

# Biodegradation of Dimethylformamide Using *Bacillus Subtilis*

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Received December 26, 2012; Revised February 02, 2013; Accepted February 28, 2013

**Abstract** The present study investigated the potential of the chosen bacterium, a natural isolate *Bacillus subtilis* isolated from textile industry effluent (textile effluent adapted bacterium) and the isolate was found to be more efficient in degrading DMF based on the assessment of physico-chemical parameters like pH, turbidity, carbon dioxide and ammonia released during the degradation process. DMF degradation has led to the accumulation of ammonia and dimethylamine contributing to the increase of pH of the medium from 7.0 to 9.2. Increase in turbidity and biomass was also observed during the treatment. The maximum release of carbon dioxide and ammonia was found during the degradation of 100 µl of DMF. HPLC analysis for 200 µl of DMF degradation by the isolate showed peaks with different retention times. Thus the results indicated that the isolate was able to degrade DMF found in the textile industrial effluents.

**Keywords:** dimethylformamide, textile effluent, bacillus subtilis, degradation, HPLC

## 1. Introduction

Industrialization is vital to a nation's economy because it serves as a vehicle for development. The increasing demand for water and the dwindling supply has made the treatment and reuse of industrial effluents an attractive option. However, there are associated problems resulting from the introduction of industrial waste products into the environment. Many of these products are problematic because of persistence (low biodegradability) and/or toxicity. The textile industry is one of the many industries that utilize large volumes of water in the manufacturing process. The textile industries produce effluents that contain several types of chemicals such as dispersants, leveling agents, acids, alkalis, carriers and various dyes [2]. Textile effluents are of concern because they color the drains and ultimately the water bodies. They also diminish the water quality. In many Nigerian cities, the textile factories daily discharge millions of litres of untreated effluents in the form of wastewater into public drains that eventually empty into rivers [14].

Being a versatile organic solvent, N, N-dimethylformamide (DMF) is widely used in several industrial applications. It is primarily used as a solvent in the production of polyurethane products and acrylic fibers. It is a colorless liquid with a faint amine odor. Manufacturers use DMF as a solvent in a variety of applications, including textile coatings, production of electronic components and pharmaceutical products [16]. As most of the DMF is released into the effluents after recovering the solute, it is regarded as one of the most common chemical found in the industrial effluents [21]. In

view of the established reports on its widespread occurrence and its adverse impacts on the environment and health, the DMF is considered to be an increasing threat both for the environment and to humans [13].

DMF is a possible product of the photochemical degradation of dimethylamine and trimethylamine [15,20]. Both are commonly occurring natural substances and are also used in industrial applications. DMF does not occur naturally. There are few data concerning environmental levels or the exposure of the general population to DMF. Concentrations in the air in the range of 0.02-0.12mg/m<sup>3</sup> have been found in residential areas, near industrial sites. DMF is released in to the environment through various ways. When emitted into air, most of the DMF released remains in that compartment, where it is degraded by chemical reactions with hydroxyl radicals. Indirect releases of DMF to air, such as transfers from other environmental media, play only a small role in maintaining levels of DMF in the atmosphere. When DMF is released into water, it degrades there and does not move into other media. Contamination of soil with DMF may occur through spillage or leakage during its production, transport, storage, or use. When released into soil, most of the DMF remains in the soil presumably in soil pore water until it is degraded by biological and chemical reactions. If DMF reaches groundwater, its anaerobic degradation will be slow [4,6].

The toxicity of DMF has been studied in many species, by various administration routes with generally similar results [9,12]. Hepatotoxicity has been reported in most species studied, including humans, following both acute and sub chronic exposure [11,17]. The toxicity of DMF following inhalation exposure has been reasonably well characterized. Rats survived a single 4-hour exposure to

saturated vapors of DMF, with a nominal concentration of approximately 5000 ppm [18]. The potential carcinogenicity of DMF recently has been evaluated. It was determined that DMF may be carcinogenic to humans (Group 2B), based on findings from epidemiological studies of an excess risk for testicular germ cell cancers and cancer of the buccal cavity among workers exposed occupationally to DMF and other materials. The available studies on the carcinogenicity of DMF in animals were considered inadequate to make an evaluation [10].

