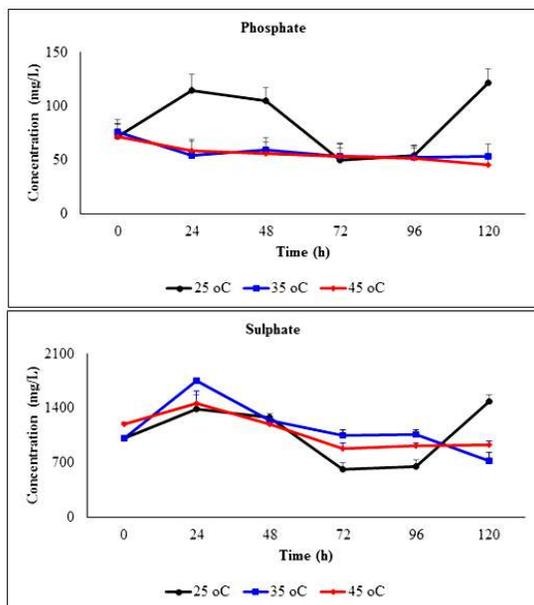


Fig. 3. Phosphate and sulphate levels in the media in presence of the agar-agar-immobilized cells at the different temperatures

As shown in Table 1, increases in pH of the media in presence of the respective immobilized cells were observed at the end of incubation. This observation was irrespective of the incubation temperatures. Highest increases in pH were observed at 35°C in presence of the alginate-immobilized and agarose-immobilized cells while in the agar-immobilized, highest pH increases were observed at incubation temperatures of 25°C and 45°C, respectively (Table 1).

During this study sulphate and phosphate removals in presence of the presence of the immobilized cells were observed at 25°C and 35°C, respectively, although the extent of removals was different at the different incubation temperatures. In a study by Brdjanovic et al. [19], incubation temperature of 20°C was observed as the optimum temperature for nutrient removal. It is indicated that polyphosphate accumulating organisms are mostly cryophiles and prevail more at 20°C and lower mesophilic ranges [20,21]. Although the effects of temperature on the reaction

performance and kinetics of biological nutrient removal systems has been reported, there still exists some controversy on its impacts on enhanced biological nutrient removal systems. It is opined that the variations in temperature reports among different researchers may be due to the differences in substrates, system configurations, analytical techniques and the application of different operational conditions. These variations could make the comparison of results, challenging [22,23]. The external carbon source used in the media for this study was sodium acetate.

Table 1. Changes in pH of the media the different incubation temperatures

Temperature	Initial	Final	% change levels
Alginate-immobilized cells			
25°C	6.50	8.62	32.62
35°C	6.49	8.92	37.44
45°C	6.46	8.75	35.45
Agarose-immobilized cells			
25°C	6.50	9.00	38.46
35°C	6.49	8.95	37.90
45°C	6.46	8.84	36.84
Agar-agar-immobilized cells			
25°C	6.50	8.93	37.38
35°C	6.49	8.91	37.27
45°C	6.46	8.85	37.00
Un-inoculated control			
25°C	6.50	6.65	2.31
35°C	6.49	6.63	2.16
45°C	6.46	6.77	4.80

Initial and final represent pH of the medium at 0 h and 120 h, respectively. All values are averages of triplicate analysis. All percentage values were increases

It has been reported that the optimum temperature for growth may not necessarily be the same for optimum nutrient reduction and oxidation [24,25,26,27]. An incubation temperature of 25°C was observed to be optimum for sulphate uptake by immobilized cells in the present study. This observation negates the findings by Al-Zuhair et al. [28], who indicated an incubation temperature of 35°C as the optimum for sulphate removal in presence of sulphate-reducing bacteria. There is the indication that despite the fact most sulphate-reducing bacteria have optimum performance at mesophilic temperature ranges, they are still efficient at a temperature range of 10-50°C [28]. Temperature is said to be an important parameter in wastewater that determines the

extent of nutrient removal by bacteria. Increase in temperature is reported to cause a corresponding increase in chemical and enzymatic reactions in the cell, thus leading to faster growth rate. In a laboratory-scale experiment with synthetic wastewater, Gonzales-Martines and Wilderer [29], reported phosphate release with decreasing temperature.

