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Determination of overall microbial activity in sewage treatment plants using FDA hydrolysis assay

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ABSTRACT

In the present study, the fluorescein diacetate (FDA) activities using hydrolysis assay as well as removal efficiencies in terms of physico-chemical and biological parameters has been investigated for some of the sewage treatment plants in Mumbai, India. Effect of various process parameters such as pH, temperature and time on FDA hydrolysis of aerated lagoon treated samples has been studied. The pH, temperature and time which executed maximum FDA hydrolysis were 7.8, 50°C and 1.25 h, respectively. For Bhandup sewage treatment plant (STP), the FDA activity of 0.01 µmole/mL/h in raw sewage was further increased to 0.11 µmole/mL/h and 0.10 µmole/mL/h during the treatment for lagoon 1 and 2 respectively. The FDA activity after aerated de-gritting chamber was found to be 0.07 µmole/mL/h which was enhanced to 0.10 µmole/mL/h and 0.08 µmole/mL/h for lagoon 1 and lagoon 2, respectively, in Versova STP. The enhanced microbial activities in aerated lagoons have clearly indicated the extent and efficiency of biodegradation – which was evidenced by the enhanced FDA activity. Therefore, in order to sense the pulse of any biological operation in a given sewage treatment plant, FDA hydrolysis assay can be used as a quick technique to assess the change in overall microbial activity.

Keywords: FDA activity; Wastewater treatment; Sewage treatment plants; Water pollution

1. Introduction

In India, during the past five decades, there has been a steady deterioration of all the environmental sub-systems. An inadequate capacity of the sewage treatment facilities has led to discharge of large volume of wastewater into the natural watercourses across the nation. Environmental degradation has been incurred due to urbanization, especially in the quality of air, water and noise. The wastes are directly channelled to the water resources or nearest river owing to poor infrastructure in houses as well as in some unlawful factories which directly pollutes the water. Degradation of water quality has been reported due to direct discharge of domestic waste, industrial effluents and other wastes into the water resources or nearest river [1,2].

Almost in all the urban centres in India, an inadequate sewage treatment exists. An illicit growth of the cities as well as resource crisis faced by the municipalities is two major causes of this miserable state of affairs. For the treatment of sewages and sullages, most cities in the country do not have proper arrangements and it is directly drained into a nearby river or water resources attributing to the contamination of water bodies [3–5].

As reported by CPCB (2013), the sewage generation from 500 Class I cities are approximately 35,000 MLD where only the 32% sewage treatment capacity exists which clearly exhibits a huge gap between generation and treatment of wastewater in India [6]. Therefore, for the treatment of wastewater, not only suitable technological alternative should be used but also insight cause and effects of various unit operations should be understood.

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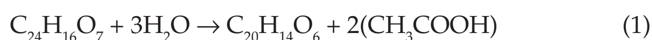
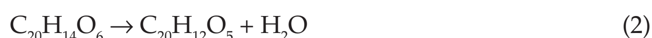
Through a set of several physico-chemical parameters such as pH, chemical oxygen demand (COD), biochemical oxygen demand (BOD), total nitrogen, total phosphorus, mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS) *etc.*, the performance assessment of sewage treatment plants has been performed during the wastewater treatment. However, these techniques are time consuming. Therefore, for assessment of any biological treatment process, the advance biotechnological techniques including FDA hydrolysis assay may provide an easy and speedy method by measuring the enzyme activity of microbial populations and ultimately providing an estimate of overall microbial activity. In the present study, efforts have been made on the performance assessment of STPs in Mumbai, India through analysing the change in the FDA activities.

