PHYSICO–CHEMICAL AND GENOTOXIC ANALYSIS OF DAIRY INDUSTRIAL EFFLUENT

AAGOSH VERMA* AND ANURADHA SINGH

Department of Zoology, Environmental Molecular Microbiology Lab, University of Rajasthan Jaipur, India

ABSTRACT

India is the highest milk producer of the world. Dairy industries discharge wastewater which is characterized by high chemical oxygen demand (COD), biological oxygen demand (COD), nutrients, and organic and inorganic contents. Such wastewaters, if discharged without proper treatment, severely pollute water bodies and disrupts complete ecosystem. This paper presents the physico -chemical characteristics of waste water from the Dairy Industry. The waste water from the Dairy Industry is characterized by pH, COD (Chemical Oxygen Demand), BOD (Biological Oxygen Demand). BOD noted in Jan' 12 and July' 12 after ETP was 320 ± 26.76 and 355 ± 78.99 while COD range from 954 ± 86.18 to 982 ± 67.57. Study is also done for their genotoxicity analysis by Ames mutagenicity assay. Mutagenecity ratio of dairy wastewater samples noted was below 2.

INTRODUCTION

India is the largest producer of milk and Rajasthan ranks II among milk producing states of the country. Among all industrial sectors, food processing units (including Dairy Industry) are major contributor of waste water generation. India, being largest producer of milk, huge amount of waste is also generated from these industries through its different operations like pasteurization, whey generation, sanitizers, detergents, washing of utensils etc. To comply with the discharge standards, the dairy projects in India are practicing an elaborate effluent treatment protocol. The main objective of treating dairy waste is reduction of organic load so that pollution load may be reduced to a considerable level and to remove pathogenic microbes so that an eco-friendly effluent could be generated. In India generally, dairy industry reported to gerate 6-10 litres of wastewater per litre of the milk processed. The waste generated is high in organic matter and can create pollution and can affect the ecosystem (Anikwe and Nwobodo, 2006). The degradation of environment results by the adverse effect of industrial waste on living organism and agriculture (Danalewich, *et al.*, 1998). Keeping all these points into consideration the study has been done to study physico – chemical properties of wastewater generated from dairy industry.

MATERIALS AND METHODS

Sample area and sample collection

Samples were collected from Jaipur Dairy directly from a discharge point in a clean plastic container, transferred to laboratory and stored at 4°C until use for analysis. Sampling was done in the month of January and July'12. Study was done to analyze dairy industrial wastewater samples physico – chemically and genotoxically through Ames mutagenicity assay.

Physico-chemical analysis

In the study, Physico – chemical parameters of dairy wastewater was measured i.e., Physical parameter including Temperature, pH, Total Dissolved Solids and Chemical parameters namely Chemical oxygen Demand (COD), Biological Oxygen Demand (BOD), Sulphates, Chloride, Oil and Grease. A multiparameter Water Analyzer Kit (Systronics Water Analyzer 371) was used to determine these following parameters except BOD and COD which were estimated by

*Corresponding authors email: aagosh.minkss@gmail.com

Membrane electrode method (APHA, 1995) and Open reflux method (APHA, 1999) while Chlorides were measured using Mohr's Method.

Genotoxicity analysis of dairy wastewater after Effluent treatment plant: Ames mutagenicity assay

A short term bacterial assay namely. *Salmonella typhimurium*/ microsomal assay is used to analyze dairy waste samples. This is a widely accepted assay for identifying substances that leads to genetic mutations and can produce genetic damage (Ames, *et al.*, 1973; Ames, *et al.*, 1975; Ames, *et al.*, 1979). The *Salmonella* or histidine reversion assay is based on the use of mutant strains of *Salmonella typhimurium* which revert from histidine dependence (auxotrophy) to histidine independence (prototrophy) at an increased frequency or rate, in presence of mutagens. This assay can be preliminary screening ideal genotoxic bioassay for complex unknown environmental wastes (Mortelmans and Zeiger, 2000).

Tester strains

Salmonella typhimurium strains viz., TA 98 and TA 100. These strains were obtained from Microbial Type Culture Collection and Gene Bank (MMTC), Institute of Microbial Technology (IMTech), Chandigarh (India). They were stored as glycerol cultures at -20°C. All tester strains were maintained and stored according to the standard methods (Ames, *et al.*, 1975; Maron and Ames, 1983). The genotypes of strains (histidine requirement, rfa mutation, uvr B, and R-factor) were confirmed immediately after receiving the cultures and every time a new set of frozen permanents was prepared and used. Plate incorporation method as described by (Ames, *et al.*, 1975) and revised by (Maron and Ames, 1983) was used. The samples were analyzed with and without hepatic S9 fraction. Introduction of mammalian liver enzymes into the prokaryotic system incorporates the aspect of mammalian metabolism into the *in vitro* test. Uninduced Swiss albino mice were used to prepare the standard S9 mixture. It was prepared according to the protocol described by (Maron and Ames, 1983).

