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Kinetics studies on biodegradability of pharmaceutical effluent by *pseudomonas aeruginosa* ES 10

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ABSTRACT

Every Industry has problems of effluent treatment. This can be revealed by the effective treatment of the effluent. The effective treatment can be done by using microorganisms to stabilize the organic and inorganic load of the effluent. The aim of the present work is to study the Industrial effluent microbiota and to identify some new active strains which can bring about fast biodegradation of the organic compounds. Studies were carried out to isolate the microorganisms from the collected effluent sample from the Industries of Bollaram, Hyderabad. An investigation was carried out to analyze Physico-chemical parameters like Total suspended solids (TSS), Total dissolved solids (TDS), Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD) of untreated effluent and to degrade the effluent using native and non-native bacteria. The results of the parameters analysis revealed that untreated effluent was black in colour with an offensive odour. pH was alkaline with a high organic load such as TSS, TDS, BOD and COD which were higher than the permissible since the effluent had a high organic load, microbes (bacteria) present within the effluent was identified and isolated. The results of the study revealed the occurrence of *Pseudomonas* spp. And named as *Pseudomonas auruginosa* ES10. The presence of bacteria indicates the pollution status of the untreated tannery effluent, suggesting that it should be treated before its disposal using the biological method, particularly native and non-native bacteria for comparing their degrading efficiency. The results of the degradation study show that native bacteria *Pseudomonas auruginosa* ES10 was found to be very much successful in the reduction of toxic substances at the percentage range of 60-90% at different pH and incubation time, the bio-treated water can be reused for the agricultural and aqua-cultural purposes.

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INTRODUCTION

Environmental pollution is one of the major ecological challenges, for the last few decades all over the world, in which developing countries are most affected. The major cause of pollution is rapid industrialization, which is progressive at breakneck speed and is suffocating the earth with the common problems of pollution. A 2007 study reports that the discharge of untreated sewage is the single most important cause for pollution of surface and groundwater in India. Among the major industries in India, dairy

is one of the industries producing wastewater rich in organic matter and thus leading to the creation of odorous and high COD containing water (Harush *et al.*, 2011). The problem is not only that India lacks sufficient treatment capacity but also that the sewage treatment plants that exist do not operate and are not maintained (Dhanam, 2009).

The dairy industry on an average has been reported to generate 6-10 litres of wastewater per litre of the milk processed (Kolhe and Pawar, 2011). It is estimated that about 2% of the total milk processed is wasted into drains (Munavalli and Saler, 2009). Due to the high pollution load of dairy wastewater, the milk-processing industries discharging untreated/partially treated wastewater cause serious environmental problems (Thambavani *et al.*, 2009; Selvi *et al.*, 2012). In recent years, urban people are facing many problems, and water pollution are one among them. Environmentalists and government are looking for efficient, cheap and long-lasting solutions for waste treatment and recycling.

Physico-chemical methods of wastewater treatment are inevitably cost-intensive and cannot be employed in all industries, especially in developing countries like India. Hence, in recent years, the importance of biological treatment systems has attracted the attention of workers all over the world and has helped in developing relatively efficient, low-cost waste treatment systems. In order to design an efficient biological wastewater treatment, it is important to know the wastewater microbiota composition and the biochemical properties correlated to the origin of pollutants, as well as the optimum metabolic activity and the physical-chemical conditions (Pandey and Carney, 1998; Khasim *et al.*, 1989).

The study of wastewater microbiota and to identify some new active strains adapted to the wastewater physical-chemical conditions, which metabolize organic compounds, similar to those which determine the pollution of wastewaters such as starch, casein, basic carbohydrates and lactic acid (Mohammed *et al.*, 2001). Microbial strains were identified for the rapid biodegradation of the organic compounds. In the present work, bacteriological studies were carried out to isolate the microorganisms from dairy effluent samples.

MATERIALS AND METHODS

Screening of bacterial isolates

The soil sample was collected from different sites of Bulk Drug Industries, Bollaram, Hyderabad, where the bulk drug industry dumps the waste. 1gm of soil was serially diluted to get 10^{-7}. This dilution series was added to 3 conical flasks containing Bushnell Haas media containing 0.05ml of toluene, benzene and phenol as carbon source respectively. Incubated the flacks at 28°C for 5 days in BOD chamber. After incubation isolated colonies were streaked on nutrient agar slants for 24-48hrs to obtain pure cultures for further studies. Characteristics of industrial wastewater vary greatly from industry to industry and within industries also.