Compared with chemical/physical methods, biological processes have received more interest because of their cost effectiveness, lower sludge production and environmental friendliness. Improvement in the ability of microorganisms to degrade a pollutant could be achieved through modification of the environment or the organisms. The ability of microorganisms to degrade and metabolize a wide variety of compounds has been recognized and exploited in various biotreatment processes. The biotreatment offers a cheaper and environmentally friendlier alternative for removal of pollutants in textile effluents [19]. One such pollutant found in the textile industry effluent is dimethylformamide. The ubiquitous nature of bacteria makes them invaluable tools in effluent biotreatment. It is proposed that the biodegradation of DMF proceeds by following two distinct pathways. One of them (pathway I) is mediated by the presence of novel enzyme N, N-dimethylformamidase (DMFase) that converts DMF to dimethylamine and formate. These two catabolic intermediates are further degraded to form ammonia and carbon dioxide. In the second pathway (pathway II), the DMF is degraded by repeated oxidative demethylations leading to the generation of formamide. The formamide is further hydrolyzed to ammonia and formate [8].

In the present study an attempt has been made to isolate a bacterial strain, capable of degrading DMF and to identify and determine its efficiency of degradation by analyzing different parameters like pH, turbidity, biomass, CO<sub>2</sub> production and ammonia production.

## 2. Materials and Methods

### 2.1 Collection of Sample

Soil samples were collected in sterile screw cap bottles from the sites contaminated with textile industry effluent containing dimethylformamide at Dindigul.

### 2.2 Isolation of DMF Degrading Bacteria

The collected soil samples were serially diluted and 0.1ml from the 10<sup>-6</sup> dilution was taken and spread on minimal medium MM1 containing 50 µl of DMF as a sole carbon source. The plates were incubated at 37 °C for a week. The physico-chemical characteristics of DMF are given in Table 1 and the structure is represented in Figure 1.

### 2.3 Identification of DMF Degrading Bacteria

From the different bacterial strains grown on agar plates, one strain was selected. Gram staining and biochemical tests like MR-VP, citrate utilization, oxidase, catalase,

indole production, urease, Triple Sugar Iron agar and nitrate reduction tests were carried out for tentative strain identification. The isolate was identified as *Bacillus subtilis*.

### 2.4 Degradation Efficiency

The bacterial isolate was inoculated on minimal broth containing different concentrations of DMF like 50, 100, 150 and 200 µl. The flasks were incubated at room temperature for a period of ten days and the degradation was confirmed by analyzing the optical density, pH, biomass, carbon dioxide and ammonia production. The above mentioned parameters were measured every 48 hours for 10 days.

### 2.5 PH

The pH of the sample was determined after 4, 6, 8 and 10 days of incubation using pH meter.

### 2.6 Biomass Estimation

Turbidometric method was followed for confirming the increase in biomass by measuring the turbidity at 600nm. The biodegraded samples were taken and centrifuged. The pelleted biomass was taken and the wet biomass was calculated. After drying it in a hot air oven, the dry biomass was determined.

### 2.7 Carbon Dioxide Estimation

The estimation of carbon dioxide was carried out by adapting the method proposed by Eaton et al. (1995) [5].

### 2.8 Ammonia Estimation

Two to three drops of Nessler's Reagent were added to one ml of the sample and the color change was noted. The concentration of ammonia was then estimated using colorimeter [1].

### 2.9 High Performance Liquid Chromatography (HPLC)

The samples containing the minimal medium, isolate and 200 µl DMF taken on the 0<sup>th</sup> day, 4<sup>th</sup> day and 10<sup>th</sup> day were subjected to HPLC analysis at CECRI, Karaikudi (Model: Shimadzu, Japan). The details about the standard conditions at which the analysis was carried out are given in the Table 1.