For measuring the total microbial activity in a range of environmental samples including soils, FDA hydrolysis assay is extensively recognized as an accurate and simple method. The FDA assay is sensitive to the activity of several enzyme classes comprising lipases, proteases and esterases [7]. The activity of both free and membrane bound enzymes attributes to the hydrolytic cleavage of colorless FDA ($C_{24}H_{16}O_7$) into fluorescein (fluorescent yellow-green, $C_{20}H_{12}O_5$) which can be measured by spectrophotometry [7,8]. At the ester linkage, the two acetate groups are hydrolysed and the lactone part of the structure is also cleaved at its internal ester link [Eq. (1)]. Therefore, a positively charged bond is created as the resultant OH group leaves. In order to satisfy this charge, the intermediary step occurs starting from a loss of H at the terminal position resulting in an overall loss of water. Hence, the hydrolysis reaction of fluorescein diacetate to fluorescein is followed by a dehydration reaction [Eq. (2)] [8].



$$C_{24}H_{16}O_7 + 3H_2O \rightarrow C_{20}H_{14}O_6 + 2(CH_3COOH) \quad (1)$$



$$C_{20}H_{14}O_6 \rightarrow C_{20}H_{12}O_5 + H_2O \quad (2)$$

In the present work, the effect of various process parameters such as pH, temperature and incubation period on the FDA activity of lagoon treated samples collected from Bhandup STP, Mumbai has been studied along with determination of physico-chemical and bacteriological parameters. At the optimized conditions, the FDA activity was investigated at different sampling points in Bhandup as well as Versova STPs, Mumbai.

2. Materials and method

2.1. Samples

The primary objective of this paper was to optimize the FDA hydrolysis assay for lagoon treated samples and further estimation of FDA activities in different samples collected from facultative aerated lagoon based STPs in Mumbai. From the facultative aerated lagoon based wastewater treatment plants in Bhandup and Versova, Mumbai, the wastewater samples were collected (Fig. 1) [9]. The treatment facility comprises of a screen chamber, de-gritting chamber and aerated de-gritting chamber followed by aerated lagoons.

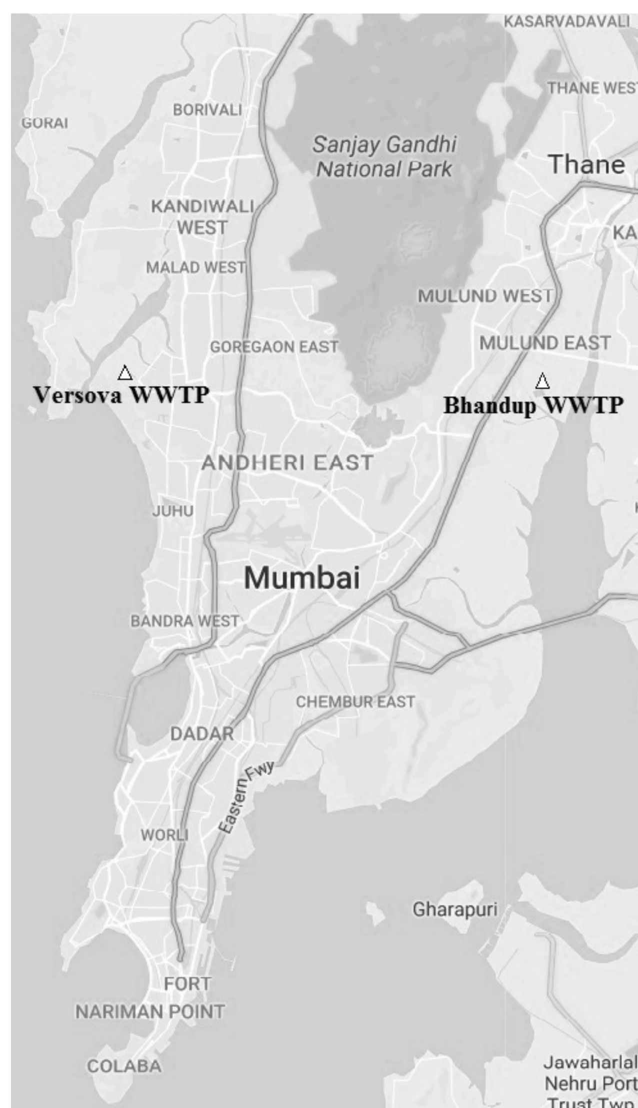


Fig. 1. Wastewater treatment plants in Mumbai.