Estimation of BOD

Industrial effluent was collected and filled into a BOD bottle without making air bubbles. 2ml of manganese sulfate was added to the BOD bottle carefully by inserting the pipette just below the surface of the water. 2 mL of alkali-iodide-azide reagent was added in the same manner. The sample was mixed by inverting many times. A brownish cloud will appear in the solution as an indicator of the presence of Oxygen. Allow the brown precipitate to settle out to the bottom. 2ml of Conc.H_2SO4 carefully was added without forming air bubbles. The solution was mixed well to dissolve the precipitate. The bottle was kept in BOD incubator for 5days of incubation. After incubation, 50 ml of sample water was titrated with 0.025N Sodium thiosulphate until to a pale -yellow colour. Then added 2ml of starch solution to it and the sample turns blue in color. The titration was continued until the sample gets clear and note the readings. The concentration of dissolved oxygen in the sample is equivalent to the number of millilitres of titrant used (Begum and Noorjahan, 2006).

Amount of titrant used = amount of dissolved oxygen present (mg/L)

Estimation of COD

Standard FAS solution was prepared and filled the burette. Pipetted out 10 ml of 0.1 N potassium dichromate solution into a clean 250 ml conical flask. 2-3 drops of ferroin indicator were added to it and kept for digestion (reflex for 30 min). Allow the flask to cool down to room temperature and titrate it against standard FAS solution. With this, we will know the conc. Of Stnd. FAS solution, where un oxidized potassium chromate reacts with FAS. Note down the reading, and repeat the process for 2-3 times to get 2 concur values. Now pipette out 10 ml water sample/ effluent and add 2-3 drops of ferroin indicator, then titrate against standard FAS solution (Singh *et al.*, 1998).

COD of sample = ————————mg/L oxygen

Total suspended solids (TSS): Total solids (TS) - Total dissolved solids (TDS)

Table 1: Colony morphology

S.NO	Source of sample	Isolate number	Shape	Size	Margin	Elevation	Color	Consistency appearance
1	Effluent Soil sample	ES 1	Circular	Small	Entire	Raised	White	Dry
2	Effluent Soil sample	ES 2	Irregular	Small	Serrate	Flat	White	Dry
3	Effluent Soil sample	ES 3	Circular	Medium	Entire	Flat	Light orange	Dry
4	Effluent Soil sample	ES 4	Circular	Small	Entire	Raised	Pale yellow	Dry
5	Effluent Soil sample	ES 5	Irregular	medium	serrate	Flat	White	Dry
6	Effluent Soil sample	ES 6	Irregular	Small	Serrate	Flat	White	Dry
7	Effluent Soil sample	ES 7	Circular	Small	Entire	Raised	White	Dry
8	Effluent Soil sample	ES 8	Irregular	Medium	Serrate	Flat	White	Dry
9	Effluent Soil sample	ES 9	Irregular	Medium	Serrate	Flat	Trans parent	Sticky
10	Effluent Soil sample	ES 10	Irregular	Medium	Serrate	Flat	White	Dry
11	Effluent Soil sample	ES 11	Circular	Small	Entire	Flat	White	Sticky
12	Effluent Soil sample	ES 12	Circular	Medium	Entire	Raised	White	
13	Effluent Soil sample	ES 13	Circular	Small	Entire	Raised	White	Dry
14	Effluent Soil sample	ES 14	Irregular	Small	Serrate	Flat	White	Dry
15	Effluent Soil sample	ES 15	Circular	Medium	Entire	Flat	Light orange	Dry
16	Effluent Soil sample	ES 16	Circular	Small	Entire	Raised	Pale white	Dry
17	Effluent Soil sample	ES 17	Irregular	medium	serrate	Flat	White	Dry
18	Effluent Soil sample	ES 18	Irregular	Small	Serrate	Flat	White	Dry
19	Effluent Soil sample	ES 19	Circular	Small	Entire	Raised	White	Dry
20	Effluent Soil sample	ES 20	Irregular	Medium	Serrate	Flat	White	Dry
21	Effluent Soil sample	ES 21	Irregular	Medium	Serrate	Flat	Trans parent	Sticky
22	Effluent Soil sample	ES 22	Irregular	Medium	Serrate	Flat	White	Dry
23	Effluent Soil sample	ES 23	Circular	Small	Entire	Flat	White	Sticky
24	Effluent Soil sample	ES 24	Circular	Medium	Entire	Raised	White	Sticky
25	Effluent Soil sample	ES 25	Irregular	Small	Serrate	Flat	White	Dry