The effects of light and temperature on microalgal growth and nutrient removal using experimental and mathematical approaches have been reported. In the report, both kinetic growth parameters and nutrient removal were observed to show similar responses to light and temperature. With an increase in temperature, higher specific growth rates, biomass productivities and nutrient removal efficiencies were achieved. The report further indicates that among the varying temperatures used for investigation, a temperature of 25°C was shown to lead to higher biomass productivities and nutrient removal efficiencies by the microorganisms. In a study on the effects of temperature (10°C, 23°C and 28°C), on nutrient removal in a green sorption media, a temperature increase was observed for nutrient removal with significant orthophosphate removal at higher temperatures in the media mixes than in the control setup [30,31,32].

3.2 Effect of pH

With respect to pH, in the presence of alginate-immobilized cells, remarkable decreases in phosphate concentrations in the media were observed at the end of incubation at pH 6 and 8. At a pH of 10, no decrease in phosphate levels in the media was observed throughout the period of incubation. Phosphate concentrations in the media after the 120 h incubation period was observed to vary from 116.90 mg/L to 52.60 mg/L, from 117.10 mg/L to 48.50 mg/L and from 110.70 mg/L to 177.30 mg/L, respectively (Fig. 4). The decreases in phosphate levels at pH 6 and 8 were observed to be significantly lower than at pH 10 ($p \leq 0.05$). In the case of sulphate concentration, in presence of alginate-immobilized cells, remarkable increases in sulphate concentration were observed throughout the period of incubation. This observation was irrespective of the initial pH of the media used for investigation. After the end of the incubation period, sulphate levels in the media were observed to increase from 276 mg/L to 1918 mg/L, from 278 mg/L to 1642 mg/L and from 288 mg/L to 1773 mg/L, at pH 6, 8 and 10,

respectively (Fig. 4). The variation in sulphate concentrations in the media at the different initial pH were not observed to differ significantly ($p \leq 0.05$).

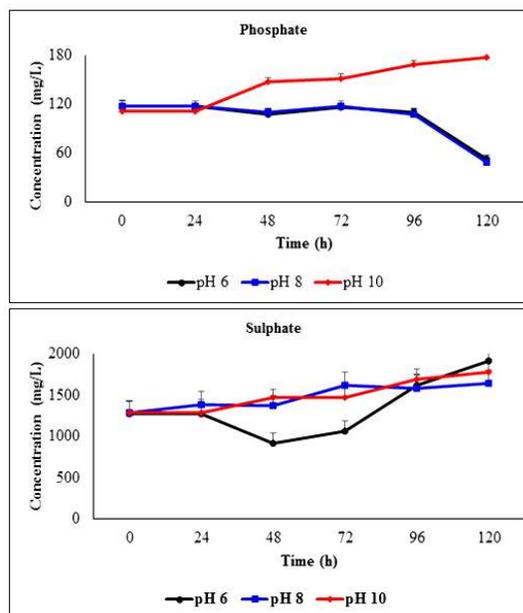


Fig. 4. Phosphate and sulphate levels in the media in presence of the alginate-immobilized cells at the different pH

In media containing the agarose-immobilized cells, significant decrease in phosphate concentration was only observed after 24 h, at pH 6, decreasing from 117.6 mg/L to 27.6 mg/L. At the other initial pH, phosphate levels were only observed to show significant decreases at the end of incubation, decreasing from 117.6 mg/L to 45.9 mg/L and 45.1 mg/L, at pH 6 and pH 8, respectively (Fig. 5). The phosphate and sulphate levels in the media did not vary significantly at the different pH ($p \leq 0.05$). For sulphate levels in the media in presence of the agarose immobilized cells, no remarkable decrease in concentration was observed throughout the period of incubation. This observation was similar at the different pH investigated. Sulphate levels at the end of incubation showed increases from 976 mg/L to 1032 mg/L, at pH 6, from 988 mg/L to 1453 mg/L, at pH 8 and from 972 mg/L to 1177 mg/L, at pH 10 (Fig. 5). Generally, the phosphate and sulphate levels in the media did not vary significantly at the different pH ($p \leq 0.05$). As shown in Fig. 6, in the presence of the agar-immobilized cells, significant decreases in