Wastewater from each sampling location was collected and settled for 20 min to avoid heavy sediment in the sample. 1 L polythene sampling cans (pre-washed with 10% HCl and distilled water) were filled with the settled samples. In order to avoid deterioration or contamination of samples before it reaches to the laboratory, the samples were preserved in the sample box containing ice cube freezer bags.

The FDA activity was determined according to the protocol given by Fontvieille et al. [10]. Optimization of process parameters of FDA assay has been carried out for the lagoon treated samples collected from Bhandup STP, Mumbai. In addition, at different sampling points in Versova and Bhandup STPs, Mumbai, the FDA activity was estimated at the optimized parameters of assay. Furthermore, the physico-chemical and bacteriological parameters such as pH, TDS, COD, BOD_5, NH_3-N *etc.* were also investigated at IIT Bombay laboratory in Mumbai. Table 1 depicts the methods and protocols used for analyzing the physical, chemical and biological characteristics of the wastewater [11].

Table 1
Methods and protocols used during analyses of samples

Sr. No.	Parameter	Method/Reference
1	pH	HACH meter with pH probe
2	TDS	HACH meter with TDS probe
3	TSS	HACH meter with TSS probe
4	COD	Standard methods (2005) [11]
5	BOD_5	Standard methods (2005) [11]
6	NH_3–N	HACH meter with NH_3–N probe

For the FDA hydrolysis assay, the sample solution (V2) was prepared by adding 2 mL sample (V1) into 10 mL phosphate buffer. For the sample tubes, 1 mL sample solution (V3) was added into the 4.1 mL phosphate buffer and further, 0.1 mL FDA substrate stock solution was added. Similarly, control tubes were prepared by adding 0.1 mL FDA substrate stock solution into the 5.1 mL phosphate buffer. Both the sample and control tubes were incubated in water bath at required temperatures. The reaction was stopped with the addition of 1 ml of $HgCl_2$ solution (400 mg/L) followed by centrifugation (10 min, 2,000 G, 4°C). Final volume in each tube becomes 6.1 mL (V4) after addition of $HgCl_2$ solution. At 490 nm, the optical density (OD) of the supernatant was measured. The FDA activity was estimated according to the formula given by Fontvieille et al. which is as follows [10]:

$$\textit{Hydrolytic activity}\left(\mu mole / mL / h\right) = \frac{OD * V2 * V4}{81.5 * t * V1 * V3}$$

where OD = optical density at 490 nm corrected for blanks (cm); 81.5 = extinction coefficient of fluorescein (μmole/mL/cm); V1, V2, V3 and V4 = volumes (mL)

2.2. Reagents

The FDA substrate stock solution ($C_{24}H_{16}O_7$, Sigma-Aldrich, USA) was prepared by dissolving it in acetone (GR grade, Merck Specialities Private Limited, India). The solution was stored in a fridge at 4°C to avoid decrease in concentration due to degradation. The sodium dihydrogen phosphate monohydrate and disodium hydrogen phosphate dihydrate (both GR grade, Merck Specialities Private Limited, India) were used to prepare phosphate buffer. The phosphate buffer (0.1 M) was used as the incubation solution and were prepared in deionized water (produced with a “Thermo Scientific Smart2Pure” purification system).

3. Results and discussion

3.1. Optimization of pH

In order to determine the optimum pH, buffers were prepared with pH ranging from 7.0 to 8.0. In slightly alkaline (pH ≥ 8.0) solutions, the spontaneous degradation of fluorescein diacetate to fluorescein has been reported in the literature. However, non-biological hydrolysis of FDA may occur at lower pH values (≤ 5.0) [12]. The H^+ concentration