Continued on next page

Table 1 continued

26	Effluent sample	Soil	ES 26	Circular	Small	Entire	Raised	Pale yellow	Dry
27	Effluent sample	Soil	ES 27	Irregular	Medium	Serrate	Flat	White	Dry
28	Effluent sample	Soil	ES 28	Irregular	Medium	Serrate	Flat	White	Sticky
29	Effluent sample	Soil	ES 29	Irregular	Medium	Serrate	Flat	White	Dry
30	Effluent sample	Soil	ES 30	Circular	Small	Entire	Flat	White	Sticky
31	Effluent sample	Soil	ES 31	Circular	Medium	Entire	Raised	Trans parent	Sticky
32	Effluent sample	Soil	ES 32	Irregular	Small	Serrate	Flat	White	Dry
33	Effluent sample	Soil	ES 33	Circular	Medium	Entire	Raised	White	Dry
34	Effluent sample	Soil	ES 34	Irregular	Medium	Serrate	Flat	White	Dry
35	Effluent sample	Soil	ES 35	Irregular	Medium	Serrate	Flat	White	Sticky
36	Effluent sample	Soil	ES 36	Irregular	Medium	Serrate	Flat	White	Dry
37	Effluent sample	Soil	ES 37	Circular	Small	Entire	Flat	Pale white	Sticky
3	Effluent sample	Soil	ES 38	Circular	Medium	Entire	Raised	White	Sticky
39	Effluent sample	Soil	ES 39	Irregular	Small	Serrate	Flat	White	Dry
40	Effluent sample	Soil	ES 40	Circular	Small	Entire	Raised	White	Dry
41	Effluent sample	Soil	ES 41	Irregular	Medium	Serrate	Flat	White	Dry
42	Effluent sample	Soil	ES 42	Irregular	Medium	Serrate	Flat	White	Sticky
43	Effluent sample	Soil	ES 43	Irregular	Medium	Serrate	Flat	Pale white	Dry
44	Effluent sample	Soil	ES 44	Circular	Small	Entire	Flat	White	Sticky
45	Effluent sample	Soil	ES 45	Circular	Medium	Entire	Raised	White	Dry
46	Effluent sample	Soil	ES 46	Irregular	Small	Serrate	Flat	White	Dry
47	Effluent sample	Soil	ES 47	Circular	Medium	Entire	Raised	White	Dry
48	Effluent sample	Soil	ES 48	Irregular	Medium	Serrate	Flat	White	Dry
49	Effluent sample	Soil	ES 49	Irregular	Medium	Serrate	Flat	White	Sticky
50	Effluent sample	Soil	ES 50	Circular	Medium	Entire	Raised	White	Dry

Measurement of total dissolved solids

Wash filter paper and dry evaporating dish & weighed. Stirred sample and Pipette 50 ml while stirring. Transferred filtrate to evaporating dish & dry. Cooled & weighed the sample.

Calculating total dissolved solids concentration

mg Dissolved Solids/L : (A-B)X100

———

ml sample

Where, A = weight of dried residue + dish, mg; B = weight of dish, mg.

Measurement of total solids

Weighed evaporating dish and stirred the sample. Pipetted 50ml into evaporating dish & dry. Cool & weighed evaporating dish.

Calculating total solids concentration

mg Dissolved Solids/L : (A-B)X100

———

ml sample where, A = weight of dish + residue, mg; B = weight of dish, mg

Chemical treatment of effluent

100ml sample A and B were treated with 200mg each of alum at pH-7.0, ferrous sulphate at pH-6 and lime for 30min under continuous stirring. After 30 min, samples were centrifuged at 10000 rpm for 15 min to remove flock formed, and the supernatant was collected for subsequent treatment.

