Decolourization of Azo Dye Direct Blue 15 Using Batch Culture of *Klebsiella* sp.

Chun Shiong Chong, Zaharah Ibrahim, Madihah Md Salleh, Noor Aini Abdul Rashid, Adibah Yahya, Wui Jin Wong

Department of Biology, Faculty of Science
Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia
Tel: +60-7-5532528, Fax: +60-7-5566162, E-mail: tccshiong80@yahoo.com

Abstract

Nowadays, the development of textile industries are mushrooming and the pollution resulting from the dye-wastewater is alarming. Colour is recognized as the first contaminant from the dye containing wastewater. The present study reports the decolourization of Direct Blue 15 by Klebsiella sp., originated from textile wastewater plant. The important parameters, including co-substrates, aeration, starting pH of culture and temperature for decolourization, were investigated. Mechanism of colour removal either by bioadsorption or biodegradation was determined. Amongst the co-substrates, glucose (0.2% w/v) was the preferred co-substrate for decolourization of Direct Blue 15. The optimal decolourization occurred under partial anaerobic condition at starting pH of about 6.0. Decolourization of Direct Blue 15 was most efficient at 45°C. Under the optimum conditions, it was found that the COD removal was 53.6%. Dye decolourization (81.9%) was successfully achieved within 24 hours; 0.03% was due to the bioadsorption and 81.87% was due to the biodegradation by Klebsiella sp., while the product of degradation of azo dye, sulfanilic acid, was detected using HPLC.

Keywords: Direct Blue 15, decolourisation, azo dye, *Klebsiella* sp., HPLC

1. Introduction

Azo dyes are extensively used in many fields such as textile industries, leather tanning industries, food industries, cosmetics industries and plastics industries [1,2]. The annual production of azo dyes, the largest group of dyes was estimated about 1 million tons [1].

Azo dyes are recalcitrant xenobiotics, thus, they are resistant to light, heat, oxidizing agent and biodegradation [3]. Thus, many of the dyes, especially synthetic azo dyes are difficult to remove by conventional waste treatment methods as they are stable due to their complex aromatic molecular structures. During the past two decades, some physico-chemical treatment methods have been suggested, however only a few of them are accepted due to low efficiency, high cost and inapplicability to a wide range of dyes [4]. Recently, Department of Environment (DOE), Malaysia maybe imposed colour as one of parameter under the regulatory lead to the requiring of more efficient and cost-effective treatment methods.

The use of microorganisms to decolour and biodegrade synthetic azo dyes from the industrial effluents offers appreciable advantages where it is relatively inexpensive and may detoxify the compounds via mineralization process [2]. It was reported that under anaerobic condition, azo dyes are readily reduced into their intermediates which are normally colourless aromatic amine compounds, cannot be further degraded under anaerobic degradation [5]. Aromatic amines on the other hand can be further degraded under aerobic condition [6].

In the present study, Direct Blue 15, a water soluble azo dye (Figure 1) was selected for carrying out microbial decolourization studies. Besides that, optimization of decolourization in different parameters, such as co-substrates, aeration, starting pH of culture and temperature, was also carried out. To confirm if decolourization process was due to the reduction of azo dyes, HPLC analysis was performed.

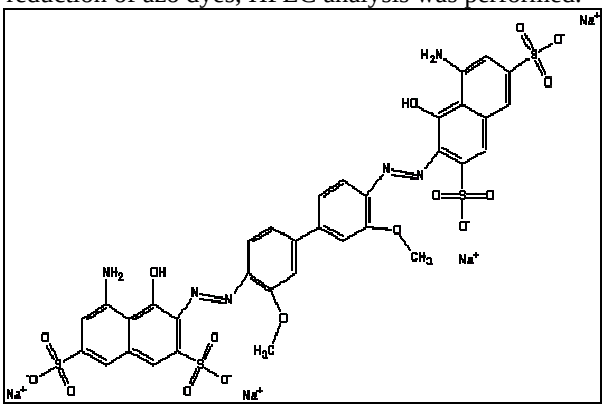

Figure 1. Structure of C.I. Direct Blue 15 [7]

* Corresponding Author. E-mail: zaharah@bio.fs.utm.my
Tel: +60-7-5534122, Fax: +60-7-5566162

2. Materials and methods

2.1 Chemical

Direct Blue 15 (C.I 24400) and sulfanilic acid (99 %) were purchased from Sigma and Fluka, respectively. All other chemical used throughout this study were of analytical grade.