Table 1. High Performance Liquid Chromatography

Model	SHIMADZU, JAPAN
Stationary Phase	SILICA GEL (Reversed phase)
Mobile phase	100% methanol
Main column	Analytical-Shim-Pack CLC-OCTA DECYL SILANE(ODS-C18) [4.6 mm ID 25cm]
Guard column	Shim-Pack G-ODS [4mm ID 1cm]
Detector	UV Spectrophotometer
Flow Rate	1ml/minute
Column head pressure	125 kgf/cm <sup>2</sup>
Injection per sample	20 µl
Wave length	254 nm

### 3. Statistical Analysis

Two way ANOVA was performed for the parameters like optical density, pH, biomass, carbon dioxide and ammonia produced using Microsoft Excel. Variability was considered significant only when the statistical value was greater than the tabulated value at P is less than or equal to 0.05.

### 4. Results and Discussion

DMF has been termed the universal organic solvent and is widely used where a low rate of evaporation is required [7]. The structure of DMF is shown in Figure 1. The overall rate of chemical degradation in surface water is expected to be very slow and so biodegradation is widely preferred for the elimination DMF from the environment. The biodegradation half-lives have been measured in the range of 18-36 hours [3]

The bacterial strain isolated from the soil was identified as gram negative rod. Then the isolate was tentatively identified as *B. subtilis* on the basis of the results obtained in the biochemical tests (Table 2). The isolate, *B. subtilis* (Plate 1) was able to grow on minimal agar containing various concentrations of DMF.

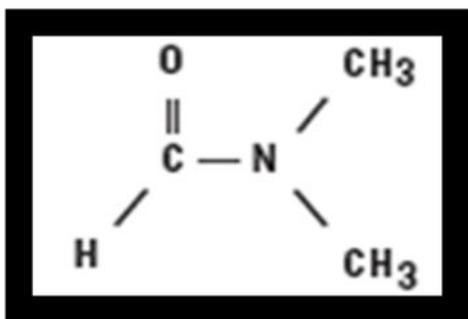


Figure 1. Structure of dimethylformamide

Table 2. Biochemical Tests for the Identification of the Isolate *B. subtilis*

S.No.	Test	Response of the organism
1.	Gram staining	+
2.	Methyl Red	+
3.	Voges proskauer	+
4.	Simmons citrate agar	+
5.	Oxidase	-
6.	Catalase	+
7.	Indole	-
8.	Urease	-
9.	Triple Sugar iron Agar	+
10.	Nitrate reduction	-

(+ = Positive reaction, - = Negative reaction)

Figure 2 illustrates the changes in the pH determined after 4, 6, 8 and 10 days of treatment with *B. subtilis*. The pH of the medium was found to be increasing from the 8th day during the degradation by *B. subtilis*. Gradual increase in the pH indicates the degradation of DMF. During the degradation of DMF, pH is found to increase gradually

from 4<sup>th</sup> day to 10<sup>th</sup> day of the treatment. This increase in the pH may be due to the constant release of dimethylamine and ammonia into the medium. A novel bacterial strain *Ochrobactrum* sp. DGVK1 has been isolated from the soil which is capable of producing dimethylformamidase to degrade DMF in which the DMF degradation witnessed concomitant increase in the growth of the culture and pH of the medium from 7 to 9.2 [22].



Plate 1. Growth of *B. subtilis* in minimal medium containing 200 µl of dimethylformamide

Changes in the turbidity of the medium during the ten days treatment of DMF by *B. subtilis* are illustrated in Figure 3. It seems to be fluctuating but the maximum growth of the bacterium was observed after sixth and eighth days of treatment. Figure 4 depicts the changes in biomass of *B. subtilis* during the study period.

Increase in turbidity and biomass during the treatment period indicates the bacterial growth due to the utilization of DMF as a source of carbon and nitrogen. Growth of the bacterium is observed to increase till the 8th day of treatment and decrease after that. This indicates the degradation of DMF increasing as the incubation period increases. The decrease in the biomass may be due to the formation of degraded products like dimethylamine and ammonia.