Primary screening method

20ml of inoculum of each isolate was added to 80ml of effluent was taken in a 500ml Erlenmeyer flask and incubated for 10days on a rotary shaker at 28°C and checked COD reduction.

Biological treatment of the effluent using screened isolates

20ml of inoculum of each isolate was added to 80ml of effluent was taken in a 500ml Erlenmeyer flask and incubated for 10days on a rotary shaker at 28°C and checked COD reduction. Culture flask without inoculum was also maintained simultaneously. pH was maintained between 7.2 - 7.5. (Evaporation losses were made by every 24hr with demineralized water. The COD analysis was carried out at 0h, 48h, 120h, 192h and 240h.

Identification of bacterial isolates

Extraction of DNA from bacterial isolates was done as per the protocol described by Atashpaz et al. [Jerin S *et al.,* 2011]. A single colony was inoculated in nutrient broth and was grown for 24 h at 37 °C. From the 5 ml of culture, the cells were harvested. 800 μL of lysing buffer (2% CTAB, 100 mM Tris-HCl, 1.4 M NaCl, 1% PVP, 20 mM Na2EDTA and 0.2% LiCl) was added to the sample and incubated at 65 °C (30 min for Gram-negative bacteria; 2 h for Gram-positive bacteria). The sample was centrifuged at 10000 rpm for 5 min at 4 °C. After the extraction of supernatant an equal volume of chloroform – isoamyl alcohol (24:1 v/v) was added to it and was centrifuged at 12000 rpm for 8 min at 4 °C. The DNA was extracted from the aqueous layer by adding cold (−20 °C) isopropanol. The dried DNA pellet was dissolved in 50 μL of 1X TE buffer. The quality and intactness of the extracted DNA were checked by running on 1% agarose gel, which contains 1 μg/ml ethidium bromide. The A260/A280 absorbance ratio was used to determine undesired contaminations (Jerin, 2011).

PCR amplification and sequencing of 16 S rRNA gene

PCR amplification and sequencing of the extracted DNA samples was done by Yaazh Genomics, Tamil Nadu. Amplification of 16 S rRNA universal primers gene fragment was done by using MJ Research Peltier Thermal Cycler. The universal primers (Forward primer 27 F AGAGTTTGATCMTGGCTCAG and Reverse primer 1492 R TACGGYTACCTTGTTACGACTT) were used.

1 μL of template DNA was added in 20 μL of PCR reaction solution. The PCR reaction was performed with the following conditions: Initial denaturation was done at 94 °C for 2 min, followed by 35 amplification cycles at 94 °C for 45 s, the annealing temperature of primers was 55 °C for 60 s, and extension at 72 °C for 60 s. The final extension was done at 72 °C for 10 min. The resulting PCR products were purified using Montage PCR Clean up kit (Millipore) and sequenced using ABI PRISM® Big Dye TM Terminator Cycle Sequencing Kits with AmpliTaq ® DNA polymerase (FS enzyme) (Applied Biosystems).

Bioinformatics protocol

The 16 S rRNA sequence was compared using NCBI blast similarity search tool. For multiple alignments of sequences, MUSCLE 3.7 program was used (Marwha *et al.*, 1998). Further, the program G blocks 0.91b was used to cure the poorly aligned regions (removes alignment noise) (Priya, 2010). Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 was used for the substitution model. The program Tree Dyn 198.3 was used for tree rendering.