2.2 Microorganism

Klebsiella sp. was isolated from a textile wastewater treatment using serial dilution method. This strain had the ability to remove some pure dyes, such as Orange II, Direct Blue 15, SF Yellow 3RS, SF Red 3BS, SF Yellow EXF and SF Black EXA, under partial anaerobic condition.

2.3 Growth medium

Filtered textile wastewater was used as medium in this study. This medium was supplemented with carbon source (sodium acetate, glycerol or glucose) and azo dye Direct Blue 15 (0.01g/L) from their respective filter sterilized stock solutions.

2.4 Optimization of decolourization

2.4.1 Effect of co-substrates

Three types of co-substrates had been used to determine their effect on decolourization process; these were glucose, glycerol and sodium acetate. Inoculum (10% v/v) was added into the textile wastewater medium containing Direct Blue 15 (0.01g/L). Decolourization under partial anaerobic condition was carried out by filling up the universal bottle, and then incubated under 37°C without shaking at pH 7 for 40 h. From these experiments, the most preferred co-substrate was used in subsequent experiments.

2.4.2 Effect of aeration

Inoculum (10% v/v) was added into the textile wastewater containing Direct Blue 15 (0.01g/L) and selected co-substrate (0.1% w/v). Decolourization under partial anaerobic condition was carried out by filling up the universal bottle and incubated at 37°C. For aerobic condition, the cultures were shaken at 100 rpm and 150 rpm at 37°C and incubated in a period of 40 h.

2.4.3 Effect of temperature

A range of temperature (27°C - 60°C) was used to study the effect of the temperature on azo dye decolourization. Inoculum (10% v/v) was added into textile wastewater medium containing Direct Blue 15 (0.01g/L) and selected co-substrate (0.1% w/v) at pH 7 and incubated under partial anaerobic condition over a period of 40 h.

2.4.4 Effect of pH

Effect of pH for decolourization of textile wastewater medium containing Direct Blue 15 (0.01g/L) was studied over a pH range of 5 to 10 by adjusting pH with HCl and NaOH. Incubation was carried out under optimized temperature, using selected co-substrate under partial anaerobic condition over a period of 40 h.

2.4.5 Effect of co-substrate concentration

Effect of selected co-substrate concentration for decolourization of textile wastewater medium was studied. Different concentrations of glucose, ranging from 0.1% to 0.5% (w/v) were used.

2.5 Batch culture experiment under optimized condition

Klebsiella sp. (10% v/v), at its active phase was transferred into medium for decolourization experiment. Incubation was done under optimized conditions, that is pH 6, glucose concentration (0.2% w/v), temperature (45^{o}C) under partial anaerobic condition. The parameters such as absorbance of dye, bioadsorption, COD and dye intermediates were monitored.

2.6 Analytical methods

2.6.1 Decolourization study

Absorbance of Direct Blue 15 measurements in medium were performed in a CARY 100 Bio spectrophotometer in the UV-Visible range. Colour was measured at the dye's optimum wavelength (584 nm). For this purpose, samples were centrifuged at 10 000 rpm for 15 min and absorbance value of supernatants were determined. The decolourization efficiency was expressed as the following Equation (1).

$$\text{Decolourization (\%)} = \frac{(I - F)}{I} \times 100 \quad (1)$$

where I = initial absorbance and F = absorbance of decolourized medium.

2.6.2 Bioadsorption study

The pellet from the centrifuged sample was suspended in 10 mL of distilled water. This was then vortexed and filtered using cellulose acetate membrane filter (0.2 µm) and the filtrate (liquid phase) was measured at 584 nm for the bioadsorption study. The decolourization efficiency due to bioadsorption was expressed as the following Equation (2).

Decolourization efficiency by bioadsorption (%) = $\frac{L}{I}$ X 100 (2)

where I = initial absorbance and L = absorbance of filtrate (liquid phase)

2.6.3 High Performance Liquid Chromatography (HPLC) and COD analysis

HPLC (Agilent 1100) equipped with a UV detector was used to determine the presence of the sulfanilic acid (dye biodegradation product) in the samples. The samples were eluted isocratically using a C_{18} reversed phase column. The mobile phase was methanol/H_3PO_4/water (50.0: 0.6: 49.7 by vol.) at 0.7 ml/min and the detection wavelength was set as 254 nm. COD values were measured according to the standard method [8].