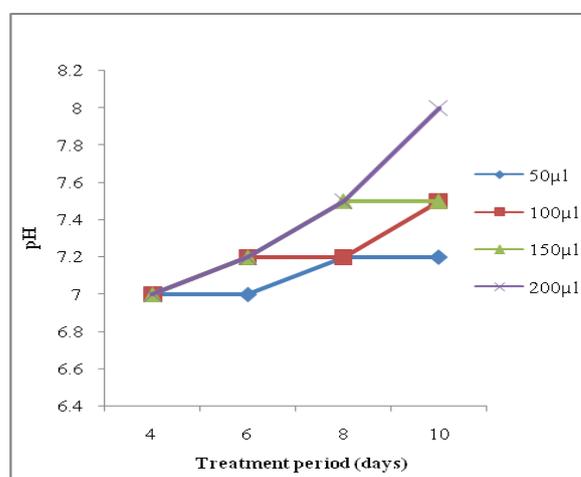
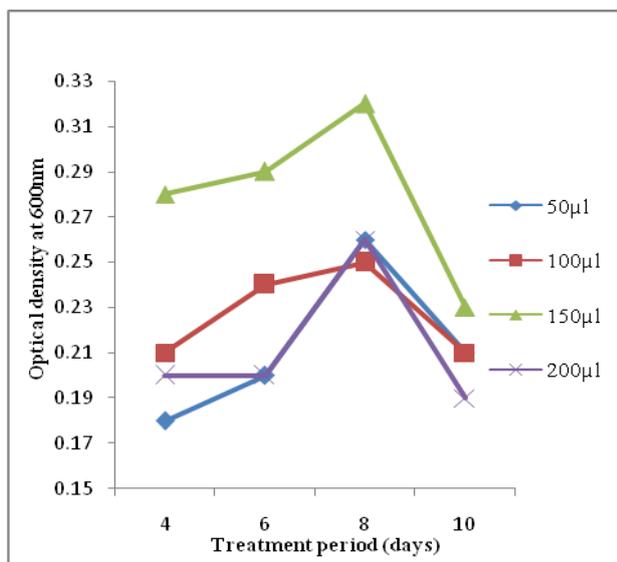
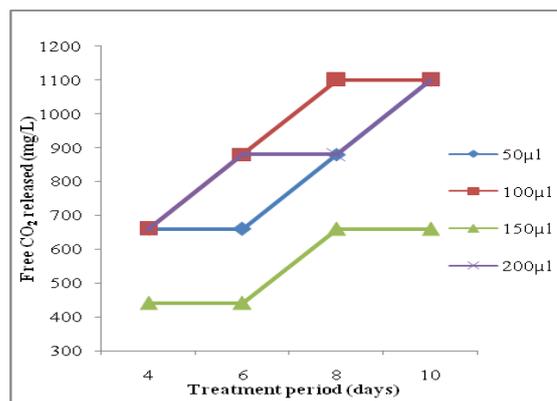


Figure 2. Changes in the pH of the medium during the degradation of DMF by *B. subtilis*



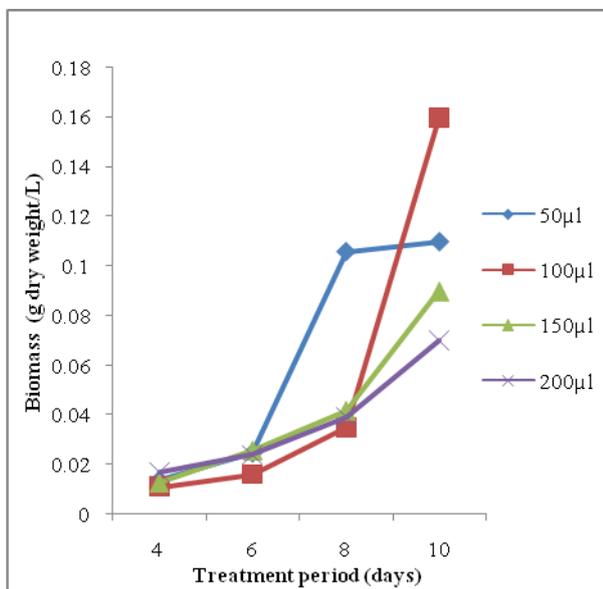
**Figure 3.** Changes in turbidity during the degradation of DMF by *B. subtilis*

Liberation of carbon dioxide during degradation of DMF can be used as an indication for the activity of bacteria in the growth medium. The maximum release of carbon dioxide was found during the degradation of 100µl of DMF by *B. subtilis*. This clearly indicates that *B. subtilis* was found to degrade DMF more efficiently. The biodegradation of DMF resulted in the production of carbon dioxide which was found to increase linearly with the increasing concentrations of DMF. The amount of carbon dioxide released during the ten days treatment of DMF by *B. subtilis* is shown in Figure 5.



