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Research Article
Effect of Nitrogen Source on Dye Decolouration by Alginate-immobilized Cells of *Pseudomonas aeruginosa* and *Bacillus subtilis*

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Abstract

Background and Objective: The discharge of dye-contaminated wastewater into receiving water bodies culminates in several health and environmental damages. The objective of this study was to investigate the effect of nitrogen source on decolouration rate of bromothymol blue, crystal violet and methylene blue by immobilized cells of *Pseudomonas aeruginosa* and *Bacillus subtilis*. **Materials and Methods:** The study was carried out under batch experimental setup made of five different nitrogen sources (casein, sodium nitrate, sodium nitrite, tryptone and urea) used at respective concentrations of 5 g L^{-1}. **Results:** In all the experimental batches, decolouration of the dyes in presence of the test bacterial cells showed progressive increase with time of incubation irrespective of the nitrogen source and the dye investigated. In presence of the *Bacillus subtilis* cells, highest decolouration rates of 59.83% (bromothymol blue), 70.19% (crystal violet) and 26.48% (methylene blue) were observed in media containing casein, sodium nitrate and sodium nitrite, respectively. In *Pseudomonas aeruginosa* inoculated cells, highest decolouration rate was observed for the bromothymol blue in media that contained the sodium nitrate. In presence of the *Pseudomonas aeruginosa* cells, only minute decolouration of the crystal violet and methylene blue was observed in media containing the respective nitrogen sources. **Conclusion:** It could thus be concluded that an appropriate nitrogen source is vital for effective microbial decolouration of the test dyes.

Key words: Decolouration, dye, inoculated cells, immobilization, nitrogen source, *Bacillus subtilis* cells

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

One of the major sources of pollution around the world is the textile industry and its dye-containing waste waters. Based on volume and composition, effluents from textile industries are classified as one of the most contaminating of all the industrial contaminants. It is opined that almost 200,000 t of dyestuff is discharged into the environment every year[1,2]. Without adequate treatment, these dyes could remain in the environment for a long period of time[3].

The release of dyes into the receiving water bodies is known to lead to several health and environmental damages. Apart from aesthetic problems, dyes disrupt light penetration and also reduce oxygen mass transfer in water bodies. They have effect on photosynthesis of aquatic plants, hence affecting the growth of primary producers and consumers[4-6]. Dyes are also indicated to be toxic to microbial population and could lead to mutagenic and carcinogenic effects in animals and humans[7].

Because of the harmful effects of dyes in receiving water bodies, several treatment methods (physical, chemical and biological) have been employed for their decolouration in water. Although physical and chemical methods are reported to be effective, they are however economically not viable with the transfer of waste from various forms, since the products that are not degraded may be more toxic than the parent compound. Generally, biological methods are mostly preferred, being environmentally friendly and cost effective[8-11]. To safeguard public health and receiving water bodies, removal of dyes from water, a number of cost effective and environmentally friendly approaches are always explored. A wide range of studies have reported the dye decolouration potential of pure and mixed cultures of bacteria. Bacteria are known to have specific ability in the production of enzymes for the degradation of several compounds, both aerobically and anaerobically. A number of bacteria species, including *Pseudomonas aeruginosa, Pseudomonas oleovorans* and *Pseudomonas putida* and *Bacillus subtilis* have been implicated as having dye decolouration ability[12-15].

During the decolouration of dye, it is opined that the presence of nitrogen sources is regarded as a vital media component that is required for NADH regeneration and serve as an electron donor for the microbial reduction of azo dyes[16]. Since microbial breakdown of contaminants is indicated to be dependent on the presence of nitrogen[17], this study sought to investigate the effect of different nitrogen sources on decolouration of three selected laboratory dyes by alginate-immobilized cells of *Bacillus subtilis* and *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Preparation of dye calibration curves: The dyes used for the study were bromothymol blue (BB), crystal violet (CV) and methylene blue (MB).The optimum wavelength for each dye was determined by scanning dilute solutions of the, respective dyes over several wavelengths, starting from 400 nm until a wavelength that gave maximum absorbance reading was obtained. Optimum wavelengths of 520, 430 and 600 nm were recorded as ideal wavelengths for maximum absorbance for CV, BB and MB, respectively.

The calibration curves for the respective dyes were prepared by taking absorbance based on the determined optimum wavelength at different concentrations (2, 4, 6, 8 and 10 mg L^{-1}) for each dye using a Jenway 6705 UV/VIS spectrophotometer. After reading the absorbance values of the different concentrations, plots of absorbance against concentrations were then made and the equation of a line was obtained and regression value of not less than 0.9 obtained for each dye.