3. Results and discussion

3.1 Effect of co-substrates

Different types of co-substrates namely sodium acetate, glycerol and glucose were used to determine their effects on decolourization of Direct Blue 15.

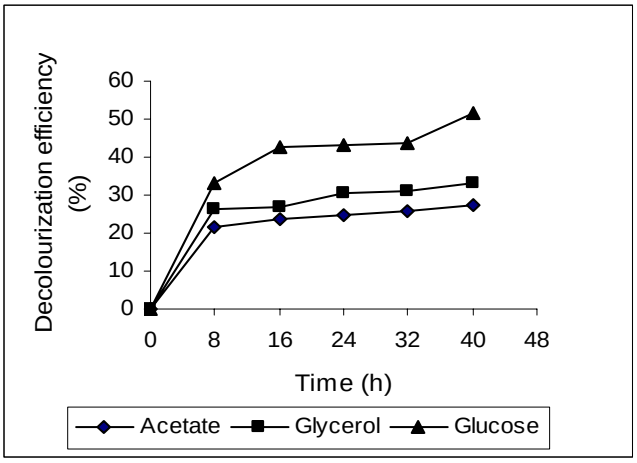

Figure 2. Effect of co-substrates on decolourization of Direct Blue 15 in wastewater medium at 37°C and pH 7

The results presented in Figure 2 indicated that glucose was the most prefered co-substrate since the presence of glucose in the medium showed highest decolourization efficiency (51. 4%), compared to glycerol (33.0%) and sodium acetate (27.2%). According to Delée *et al.* (1998), lactose, glucose, glycerol and yeast extract had been reported to be essential co-substrate to improve the decolourization efficiency [5]. A study carried out by Nigam *et al.* (1996) demonstrated that glucose gave the best result in terms of colour removal efficiency (82%) and this was in good agreement with the results obtained in this study [9]. Subsequently, glucose was used as co-substrate for the following experiment.

3.2 Effect of aeration

Different aeration rates ranging from 0 rpm to 150 rpm was used to measure its effect on decolourization. It was found that non-aerated (0 rpm) condition showed highest decolourization efficiency.

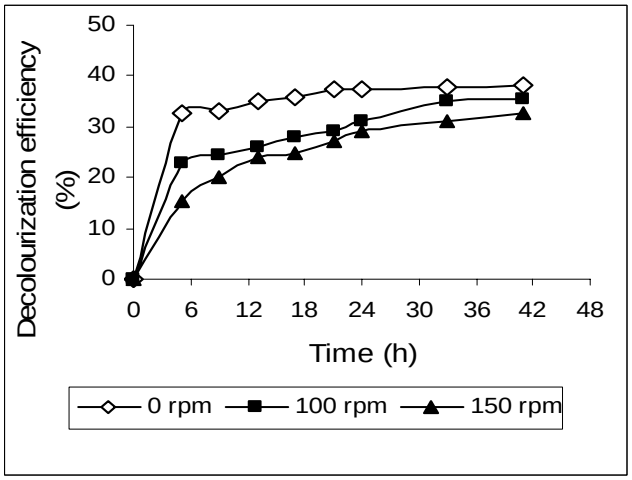

Figure 3. Effect of aeration on decolourization of Direct Blue 15 in wastewater medium containing glucose (0.1% w/v) under 37°C at pH 7

Figure 3 also indicated that the higher aeration rate, the lower decolourization rate. This means the reduction of Direct Blue 15 occurred in the absence of oxygen. This was due to the fact that the presence of oxygen would have a significantly negative effect on the dye reduction, where oxygen may act as high-redox-potential electron acceptor, thus, it would accept the electron from the electron donors rather than azo dye [10]. A wide range of dyes were reported to be decolourized anaerobically [5]. Under anaerobic conditions, azo dyes are used as terminal electron acceptor in electron transport chains and breakdown of azo bond, resulted in decolourization of wastewater [11]. This process however, was inhibited by the presence of oxygen, as oxygen is thermodynamically favorable oxidizing agent [12].

3.3 Effect of temperature

Results obtained for the effect of temperature on decolourization is shown in Figure 4.