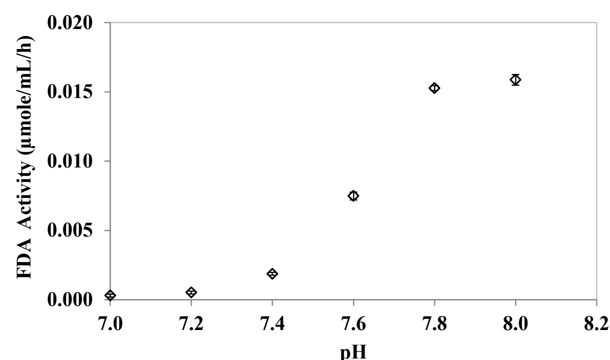


Fig. 2. Effect of pH on the release of fluorescein during the hydrolytic activity in the lagoon treated samples of Bhandup STP, Mumbai (Reaction conditions: 100 mM phosphate buffer of pH ranging from 7 to 8, 4.8 mM FDA as a substrate solution, temperature 30°C, time 1 h).

in the reaction solution influences the substrate's ionization state by affecting the ionization groups of the enzyme protein. In order to maintain the correct conformations, the ionizable groups of both the active site of the enzyme and substrate must be in their proper states for the effective interaction between them [12,13]. Therefore, the effect of pH buffer on FDA hydrolysis assay is considered to be most critical factor.

The maximum hydrolysis of fluorescein compound occurs in the pH range between 7.0 and 8.0 [8]. In the present study, the maximum rate of hydrolysis is exhibited by fluorescein diacetate at pH 7.8 (Fig. 2) as the maximum activity was observed. Additionally, several authors have been reported the pH range of 7.0–8.0 suitable for the FDA assay as the solubilisation of organic matter in the samples causes interference problems with the measurement of fluorescein released which creates blank with very high background absorbances at both high as well as low pH values [8]. Therefore, the optimum pH of 7.8 was selected for further experiments where maximum extent of FDA activity was attained.

3.2. Optimization of temperature

Temperature is another significant parameter in the FDA hydrolysis assay affecting the activity of the enzymes. The interactions between enzyme and substrate get enhances by reducing the viscosity of the reaction mixture at higher temperature which ultimately leads to higher reaction rate. However, at elevated temperature, the disruption of the active conformation of enzyme takes place. Therefore, when the temperature rises above the optimum temperature of enzyme, the loss of activity and selectivity of enzyme occurs due to denaturation [14–16]. Hence, optimum temperature plays an imperative role in the enzyme assay.

In order to achieve maximum hydrolysis activity, it is likely that the reaction must be carried out at an optimum temperature considering the enzyme feasibility. Effect of reaction temperature on the FDA activity was carried out at different operating temperatures ranging from 30°C to 70°C. The other parameters such as pH and incubation

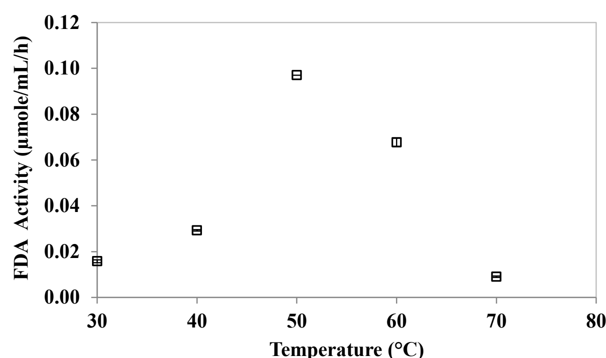


Fig. 3. Effect of temperature on the release of fluorescein during the hydrolytic activity in the lagoon treated samples of Bhandup STP, Mumbai (Reaction conditions: 100 mM phosphate buffer of pH 7.8, 4.8 mM FDA as a substrate solution, temperature 30°C–70°C, time 1 h).