Wastewater media: The media used for the study was wastewater obtained within the vicinity of Landmark University, Omu-Aran, Kwara State, Nigeria. Before usage, the wastewater was filtered using Whatman No. 1 filter paper after which 5 g L^{-1} of glucose was added to serve as carbon source and 5 g L^{-1} of the respective nitrogen source (casein, sodium nitrate, sodium nitrite, urea and tryptone) before adding a respective dye at known concentration.

After preparation of the media, 200 mL quantities were dispensed in 250 mL capacity conical flasks and autoclaved (at 121°C, 15 psi) for 15 min. To ascertain the effectiveness of the sterilization process, the sterilized media was left on the study bench overnight. Flasks that showed no turbidity the next day were used for further studies.

Preparation of immobilized cells: The bacteria used for the study were *Pseudomonas aeruginosa* and *Bacillus subtilis*. For immobilization, 100 mL of the suspended cells of each of the respective test bacterial species in normal saline were mixed with 300 mL sterile 5% sodium alginate and agitated in a shaker for 2 h at a shaking speed of 100 rpm. After shaking, the microbe-alginate mixture was dispensed in a sterile burette attached to a retort stand. The mixture was allowed to flow dropwise from the burette into a flask containing sterile 2.5% calcium chloride for bead formation. The beads were allowed to stand for 2 h to harden before washing out the excess media with sterile distilled water. Before usage, selected beads were cultured on sterile nutrient agar to

ensure viability. Only immobilized beads in flasks whose beads showed growth after incubation were used for further studies. All immobilized beads were refrigerated at 0±2°C until required for further analyses.

Dye decolouration study: For decolouration experiment, a known quantity of the respective immobilized cells was inoculated into the respective dye-containing media under aseptic conditions before incubation at a 30±2°C in a shaker at a shaking speed of 120 rpm. Immediately after inoculation and at every 24 h for 144 h, aliquot samples were withdrawn from each flask, centrifuged at 5000 rpm for 10 min and the absorbance read at the recorded wavelength for the respective dye and concentration estimated from the calibration curve.

In all experimental setups, concentrations obtained were converted to decolouration rates. Decolouration rate was calculated as:

$$\text{Decolouration rate}(\%) = \left[\frac{A-B}{A}\right] \times 100$$

where, 'A' and 'B' represent initial absorbance and absorbance after reaction, respectively. All experiment setups and assays were carried out in duplicates. In all experimental, uninoculated controls were ran alongside the inoculated setups.

Statistical analysis: Statistical analysis was carried out using the SPSS statistical software. The One-Way Analysis of Variance (ANOVA) was used to determine the comparison of mean sat% confidence interval.

RESULTS

As shown in Fig. 1, in presence of the respective nitrogen sources, the media inoculated with the *Bacillus subtilis* showed consistent increase in decolouration of BB with time. After 144 h incubation, decolouration rates of 59.83, 33.73, 46.76%, were observed in media containing casein, sodium nitrate, sodium nitrite, tryptone and urea, respectively (Fig. 1). With the exception of media that contained tryptone, when compared with the control (setup that contained no added nitrogen source), decolouration rates of the BB were significantly higher in media with the respective nitrogen sources. A comparison of decolouration rates in media with the respective nitrogen sources revealed significantly ($p<0.05$) higher decolouration rates in the casein or sodium nitrite-containing media.

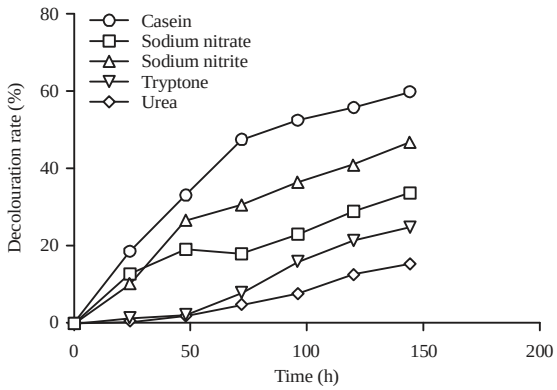

Fig. 1: Decolouration rate of the bromothymol blue in presence of the *Bacillus subtilis* at the different nitrogen sources