Kinetic studies of COD reduction by using ES 10

20ml of inoculum of each isolate was added to 80ml

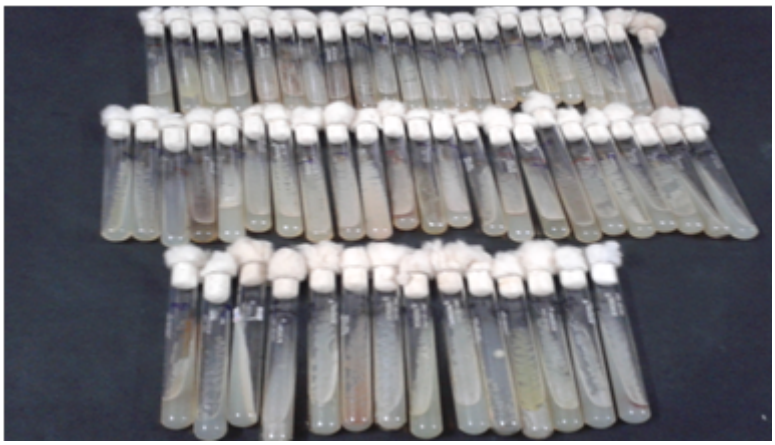

Figure 1: Pure culture was obtained by sheaths on nutrientagar

of effluent was taken in a 500 ml Erlenmeyer flask and incubated for 10days on a rotary shaker at 28°C and checked COD reduction. Different pH Sample A and B (Sample A and B pH adjusted n as 6.0, 6.5,7.0, 7.5 and 8) (control effluent with different pH test inoculated with ES and incubated at 240 hours (Karthikeyan *et al.*, 2010).

Different incubation period sample A and B

20ml of inoculum of each isolate was added to 80ml of effluent was taken in a 500ml Erlenmeyer flask and incubated for 10days on a rotary shaker at 28°C and checked COD reduction. pH maintained 7.5 and incubation period 0, 120 and 240 respectively.

RESULTS AND DISCUSSION

Colony characteristics

Observations about colony characteristics of the isolates were presented in Table 1. The colonies of the isolates were circular to irregular. The color of colonies was generally pale white. The shape varied from regular to irregular with entire to undulate margins. The bacterial isolates were stained to observe their morphological characters and the observations are presented in Table 1. Out of the 50 isolates isolated from industrial effluent, twenty strains were Gram-negative, and these were rods, cocci and coccobacilli. The arrangement of most of the cells was in pairs and in chains. Twenty strains were found to be Gram-positive with coccobacilli morphological character. These cells were mostly present in pairs. Among 50 isolates, obtained from dairy sludge samples, Gram-negative character was exhibited by twenty isolates. They were mainly rods. The gram-positive character was exhibited by thirty-five isolates. These were rods and coccobacilli. The cells were present singly, pairs, in chains and in clusters. The microscopic characteristics of the ten most efficient bacterial isolates are shown in Figure 1.

Chemical treatment of effluent

TSS ranged from 14560mg/l to 21210 mg/l, which was found to be beyond the permissible limits of CPCB. High amounts of suspended particles have detrimental effects on aquatic flora and fauna and reduce the diversity of life in the aquatic system and promote depletion of oxygen and slitting in ponds during the rainy season. TDS ranged from 13550 mg/l to 20200 mg/l which surpassed the CPCB permissible limits (2100 mg/l) (Sukumaran *et al.*, 2008; Chukwu, 2006). BOD has a minimum range of 5160 mg/ml and a maximum range of 9890 mg/ml, which was beyond the permissible limit of CPCB (Biswas *et al.*, 1995). Increase in BOD, which is a reflection of microbial oxygen demand leads to depletion of DO, which may cause hypoxia conditions with consequent adverse effects on aquatic biota (Noorjahan *et al.*, 2004). Oxygen depletion could be followed by anaerobic conditions, which would result in reduced diversity and distribution of aquatic fauna. Further, the presence of organic matter will promote anaerobic action leading to the accumulation of toxic compounds in water bodies. Oxygen depletion could be followed by anaerobic conditions, which would result in reduced diversity and distribution of aquatic fauna.

COD ranged from 10488 to 14076, which has exceeded the permissible limit of CPCB. COD test is the best method for organic matter estimation and rapid test for the determination of total oxygen demand by organic matter present in the sample. The present investigation revealed high levels of COD. This indicates that the effluent is unsuitable for the existence of aquatic organisms due to the reduction in DO content (Table 2).