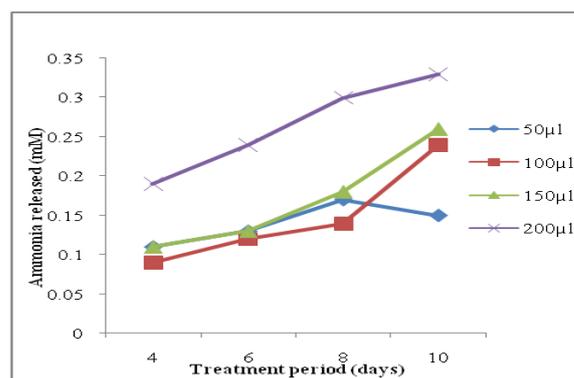
**Figure 5.** Carbon dioxide released during the degradation of DMF by *B. subtilis*

HPLC analysis of 200µl of DMF before and after the treatment with *B. subtilis* is shown in figures 7, 8 and 9. Figure 7 shows the HPLC analysis for 200µl of DMF on 0<sup>th</sup> day of treatment. The peaks observed here were missing in the HPLC analysis of the same concentration of DMF on the 4<sup>th</sup> and 10<sup>th</sup> day of degradation by *B. subtilis* (Figure 8 and Figure 9). There were few new peaks with different retention times indicating the formation of intermediates.



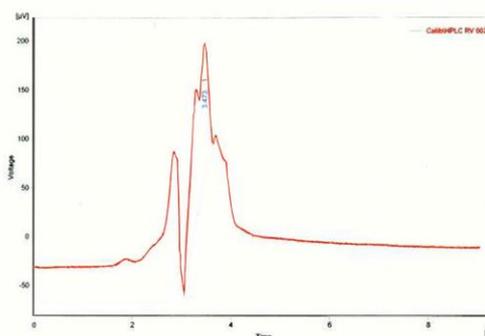
**Figure.4.** Changes in the biomass (g dry wt/l) during the degradation of DMF by *B. subtilis*

Release of ammonia as a result of biodegradation of DMF by *B. subtilis* is exhibited in Figure 6. The concentration of liberated ammonia was found to increase till the 10<sup>th</sup> day treatment of various concentrations of DMF by *B. subtilis*. The maximum release of ammonia was observed during the 10<sup>th</sup> day of treatment of DMF by *B. subtilis*. Ammonia concentration during the ten days of treatment by *B. subtilis* increased linearly and the highest concentration was recorded in the case of 200µl of DMF.



**Figure 6.** Ammonia released during the degradation of DMF by *B. subtilis*

HPLC analysis for 200µl of DMF degradation by the isolate showed peaks with different retention time. The report showed the difference in retention time between control and the biodegraded samples. The peaks obtained on the 10<sup>th</sup> day of treatment were different in their retention time from those on the 4<sup>th</sup> day of treatment indicating the mineralization of DMF into new intermediates. There was formation of several intermediates as well as disappearance of compounds.



**Figure 7.** HPLC analysis report for 200µl of dimethylformamide (0<sup>th</sup> day)

Table 3 exhibits the two way analysis of variance for the factors such as pH, turbidity, biomass, carbon dioxide and ammonia with the variables, treatment period and DMF concentration. In Table 3 where the two way analysis of variance results are given, the factors turbidity, CO<sub>2</sub> and ammonia production exhibited the variables statistically significant variations due to the variables treatment period and dimethylformamide concentration. In the factors pH and biomass, variations due to treatment period are not statistically significant while they were significant for dimethylformamide concentration. Based on the assessment of different parameters, the isolate *B. subtilis* was found to be more effective in degrading DMF.