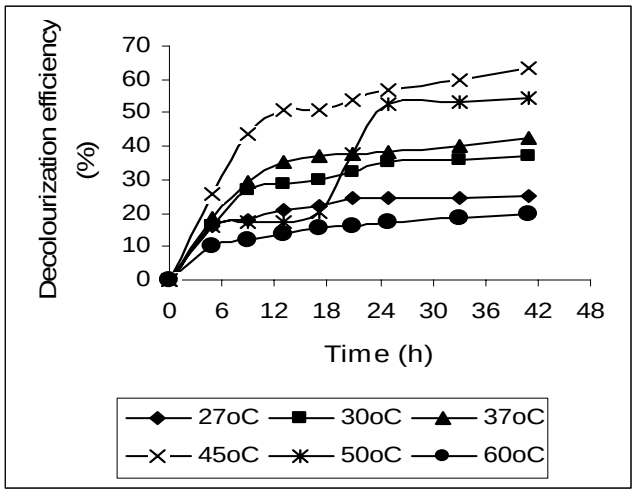

Figure 4. Effect of temperature on decolourization of Direct Blue 15 in wastewater medium containing glucose (0.1% w/v) under partial anaerobic condition at pH 7

The decolourization of Direct Blue 15 was most efficient at 45°C, with 63.3% of colour removal, followed by 50°C, 37°C, 30°C and 27°C. As indicated in Figure 4, at 60°C, the colour removal percentage was only 19.9%. The decline of microbial activity on decolourization could be due to the effects on enzyme denaturation that led to inactivation of the enzyme [13]. Similarly, Pearce *et al.* (2003) also reported that the decline decolourization efficiency might be attributed to loss of cell viability and denaturation of azoreductase enzyme [10].

Temperature influences the metabolic activities of the microbial population. It was noted that temperatures below the optimum typically have a more significant effect on growth rate than temperatures above the optimum [14]. In this study, the optimum growth temperature for *Klebsiella sp* was 37°C while the optimum temperature for colour removal was 45°C. Hence, the starter culture for the bacteria were grown at 37°C while the experiments for colour removal were carried out at temperature 45°C.

3.4 Effect of pH

The optimum pH for decolourization in this study were pH 5 and pH 6, in which decolourization efficiency was 49.9%.

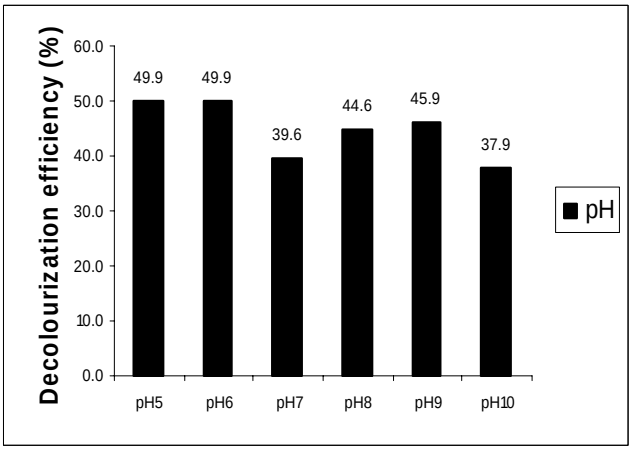

Figure 5. Effect of pH on decolourization of Direct Blue 15 in wastewater medium containing glucose (0.1% w/v) under partial anaerobic condition at 45^{o}C

Klebsiella sp. performed better under slightly acidic condition. It was reported that the decolourization rate increased nearly 2.5-fold as the pH was raised from 5.0-7.0. However the decolourization rate becomes insensitive to pH ranging from 7.0-9.5 [10]. Amongst both optimum pH, pH6 was used for subsequence experiment as it was near to neutral and suitable for industrial application.

3.5 Effect of glucose concentration

Different initial glucose concentrations of glucose (0.1-0.5% w/v) in wastewater medium were used to determine their effect on decolourization efficiency of Direct Blue 15.

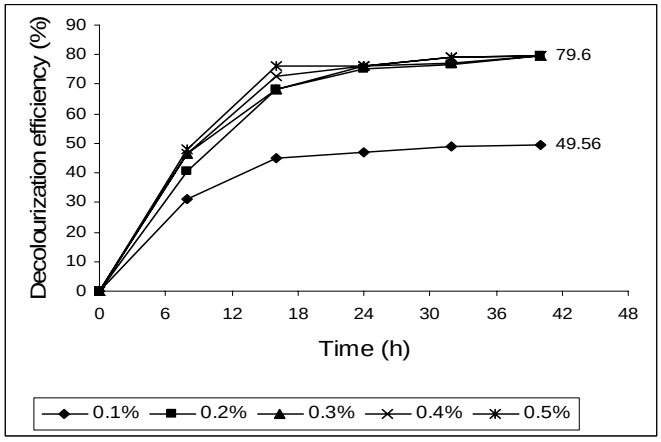

Figure 6. Effect of glucose concentration on decolourization of Direct Blue 15 in wastewater medium under partial anaerobic condition at 45^{o}C and pH 6