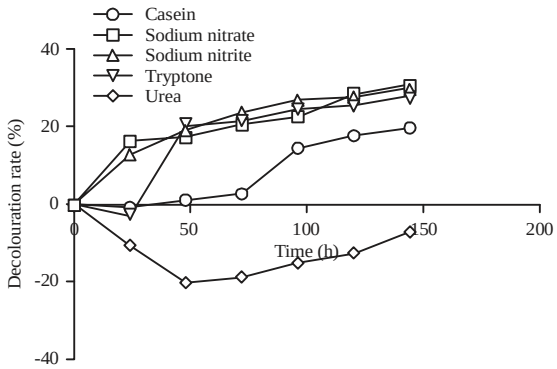

Fig. 2: Decolouration rate of the bromothymol blue in presence of the *Pseudomonas aeruginosa* at the different nitrogen sources

In the *Pseudomonas aeruginosa* cells, at the end of the 144 h incubation period, BB showed zero decolouration in media containing urea as nitrogen source. In media containing the other respective nitrogen sources, decolouration rate of BB was observed to increase progressively with time of incubation. After the 144 h period of incubation, decolouration rate of BB in media containing casein, sodium nitrate, sodium nitrite and tryptone was observed to be 19.63, 30.34 and 27.74%, respectively (Fig. 2). In the presence of the *Pseudomonas aeruginosa*, decolouration of the BB showed significantly ($p<0.05$) higher rates in media that contained the test nitrogen sources compared to the control. Generally, significantly ($p<0.05$) higher decolouration rates were observed in media containing either sodium nitrate, sodium nitrite or tryptone compared to media with casein or urea.

In presence of the *Bacillus subtilis* cells, decolouration rates of -0.13, 39.31, 23.50, 3.68 and 10.64% were observed

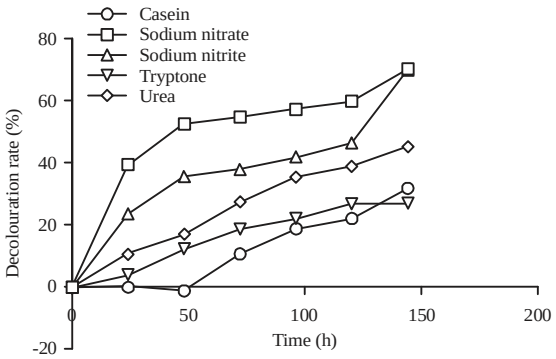

Fig. 3: Decolouration rate of the crystal violet in presence of the *Bacillus subtilis* at the different nitrogen sources

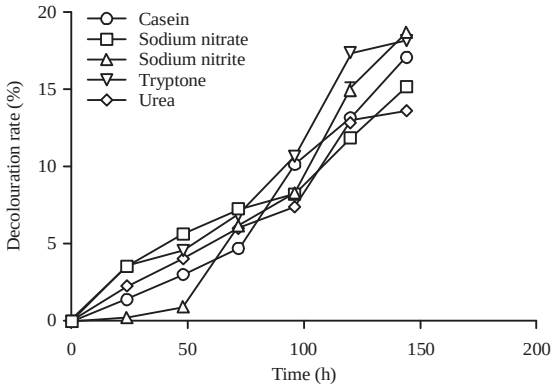

Fig. 4: Decolouration rate of the crystal violet in presence of the *Pseudomonas aeruginosa* at the different nitrogen sources

for the CV after 24 h incubation, when casein, sodium nitrate, sodium nitrite, tryptone and urea were used as respective nitrogen sources. However, at the expiration of the incubation time, decolouration rates were observed to increase to 31.62, 70.19, 69.97, 26.70 and 45.21%, respectively (Fig. 3). Generally, significantly ($p<0.05$) higher decolouration of the CV was observed in media that contained the respective nitrogen sources compared to control. Among the nitrogen sources, decolouration of the CV in media that contained either sodium nitrate or sodium nitrite were observed to be significantly ($p<0.05$) higher than media that contained the other nitrogen sources.