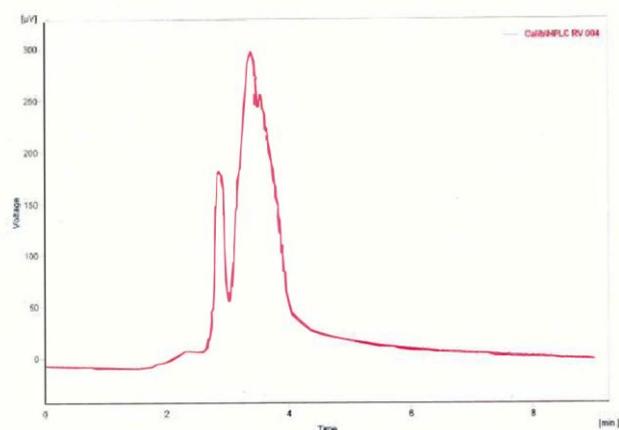


Figure 8. HPLC analysis report for 200 µl of dimethylformamide treated with *Bacillus subtilis* (4<sup>th</sup> day)

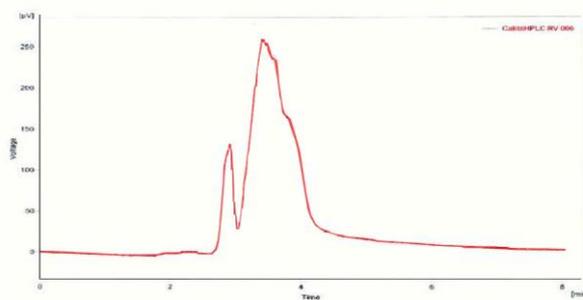


Figure 9. HPLC analysis report for 200 µl of dimethylformamide treated with *Bacillus subtilis* (10<sup>th</sup> day)

## 5. Conclusion

Extensive use of DMF as a versatile organic solvent in various processes makes its way into the environment. Though there are no reports on accidental release of DMF, the major route of DMF contamination is through the release of DMF into industrial effluents. As biodegradation is the major route of degrading DMF, several attempts have been made to isolate suitable strains that grow using DMF as the sole source of carbon and nitrogen. However, only a limited number of bacterial strains have been shown to utilize DMF as the source of carbon and nitrogen. From the assessment of various parameters, it can be concluded that *B. subtilis* can efficiently degrade the DMF and certainly forms basis for generation of novel bioremediation strategies for effective removal of DMF from the industrial effluents.

Table 3. Two way analysis of variance for the factors with the variables, treatment period and DMF concentration for *P. aeruginosa*

Factor	Source of variation	df	MS	Calculated F value	Table F value	Level of Significance at 5% level
pH	Treatment period	3	0.027292	1.686695	3.862548	Not significant
	Concentration	12	0.302292	18.6824	3.862548	Significant
Turbidity	Treatment period	3	0.00495	13.5	3.862548	Significant
	Concentration	12	0.00195	5.318182	3.862548	Significant
Biomass	Treatment period	3	0.000769	0.936131	3.862548	Not significant
	Concentration	12	0.006095	7.414234	3.862548	Significant
Carbon dioxide	Treatment period	3	124025	21.70588	3.862548	Significant
	Concentration	12	107891.7	18.88235	3.862548	Significant
Ammonia	Treatment period	3	0.013306	16.97166	3.862548	Significant
	Concentration	12	0.01096	13.91054	3.862548	Significant

## Acknowledgement

The authors thank the authorities of the American College, Madurai for the facilities and encouragement.