phosphate concentrations were observed at the different pH at the end of incubation. At the expiration of the incubation periods, phosphate levels at the different pH were found to decrease from 118.80 mg/L to 58.6 mg/L, from 122.40 mg/L to 46.7 mg/L and from 112.90 mg/L to 52.2 mg/L at pH 6, 8 and 10, respectively. In the case of sulphate, slight decrease in concentration was only observed at pH 6 after 72 h incubation. At the other pH, sulphate levels were either to show mainly increases. No significant decreases were observed at pH 8 and 10. From an initial concentration of 1804 mg/L, sulphate levels were observed after 72h to be 814 mg/L at pH 6, 1017 mg/L at pH 8 and 1308 mg/L at pH 10 (Fig. 6). The phosphate and sulphate concentrations in the media at the different pH were not observed to vary significantly ($p \leq 0.05$).

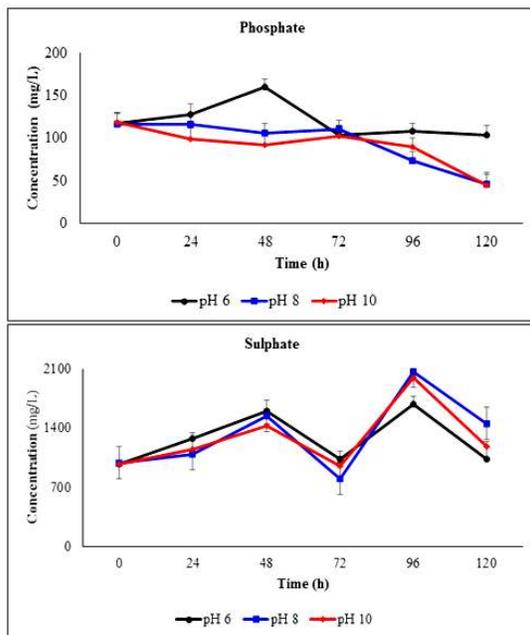


Fig. 5. Phosphate and sulphate levels in the media in presence of the agarose-immobilized cells at the different pH

As shown in Table 2, an increase in pH of the media was observed at pH 6 and 8. At pH 10, decreases in pH were observed at the expiration of incubation. This trend was also evident in presence of the immobilized or free cells.

The present study revealed an optimum pH of 6 for phosphate uptake in presence of the immobilized cells. It is indicated that biological wastewater treatment is affected by the acidity or alkalinity of wastewater. pH is known to have

effect on enzymes, microbial affinity for substrates, substrate availability, and substrate or product production [33,34]. The pH range of most wastewaters is reported to range from 6 to 9 [35]. Widdel [36], postulated that the optimal initial pH for remarkable phosphate removal is likely in the range of 6.4 and 7.2. There is however the indication that a higher pH could lead to greater phosphate uptake in wastewater containing acetate, which may be due to increased energy requirement for the transportation of acetate. When investigating the effects of pH on enhanced biological phosphorus removal metabolisms, Schuller and Jenkins, [37] have reported anaerobic P release increasing with increasing pH. The optimum pH during biological removal is usually different from optimum pH during chemical removal processes. It is reported that during phosphate removal from wastewater using chemical precipitation, the optimum conditions for the removal is achieved with percentage removal of 90 % with CaO concentration of 40 mg/L at pH value of (8.5-10). With aluminum sulphate the percentage removal was indicated to be 85% at pH value of 4, while with iron sulphates the best removal of 80% was achieved at a pH range of 8.5-10 [38].