In the *Pseudomonas aeruginosa* cells, the observed decolouration rate for CV was minute throughout the period of incubation. Although decolouration rates after 24 h incubation were 1.34, 3.54, 0.21, 3.54 and 2.2%, at the expiration of the 144 h incubation time, decolouration rates of 17.02, 15.12, 18.63, 18.09 and 18.55% were observed in media containing casein, sodium nitrate, sodium nitrite,

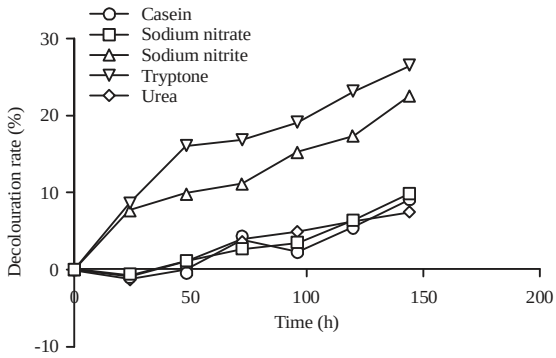

Fig. 5: Decolouration rate of the methylene blue in presence of the *Bacillus subtilis* at the different nitrogen sources

tryptone and urea, respectively (Fig. 4). No significant difference in decolouration of the CV was observed in presence of the *Pseudomonas aeruginosa* between media containing the respective nitrogen sources rate. Decolouration in media containing the respective nitrogen sources did not also differ significantly ($p>0.05$) from that of the control.

For the MB dye, decolouration rates in presence of the *Bacillus subtilis* cells, no remarkable decolouration was observed throughout the period of incubation, although there was progressive increase in decolouration rate with time. This trend was irrespective of the different nitrogen sources used in the media. From decolouration rates of -1.03, -0.88, 7.75, 8.66 and -1.33% at 24 h incubation, decolouration rates showed increases to 9.11, 9.82, 22.47, 26.48 and 7.46% in media containing casein, sodium nitrate, sodium nitrite, tryptone and urea, respectively (Fig. 5). Decolouration of the MB in presence of the *Bacillus subtilis* was observed to be significantly ($p<0.05$) higher in media that contained either sodium nitrite or tryptone than the control setup and media containing the other nitrogen sources.

In presence of the *Pseudomonas aeruginosa* cells, no remarkable decolouration of the MB was observed throughout the period of incubation. Although decolouration rates of -0.17, 1.41, -1.01, 3.35 and 6.39% were observed at 24 h incubation, at the expiration of the period of incubation, decolouration rates of 5.46, 4.95, 4.50, 12.04 and 16.24% were observed in media containing casein, sodium nitrate, sodium nitrite, tryptone and urea, respectively (Fig. 6). Generally, significantly ($p<0.05$) higher decolouration of the MB in presence of the *Pseudomonas aeruginosa* was observed in media that contained either tryptone or urea compared to the control and media that contained the other respective nitrogen sources.

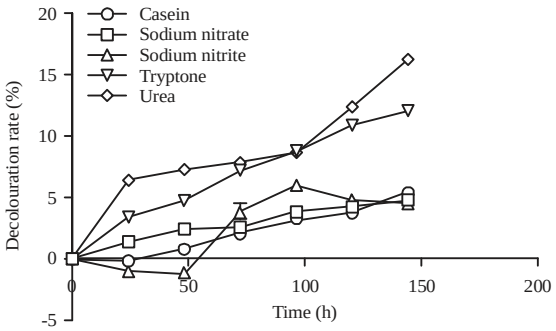

Fig. 6: Decolouration rate of the methylene blue in presence of the *Pseudomonas aeruginosa* at the different nitrogen sources

DISCUSSION

The dyes used in this study were CV, BB and MB. The dyes were reported to represent a large group of dyes that cause environmental concern, due to colour, bio-recalcitrance, potential toxicity and carcinogenicity to animals and also humans[18,19].

In this study, dye decolouration was measured as reduction in absorbance, which was attributed to the metabolic activities of the inoculated bacterial cells. Reduction in absorbance or observed decrease in visible and UV absorbance peaks have been reported by earlier workers to suggested degradation of dye and their subsequent aromatic compounds[14].

The present study used immobilized cells of the test bacteria species for investigation. The choice of immobilized cells was intentional. It is opined that cell immobilization could increase the degree of pollutant degradation and removal in wastewater[20,21]. Immobilized cells are reported to be more advantageous than free cells because of their higher cell density per reactor volume, ease of separation from reaction medium, possibility of use for continuous operation without the risk of cells being carried away downstream, decrease in lag phase, increased substrate conversion, decreased possibility of inhibition by products, reduction in retention time and control of cell growth[22].

The immobilization matrix used in this study was sodium alginate-calcium chloride mixture. Although several other immobilization matrices (carrageenan, agar, cellulose, polyacrylate and polyamide) have been reportedly used in the immobilization of various microbial cells, the choice of sodium alginate-calcium chloride mixture as the immobilization matrix for the cells used in this study was deliberate. Besides being one of the most commonly used support for cell immobilization, sodium alginate-calcium chloride mixture is indicated to be low cost, easily available, easy to prepare and good in terms of biocompatibility[23].