Primary screening method

Microbes especially bacteria act as bio-indicator of high polluted effluents, which prompted to analyse

Table 2: Chemical characteristics of sample A&B before and after chemical treatment

Parameter	Sample A		Sample B	
	Before treatment (mg/l)	After treatment (mg/l)	Before treatment (mg/l)	After treatment (mg/l)
TS	21210	19200	14560	12610
TDS	20200	18500	13550	10610
TSS	910	472	1080	642
BOD	5160	4870	9890	9043
COD	10488	10200	14076	13064

the native bacterial population in tannery effluent and to use it for bio degradation. The results of the analysis of isolation and identification of microbes (bacteria) present in untreated industrial effluent. The results of the study revealed the occurrence of Pseudomonas sp. The presence of different strains ES10, ES17, ES24, ES28 and ES37 Pseudomonas species in the effluent as reported in the present study has significance in their utility as biological indicators (Goudar and Subramanian, 1996). Based on the biological treatment of eluent, the top 5 were selected further studies (Table 3).

Table 3: Strains selected for the studies

S.No	Isolate
1.	ES 10
2.	ES 17
3.	ES 24
4.	ES 28
5.	ES 37

Biological treatment of the effluent using screened isolates

Percentage of COD reduction is ranging between 70-80% by using the selected strains. COD of effluent was reduced to 80% by ES 10 strain and 70% by ES 24 strain in Sample A (Figure 2). This is supported by the work of (Farag and Zaki, 2010). Percentage of COD reduction is ranging between 62-80% by using the selected strains. COD of effluent was reduced to 80% by ES 10 strain and 62% by ES 24 strain in Sample B (Figure 3).

Identification of Bacterial isolates

Sequencing results

For bacterial classification, generally, sequencing of 16 S rRNA gene was used as an important identification tool (Maghsoodi *et al.*, 2007). The reasons include its presence in almost all bacteria; its function has not changed over time, and the 16 S rRNA gene (1,500 bp) is large enough to provide a genus and species identification for isolates. The DNA samples of all the bacterial isolates were run on the agarose gel, and the bands were visualized when observed under the Gel doc. The sequencing of the 16 S rRNA gene was done. Based on the 16 S rRNA sequences, phylogenetic dendrograms were constructed to know the genetic relationship between the bacterial isolates. The identification of the isolates phylogenetic dendrograms was represented in Figure 4.

Kinetic studies of COD reduction by using ES 10

In sample A, the pH range from 6-8 was selected for the study of COD by untreated and *Pseudomonas aureginosa* ES10 strain. In control the COD range was 9500 mg.ml to 10500 mg/ml whereas in *Pseudomonas aureginosa* ES10 treated COD range was 3000 mg/ml to 6000 mg/ml. *Pseudomonas aureginosa* ES10 showed the highest reduction of COD at pH 7.5 (Figure 5). In sample B, the pH range from 6-8 was selected for the study of COD by untreated and *Pseudomonas aureginosa* ES10 strain. In control the COD range was 12000 mg.ml to 13000mg/ml whereas in *Pseudomonas aureginosa* ES10 treated COD range was 3800 mg/ml to 8000 mg/ml. *Pseudomonas aureginosa* ES10 showed the highest reduction of COD at pH 7.5 (Figure 6).

Different incubation period sample A and B

In sample A, the pH range from 0-240h was selected for the study of COD by untreated and *Pseudomonas aureginosa* ES10 strain. In control the COD range was 9500 mg.ml to 10500 mg/ml whereas in *Pseudomonas aureginosa* ES10 treated COD range was 3000 mg/ml to 10000 mg/ml. *Pseudomonas aureginosa* ES10 showed the highest reduction of COD at 240h (Figure 7), (Fantroussi and Agathos, 2005). In sample A, the pH range from 0-240 h was selected for the study of COD by untreated and *Pseudomonas aureginosa* ES10 strain. In control the COD range was 12000 mg.ml to 13000mg/ml whereas in *Pseudomonas aureginosa* ES10 treated COD range was 3000 mg/ml to 12800 mg/ml. *Pseudomonas aureginosa* ES10 showed the highest reduction of COD at