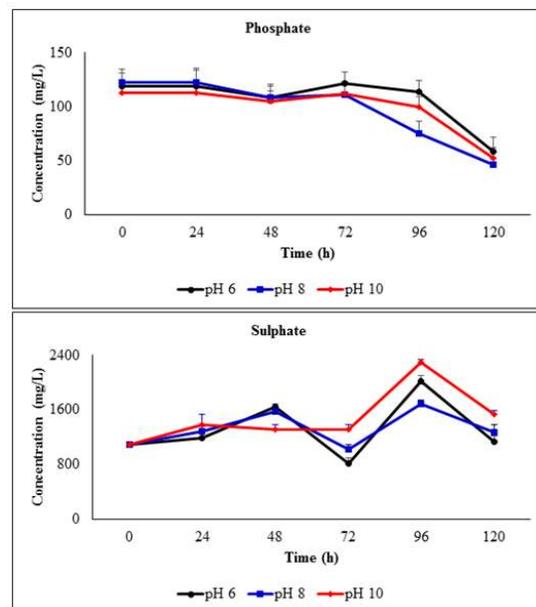


Fig. 6. Phosphate and sulphate levels in the media in presence of the agar-agar-immobilized cells at the different pH

This study revealed optimum pH for sulphate removal by the cells to be 8. At pH 6 and 10, only slight decreases in sulphate levels were

observed at the end of incubation in the presence of the test bacterial species. In similar studies conducted by Al- Zuhair et al. [28], their findings indicated an optimum pH of 7. Zhao [39], reported that sulphate reducing bacteria are usually suitable for growth in the neutral conditions of pH 6–8. It has been observed that for sulphate reducing bacteria to thrive, they require a pH range of 5 to 8. At a different pH range, the rate of microbial sulphate reduction is indicated to be reduced. A pH less than 5 is said to inhibit sulphate reduction [40]. In a study on the effect of pH on sulfate removal from wastewater using a bio-electrochemical system at pH range between 2.5 and 10.5, the optimum condition for sulfate removal from wastewater was reported at pH 4.5. This finding was however different from some reports that have indicated neutral condition as being suitable for sulphate reducing bacteria to treat pollutants. Their finding indicated that sulfate removal at systems operated at pH 4.5, 6.5 and 8.5 were higher than that of 2.5 and 10.5 [41].

Table 2. Variation in pH of the media at the different initial pH

pH	Initial	Final	% change
Alginate immobilized cells			
pH 6	6.05	8.85	-46.28
pH 8	7.18	8.83	-22.98
pH 10	9.47	9.25	2.32
Agarose immobilized cells			
pH 6	6.04	8.73	-44.54
pH 8	7.67	8.65	-12.78
pH 10	9.55	8.87	7.12
Agar immobilized cells			
pH 6	6.12	8.49	-38.73
pH 8	8.05	9.02	-12.05
pH 10	9.40	9.09	3.30
Un-inoculated control			
pH 6	6.05	5.91	2.31
pH 8	7.67	8.64	-12.65
pH 10	9.7	8.35	13.92

Initial and final represent pH of the medium at 0 h and 120 h, respectively. All values are averages of triplicate analysis. Positive and negative values represent % decreases and increases, respectively

4. CONCLUSION

This study, which was aimed at investigating the effects of temperature and pH on the phosphate and sulphate uptake ability of the immobilized cells of *Pseudomonas aeruginosa* showed remarkable phosphate uptake occurring at incubation temperatures of 35°C. The optimum

temperature for sulphate removal was however observed to range from 25°C to 35°C, with maximum removal observed at 25°C. This trend was irrespective of the immobilized or free cells used for inoculation.

In the case of pH, remarkable phosphate and sulphate removal was only observed at 6 and 8, respectively, a trend that was irrespective of the immobilized or free cells. Moreover, this study has given an insight into the phosphate and sulphate uptake potential of the immobilized of the *Pseudomonas aeruginosa* under the experimental conditions investigated.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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