The bacterial species (*Pseudomonas aeruginosa* and *Bacillus subtilis*) used in the study have been previously implicated in the breakdown and mineralization of reactive dyes[24-27]. The role of *Pseudomonas aeruginosa* strain in effective decolouration of dyes has been reported by earlier investigators[26,28]. In addition, Saratale *et al*.[29] have reported the use of *Bacillus*, *Klebsiella* and *Pseudomonas* species in dye decolorization in previous studies. In their study on the decolouration of orange 3R, *Pseudomonas* and *Bacillus* exhibited the highest dye decolouration rate of 92% at the end of incubation. When using free and immobilized cells of *Pseudomonas luteola*, decolouration rates of 50% and 85%, respectively have been reported[30].

Data obtained in this study showed that, in presence the *Bacillus subtilis* cells, highest decolouration of the bromothymol blue was observed in media that contained either casein or sodium nitrite as nitrogen source while in the presence of crystal violet, highest decolouration was observed in media that contained either sodium nitrite or sodium nitrate as nitrogen source. In the case of the methylene blue dye, highest decolouration was observed in media that contained either sodium nitrite or tryptone as nitrogen source. When the *Pseudomonas aeruginosa* was used for inoculation, remarkable decolouration of the bromothymol blue was observed when sodium nitrate or sodium nitrite or tryptone was used as nitrogen source. No remarkable decolouration was however observed for the crystal violet and the methylene blue in presence of any of the nitrogen sources.

The use of sodium nitrate as a nitrogen source has been reported by earlier workers. In study carried out using *Bacillus pumilis*, a dye decolorization rate of 87.21% was observed within 24 h when nitrate was used as nitrogen source[15]. The presence of nitrogen source is indicated to be a limiting factor in the breakdown of pollutants in wastewater. This is because organisms may tend to have inadequate source at a concentration that is low of nitrogen source or may impact the alteration in pH at a nitrogen content that is high. In a study on the effect of carbon and nitrogen sources on *Escherichia coli* bacteria in removing dyes, glucose and ammonium sulphate were reported to be to be the best carbon and nitrogen sources respectively[31].

In a related study, the presence of yeast extract and beef extract in media was reported to increase dye decolouration while the presence of peptone, urea, ammonium sulphate and

ammonium nitrate revealed poor decolorization rate[32]. A study has reported significant decolouration of Ranocid fast blue dye in media that contained yeast extract as nitrogen source[33]. Andrade *et al.*[34] in their study reported on azo dye degradation by *Phanerochaete chrysosporium* in medium enriched with nitrogen in the presence of primary co-substrate, indicated optimum degradation occurring in reactors that contained glucose and ammonium sulphate as carbon and nitrogen sources, respectively. Furthermore, they observed that when glucose was substituted with saccharose, reactors containing ammonium nitrate as nitrogen source revealed the highest dye removal of 86%. Also, an investigation on the effect of carbon and nitrogen sources on biodegradation of textile azo dye Reactive Violet 5 by *Pseudomonas aeruginosa* GSM3 revealed glucose and yeast extract as the best carbon and nitrogen sources, respectively[35].

CONCLUSION AND FUTURE RECOMMENDATIONS

Overall, data generated in this study showed the dye degradative ability of the test organisms is influenced by the nitrogen source utilized. This further reinforced the earlier observation by some authors that nitrogen source is vital for microbial decoulouration of dyes. However, further studies are needed to ascertain the optimum concentration of nitrogen source that will enhance maximum decolouration. There is also a need for scale up studies in laboratory-scale microcosm and application in situ situation, which is the focus of the next study.

In general, this study discovered that alginate-immobilized cells of the test bacterial species are effective in decolouration of the test dyes in wastewater. The findings of this study could be beneficial in implementation of evidenced-based strategy for biological treatment of textile effluents.

SIGNIFICANCE STATEMENT

This research found that, highest decolouration of bromothymol blue was observed in the presence of *Bacillus subtilis* cells and in media that either contained casein or sodium nitrite as nitrogen source. On the other hand, media that contained either sodium nitrite or sodium nitrate as nitrogen source, highest decolouration was observed in the presence of crystal violet. The findings of this investigation could be applied in scale up studies and continuous process, for implementation in biological decolouration of dye effluents.

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