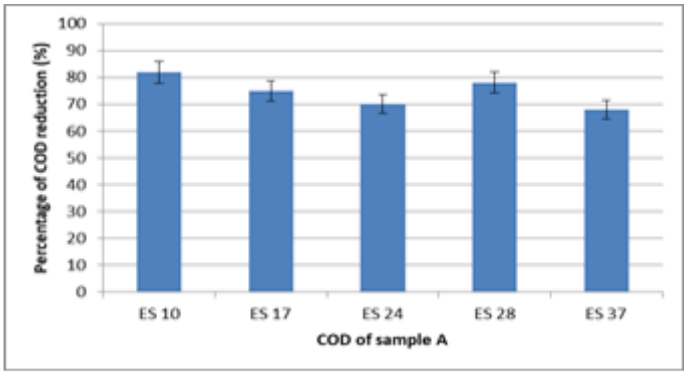

Figure 2: COD different isolates for sample A

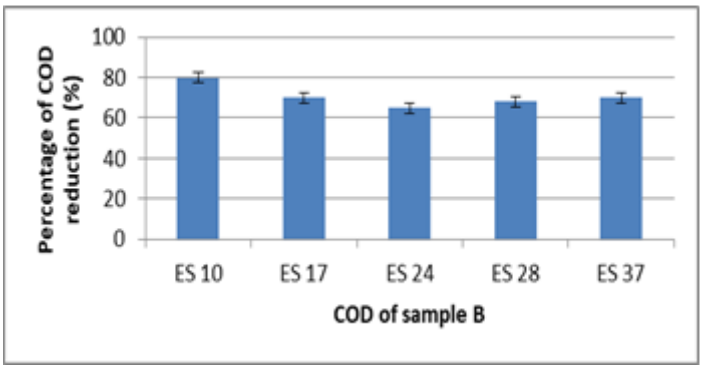

Figure 3: COD different isolates for Sample B

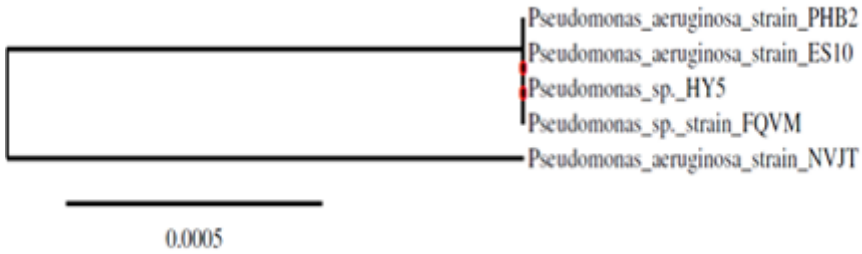

Figure 4: COD different isolates for Sample B

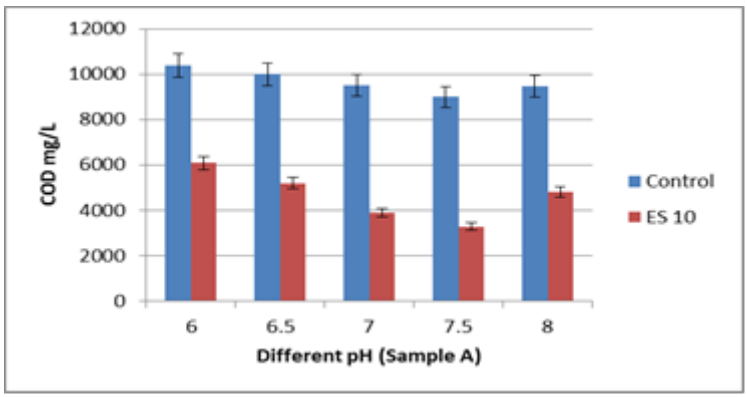

Figure 5: Studies of COD by Pseudomonas ES10 on Sample A at different pH

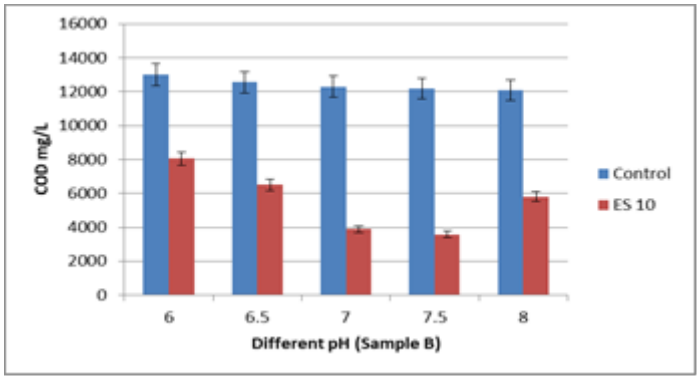

Figure 6: Studies of COD by Pseudomonas ES10 on Sample B at different pH

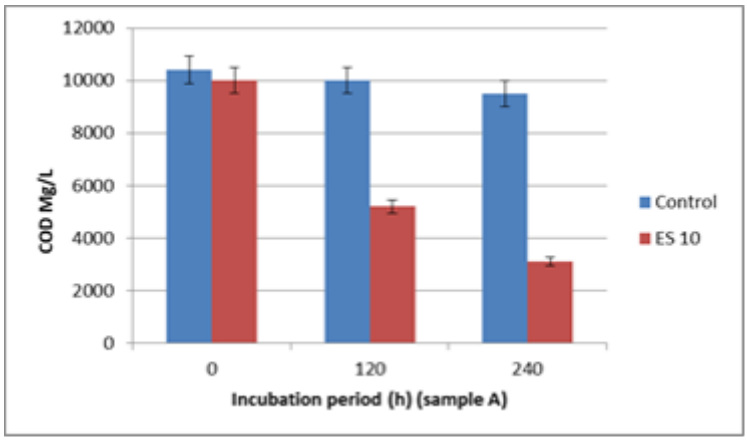

Figure 7: Studies of COD by Pseudomonas ES10 on Sample A at different incubation period

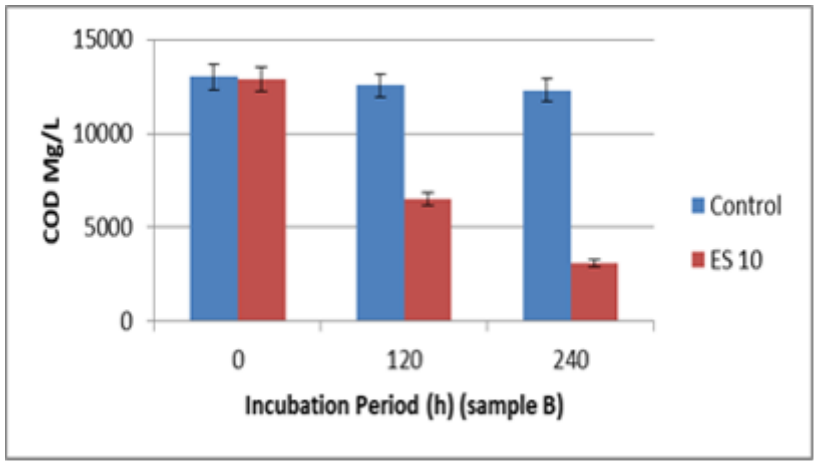

Figure 8: Studies of COD by Pseudomonas ES10 on Sample B at different incubation period

240 h (Figure 8).

CONCLUSION

In any Industrial plant, the quantity and characteristics of effluent depend upon the extent of production activities. The above results, it can be inferred that the maximum reduction of toxic substances was recorded in a bio-treated sample using *Pseudomonas aureginosa* ES10 compared to untreated effluent. Thus, from the foregoing discussion, it is very clear that microbes play an important role in the biodegradation of organic and inorganic matter. They have enzymes that allow them to use environmental contaminants as food and hence make them ideal for biodegradation. Besides their characteristics like rapid growth, metabolism and a remarkable ability to adjust to a variety of environments make them very useful in biodegradation. How successful are the micro-organisms in degrading the environmental contaminants depends on the type of microbes, contaminant and on the nature of the con-

taminated site? From the present study, native *Pseudomonas aureginosa* ES10 showed efficient degrading capabilities by degrading the contaminants as they use it for their growth and reproduction. Thus, degradation by microbes seems to be a most promising technique for 100% untreated tannery effluent as evidenced in the present investigation. It is well-known that water of good quality and free of pollutants are primary requirements for agricultural and piscicultural practice. After degradation, the treated water could be used for crop cultivation or irrigation and aquaculture purpose.

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