The results presented in Figure 6 indicated that decolourization efficiency increased from 0.1% (w/v) to 0.2% (w/v) of glucose. However, further increased in glucose concentration up to 0.5% (w/v) had no significant effect on

decolourization rate of dye. Thus, 0.2% (w/v) of glucose in wastewater medium was optimum to achieve efficient decolourization. According to Sponza and Isik, (2002), a sequential anaerobic/aerobic system, which the medium supplemented with glucose (as 3000 mg/L COD), was efficient to remove colour (Reactive Black 5) up to 98% in anaerobic stage [12].

3.6 Batch culture experiment under optimized condition

Under optimized condition, the results showed the decolourization efficiency was 81.90%.

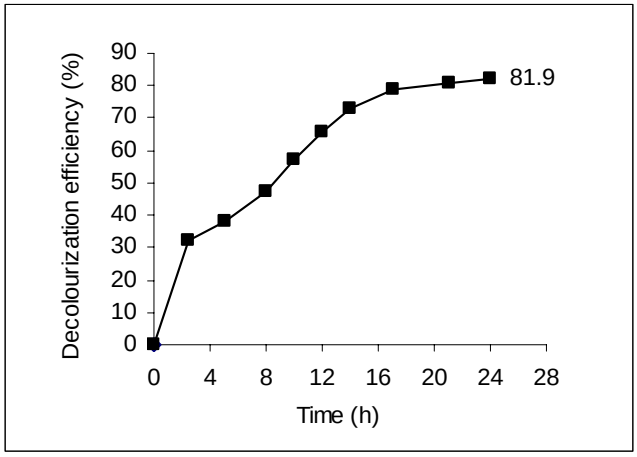

Figure 7. Decolourization of Direct Blue 15 under optimized condition

It should be noted that decolourization process might occur by bioadsorption or/and biodegradation. Thus, it was interesting to determine the percentage colour removal that contributed by bioadsorption and biodegradation. Results indicated that the decolourization due to bioadsorption and biodegradation were 0.03% and 81.87%, respectively. Pearce *et al.* (2003) reported that adsorption of dye onto the biomass was the simplest mechanism to remove colour [10]. However, as similar with the physical adsorption mechanisms for colour removal, this process would become a stage, called saturated, and hence, it was not a good process for long term of colour removal. On the other hand, colour removal via biodegradation was preferred as it remove colour by degrading the dyes into simpler compounds, which is less toxic than the parent compounds.

To further confirm that decolourization had occurred by biodegradation process, HPLC analysis was used to detected products of degradation. It is known that sulfanilic acid was one of the products for degradation of Direct Blue 15. Hence, the presence of sulfanilic acid in the decolourized sample can confirm that decolourization process occurred by biodegradation.

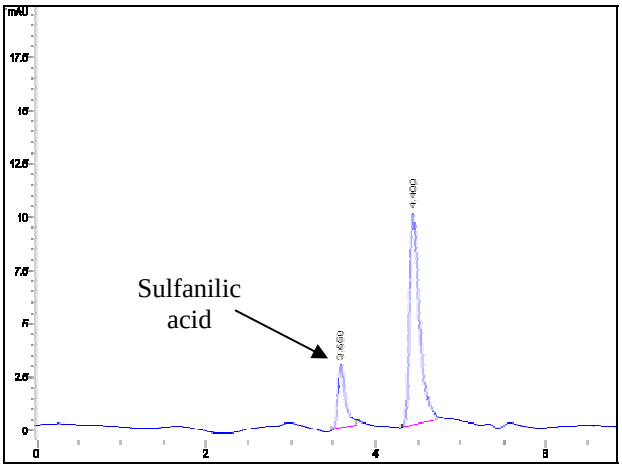

Figure 8. Chromatogram for sample after 24 h of incubation time under optimized condition

As shown in Figure 8, sulfanilic acid peak was present at retention time 3.55 minutes. Therefore, it could be concluded that biodegradation was one of the mechanisms involved in decolourization of Direct Blue 15.