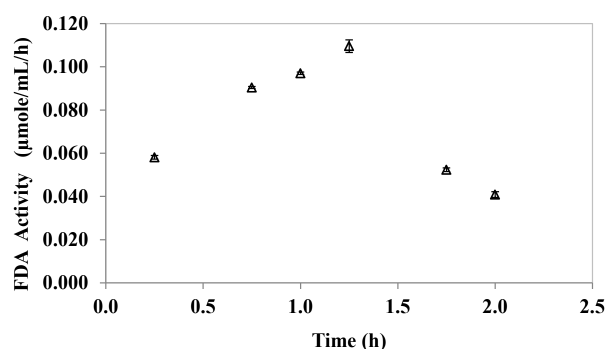


Fig. 4. Effect of incubation period on the release of fluorescein during the hydrolytic activity in the lagoon treated samples of Bhandup STP, Mumbai (Reaction conditions: 100 mM phosphate buffer of pH 7.8, 4.8 mM FDA as a substrate solution, temperature 50°C, time 0.25–2 h).

period were kept constant. The results obtained have been shown in Fig. 3 which depicts that at lower temperature the FDA activity is lower in comparison with the higher temperature range activity.

With an increase in reaction temperature from 30°C to 50°C, the FDA activity was increased from 0.015 µmole/mL/h to 0.097 µmole/mL/h. As compared to other temperatures, the FDA activity was reduced at reaction temperature of 70°C due to thermal deactivation of enzyme at higher temperature. Hence, 50°C was considered as optimum temperature for further experiments in order to avoid the denaturation of enzymes at elevated temperatures leading to reduced activity.

3.3. Optimization of incubation period

Typically, enzyme-catalysed reactions show linear relationships between the time of incubation and the amount of products formed [12]. With an increase in incubation time, the risk of error through microbial proliferation increases. Therefore, an assay for enzymes should not necessitate a prolonged incubation time [8,12]. In order to investigate the effect of incubation time on the FDA assay, the reaction was carried out with the incubation time ranging from 0.25 h to 2 h at the temperature of 50°C and pH 7.8. The obtained FDA activity is depicted in Fig. 4 for different incubation periods.

It has been observed that with an increase in time periods, the activity get increases initially. A good linearity between incubation time and generated fluorescein was observed within the first hour in the light of increasing trend. The maximum extent of FDA activity was found to be at 1.25 h. However, with further increase in incubation time, decreased activity was observed. As reported by several authors, longer incubation time should be avoided owing to consumption of substrate as well as decrease in average hydrolysis rate [12,13]. The reduction in the activity has been observed beyond 1.25 h of incubation which signifies that the substrate may be limiting the reaction beyond 1.25 h. Hence, an incubation time of 1.25 h was chosen for the final assay procedure.

Table 2
Description of sampling points from Versova and Bhandup WWTP, Mumbai

Location	Nomenclature	Sampling points
Versova	V1	Aerated de-gritting chamber
	V2.1	Lagoon 1
	V2.2	Lagoon 2
Bhandup	B1	Raw Sewage
	B2.1	Lagoon 1
	B2.2	Lagoon 2

3.3. FDA activity in samples of Versova and Bhandup STPs in Mumbai

The description of sampling points from Versova and Bhandup STP, Mumbai are depicted in Table 2. The comparative performance of lagoon 1 versus lagoon 2 in Versova STP, Mumbai has been shown in Table 3. At the optimized parameters, the FDA activity was estimated in the samples collected from the Versova and Bhandup STP, Mumbai. As depicted in Fig. 5 as well as Fig. 6, the FDA hydrolysis assay undoubtedly designates the change in overall microbial activity during the course of treatment in aerated lagoons in both the Versova as well as Bhandup STPs in Mumbai. For Versova STP, the FDA activity after aerated de-gritting chamber was found to be 0.07 µmole/mL/h which was enhanced to 0.10 µmole/mL/h and 0.08 µmole/mL/h for lagoon 1 and lagoon 2 respectively (Fig. 5). The enhanced FDA activity may be directly associated with the higher removal efficiencies of lagoon 1 for BOD and COD as compared to lagoon 2 as depicted in Table 3 [17].