## References

- [1] APHA, Standard methods for the examination of water and wastewater, 18<sup>th</sup> edition, 1992.
- [2] Bromley-Challenor, K.C.A., Caggiano, N. and Knapp, J.S., "Bacterial growth on N,N-dimethylformamide: Implications for the biotreatment of industrial wastewater", *Journal of Industrial Microbiology and Biotechnology*, 25, 8-16, 2002.
- [3] Cooper, P., Color in dye house effluent. Bradford *Society of Dyers and Colourists*, Bradford, on behalf of the Dyers' Company Publications Trust, 1995.
- [4] Dojlido, J.R., Investigations of biodegradability and toxicity of organic compounds. Washington, DC, US Environmental Protection Agency (EPA-600/2-79-163) [cited in Howard, 1993], 1979.
- [5] Eaton, A.D., Clesceri, L.S. and Greenberg, A.L., Standard examination of water and wastewater, 19<sup>th</sup> Edition, 4.17, 1995.
- [6] European Chemicals Bureau, Trimethylamine. IUCLID (International Uniform Chemical Information Database), 1996a.
- [7] European Chemicals Bureau, Dimethylamine. IUCLID (International Uniform Chemical Information Database), 1996b.
- [8] Ghisalba, O., Cevey, P., Kuenzi, M. and Schär, H.P., "Biodegradation of chemical waste by specialized methylotrophs, and alternative to physical methods of waste disposal", *Conserv Recycl*, 8, 47-71, 1985.
- [9] Howard, P.H., Handbook of Environmental Fate and Exposure Data for Organic Chemicals 7(2), Chelsea, MI: Lewis Publishers Inc, 1993.
- [10] IARC, Evaluations-in press. IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man. Lyon, International Agency for Research on Cancer, 1988.
- [11] IARC, Dimethylformamide. In: Some organic solvents, resin monomers and related compounds, pigments and occupational

- exposures in paint manufacture and painting. Lyon, International Agency for Research on Cancer, 171-197. 1989.
- [12] IARC, Dimethylformamide. In: Re-evaluation of some organic chemicals, hydrazine and hydrogen peroxide. Lyon, International Agency for Research on Cancer, 545-574. 1999.
- [13] Kennedy, G.L., and Sherman, H., "Acute and subchronic toxicity of dimethylformamide and dimethylacetamide following various routes of administration", *Drug and chemical toxicology*, 9. 147-170. 1986.
- [14] Long, G., and Meek, M.E., Concise international chemical assessment. Document 31. World Health Organization, Geneva, 1-61. 2001.
- [15] Olayinka, K.O., and Alo, B.I., "Studies on industrial pollution in Nigeria: The effect of Textile effluent on the quality of ground waters in some parts Lagos", *Nigerian Journal of Health and Biomedical Sciences*, 22-25. 2004.
- [16] Pellizzari, E.O., The measurement of carcinogenic vapors in ambient atmosphere. Research Triangle Park, NC, US Environmental Protection Agency, Washington DC, 1977.
- [17] Redlich, C., Beckett, W.S., Sparer, J., Barwick, K.W., Riely, C.A., Millersigal, H., Shalat, S.L. and Cullen, M.R., "Liver disease associated with occupational exposure to the solvent dimethylformamide", *Annals of Internal medicine*, 108. 680-686. 1988.
- [18] Scailteur, V., and Lauwerys, R.R., "Dimethylformamide (DMF) Hepatotoxicity", *Toxicology*, 43. 231-38. 1987.
- [19] Smyth, H.F., and Carpenter, C.P., "Further experience with the range-finding test in the industrial toxicology laboratory", *Journal of industrial hygiene and toxicology*, 30(1). 63-68. 1948.
- [20] Stolz, A., "Basic and Applied Aspects in the Microbial Degradation of Azo Dyes", *Applied microbial biotechnology*, 56. 69-80. 2001.
- [21] US EPA, United States Environmental Protection Agency, Superfund Public Health Evaluation Manual. EPA/5401/1-86/060, US Environmental Protection Agency, Washington DC, 1986.
- [22] Veeranagouda, Y., Emmanuel Paul, P.V., Gorla, P., Siddavattam, D., and Karegoudar, T.B., "Complete mineralisation of dimethylformamide by *Ochrobactrum* sp. DGVK1 isolated from the soil samples collected from the coalmine leftovers", *Applied Microbiology and Biotechnology*, 71. 369-375. 2006.