3.7 Removal of COD

A total amount of 53.6% of COD was successfully removed after 24 hours of incubation time. The initial COD level in the wastewater medium was 1815 ppm. During the first 5 hours of incubation time under the optimum condition, the COD level decreased by 51.9%, from 1815 ppm drastically dropped to 873 ppm. However, after that, the COD reduction rate became slow. This may be due to the accumulated of the aromatic amines which are anaerobic recalcitrant inhibited the reduction of the COD level [15]. Furthermore, the change of pH in wastewater medium into more acidic condition might possible affect the reduction of COD.

4. Conclusion

The optimum parameters for Direct Blue 15 decolourization, such as co-substrates, aeration, starting pH of culture and temperature, were of glucose (0.2% w/v), under partial anaerobic condition, pH6 and 45^{o}C respectively. Under these optimized conditions, 81.9% of colour removal was successfully achieved. It was found that both bioadsorption and biodegradation were involved in the colour removal process. Sulfanilic acid as one of the biodegradation product was successfully detected by using high performance liquid chromatography. Under the optimized condition, the COD removal was 53.6%.

Acknowledgements

This work was funded by Malaysian Ministry of Science, Technology and Innovation under IRPA, vote 74053.

References

[1] Stolz, A. 2001. Basic and Applied Aspects in the Microbial Degradation of Azo Dyes. *Appl Microbiol Biotechnol* 56:69-80.

[2] Forgacs, E., Cserháti, T., Oros, G. 2004. Removal of Synthetic Dyes from Wastewaters: A Review. *Environ Int* 30:953-971.

[3] Manu. B, Chaudhari, S. 2001. Anaerobic Decolourisation of Simulated Textile Wastewater Containing Azo Dyes. *Bioresource Technol* 82:225-231.

[4] Banat, IM., Nigam, P., Singh, D., Marchant, R. 1996. Microbial Decolourisation of Textile Dye Containing Effluents: A Review. *Bioresource Technol* 58:217-227.

[5] Delee, W., O'Neill, C., Hawkes, F.R., Pinheiro, H.M. 1998. Anaerobic Treatment of Textile Effluents: A Review. *J Chem Technol* 73:323-335.

[6] Supaka, N., Juntongjin, K., Damronglerd, S., Delia, M-L., Strehaiano, P. 2004. Microbial Decolourization of Reactive Azo Dyes in A Sequential Anaerobic-aerobic System. *Chemical Engineering Journal* 99:169-176.

[7] Alden, C.J., Boorman, G.A., Bristol, D.W., Dunnick, J.K., Eustis, S.L., Goehl, T.J., Griesemer, R.A., Haseman, J.K., Jokinen, M.P., Rao, G.N., Walters, D.B., Witt, K.L. 1992. Toxicology and Carcinogenesis Studies of C.I. Direct Blue 15 in F344/N Rats." *National Toxicology Program Reports* 14.

[8] Water Analysis Handbook. (2002). 4th edition. HACH Company.

[9] Nigam, P., McMullan, G., Banat, I. M., Marchant, R. 1996. Decolourization of Effluent from the Textile Industry by A Microbial Consortium. *Biotechnology Letters.* 18:117-120.

[10]Pearce, C.I., Lloyd, J.R., Guthrie, J.T. 2003. The Removal of Colour from Textile Wastewater using Whole Bacterial Cells: A Review. *Dyes and Pigments* 58:179-196.

[11]Isik, M. 2004. Efficiency of Simulated Textile Wastewater Decolorization Process Based on the Methanogenic Activity of Upflow Anaerobic Sludge Blanket Reactor in Salt Inhibition Condition. *Enzyme and Microbial Technology* 35 (5):399-404.

[12]Sponza, D.T. and Isik, M. 2002. Decolorization and Azo Dye Degradation by Anaerobic/aerobic Sequential Process. *Enzyme and Microbial Technology* 31:102-110.

[13]Shuler, M.L., Kargi, F. 2nd edn. 2002. *Bioprocess engineering basic concepts.* Prentice Hall, NJ. USA.

[14]Mulkerrins D., Dobson D.W., Colleran.E. 2004. Parameters Affecting Biological Phosphate Removal from Wastewaters, *Environment International* 30(2):249-259.

[15]O'Neill, C., Hawkes F.R., Hawkes, D.L., Esteves, M.S. and Wilcox, S.J. 2000. Anaerobic–aerobic Biotreatment of Simulated Textile Effluent Containing Varied Ratios of Starch and Azo Dye." *Water Research* 34(8):2355-2361.