The FDA activity of 0.01 µmole/mL/h in raw sewage was further increased to 0.11 µmole/mL/h and 0.10 µM/mL/h during the treatment for lagoon 1 and 2 respectively in Bhandup STP (as shown in Fig. 6). Owing to consumption of substrate, the microbial activity is expected to increase as the treatment proceeds. From the results, it is clear that an increasing trend in FDA activity has been

Table 3
Comparative performance of Lagoon 1 versus Lagoon 2 in Versova WWTP, Mumbai

Parameter	Units	Primary treated	Lagoon 1 outlet	% Removal	Lagoon 2 outlet	% Removal
pH	–	6.94	7.06	–	7.28	–
TDS	mg/L	532	483	–	474	–
TSS	mg/L	38	27	–	34	–
COD	mg/L	80	28	65	69	14
BOD_5	mg/L	33	13	61	28	15
NH_3–N	mg/L	11.4	9.57	16	8.62	24

TDS = Total dissolved solids; TSS = Total suspended solids; COD = Chemical oxygen demand; BOD_5 = Biochemical oxygen demand.

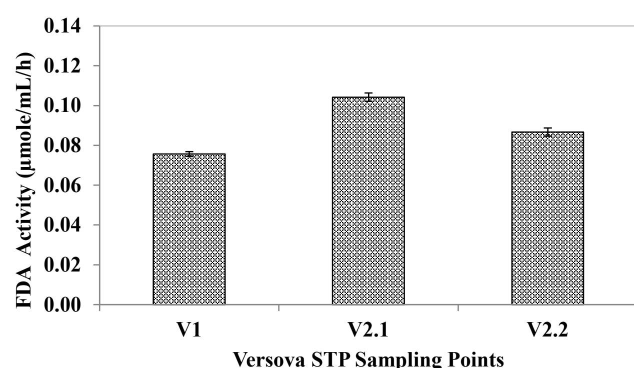


Fig. 5. FDA activity in samples collected from Versova STP, Mumbai (Reaction conditions: 100 mM phosphate buffer of pH 7.8, 4.8 mM FDA as a substrate solution, temperature 50°C, time 1.25 h).

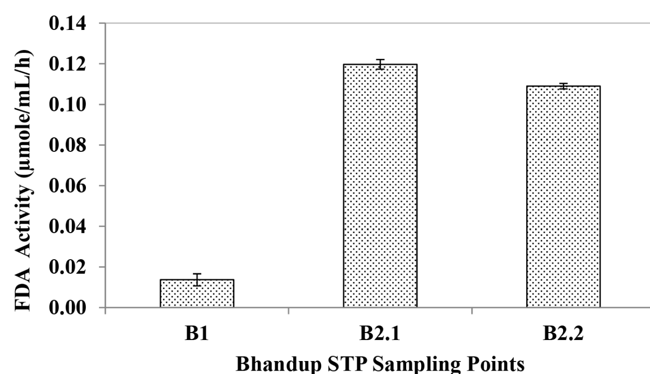


Fig. 6. FDA activity in samples collected from Bhandup STP, Mumbai (Reaction conditions: 100 mM phosphate buffer of pH 7.8, 4.8 mM FDA as a substrate solution, temperature 50°C, time 1.25 h).

observed during the course of water treatment. In the samples of both Versova as well as Bhandup STPs in Mumbai, the enhancement in the FDA activity has been observed during the course of treatment. Additionally, the physico-chemical parameters also exhibited the significant removal of pollutants in Versova STP, Mumbai with respect to the enhanced FDA activity.

4. Conclusions

Considering the several challenges including labour extensive, resource and time consuming tasks in routine evaluation of biological treatment units through physico-chemical and biological removal performance, the FDA hydrolysis assay seems to be better alternative due to easy, rapid and representative approach.

In the present study, several process parameters affecting the maximum extent of FDA activity were optimized along with investigation of FDA activity at different sampling points in Bhandup and Versova STPs in Mumbai. It has been observed that with various optimized parameters such as pH 7.8, 50°C temperature and with incubation time of 1.25 h; the maximum extent of FDA activity was obtained. Also, during the course of wastewater treatment, the FDA activity clearly shows the increasing trend. Thus, it is argued in this paper that the "FDA activity estimates" can be successfully used for diagnosis and performance optimization of biological treatment of sewages. Therefore, in order to determine any change in biological unit operations, the FDA activity may be routinely used as an indicator method.

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