Positive control for

TA 98: 4-nitro-o-phenylenediamine

TA 100: Sodium azide

Mutagenicity assay and Methodology

The *Salmonella typhimurium* strains were grown at 37°C, with shaking, for 10 hrs to obtain final cell concentration of 10^9 bacterial cells. 0.1 ml of this fresh culture + 0.2 ml of his/bio solution $_+$ 0.1 ml or less of

the test chemical + 0.5 ml of buffer or 0.5 ml of S9 mix was mixed and the total volume was made up to 1.0 ml by autoclaved distilled water. This mixture was gently shaken and poured on plates containing about 25 ml of minimal glucose agar medium. Each set of experiments was repeated twice. Average numbers of spontaneous revertants per plate for TA98 and TA100 without metabolic activation were counted to be 54 - 101 CFU, respectively, and with metabolic activation, spontaneous revertants were 80 - 92 CFU, respectively. The test concentrations were selected from a set of standard test doses for liquids i.e., 2 µl, 5 µl, 10 µl, 50 µl and 100 µl (Hayes, 1982). All glassware, reagents, media and petri plate used were sterile. The plates were immediately covered with paper to protect photosensitive chemicals present in the test compounds. Plates were inverted and placed in a dark incubator for 48 hrs at 37°C. After 48 hrs, the revertant colonies on the test and control plates were counted manually and presence of the background lawn on all the plates was confirmed.

RESULTS AND DISCUSSION

Temperature

It is a first important parameter to be measured in physico-chemical analysis. It is important as its effects the chemistry and biochemical reactions in organisms, it also affects the efficiency of treatment units (Jayalakshmi, *et al.*, 2011), for example, viscosity increases in cold temperature. This in turn, diminishes the efficiency of settling of the solids present in water because of the resistance offered by high viscosity to downward motion of the particles as they settle. Further, it is an important factor for calculating solubility of oxygen, carbon dioxide, bicarbonates and carbonates.

In the present study, temperatures noted were 27 ± 2.08°C and 31 ± 1.53°C in months of January and July'12 (Table 1). There is slight change in the values of temperatures were due to seasonal variations. During the summer, water temperature is higher because of decrease in water level, clear atmosphere and great solar radiation. While in rainy and winter season there is cloudy atmosphere, high percentage of humidity and high-water levels.

Turbidity

Turbidity noted was within the range of 20 - 23 NTU for treated effluent in July and 23 ± 24 in months of Jan' 2012 (Table 1). A study reported turbidity fluctuates from 35.9 – 97.1 NTU in dairy waste water which is much higher by (Ashish and Omprakash, 2014; Carawan, *et al.*, 1979; CPCB, 1995).

Table 1. Observed values of physico-chemical parameters for dairy industrial effluent samples after ETP collected in month of January and July in year 2012.

Dairy Effluents	January	July	Standards
Colour	Colourless	Colourless	-
Temperature (^{O}C)	27 ± 2.08	31 ± 1.53	Shall not exceed 5°C above the receiving water temp
Turbidity (NTU)	23 ± 1	20 ± 0.06	-
TDS (ppt)	1.2 ± 0.25	1.28 ± 0.25	-
Salinity (ppt)	1.4 ± 0.24	1.3 ± 0.40	-
Conductivity (mS)	3.5 ±0.36	3 ± 0.29	-
pH	6.8 ± 0.64	6 ± 0.69	6.5- 8.5
BOD	320 ± 26.76	**355 ± 78.99**	350[1]/100[2]
COD	**954 ± 86.18**	**982 ± 67.57**	250[3]
Chlorides	230 ± 0.2 mg/l	241 ± 1 mg/l	600
Sulphate	81 ± 0.2 mg/l	74 ± 1 mg/l	Not above 100
Oil and grease	1.9 ± 0.1 mg/l	2.3 ± 0.2 mg/l	10

*Values in Bold are exceeding the limits of General Indian Standards for Discharge of Environmental Pollutants IS: 10500. For effluent discharge into inland surface waters BOD limit shall be made stricter to 30 mg/l by the concerned State Pollution Control Board

Total dissolved solids

Usually TDS (total dissolved solids) is measured in ppt (part per trillion). In the present study, the total dissolved solid of treated effluent in the month of January'12 was found to be 1.2 ± 0.25 ppt and in July'12 it was 1.28 ± 0.25 ppt. (Kolhe, *et al.,* 2009; Rao, *et al.,* 1993) studied dairy industrial effluent and recorded total dissolved solid value, which ranged from 1000 mg/l for untreated effluent and 480 mg/l for treated effluents. Maximum value of total dissolved solids were found in rainy season (dilution of waste effluents in water bodies) than summers (due to decaying vegetation) while minimum value was found in winters due to stagnation. Similar TDS values were obtained by (Khojare, *et al.,* 2002; Dharam, 2009; Farizoglu and Uzuner, 2011; Gaiker, *et al.,* 2010) for treated waste water from milk processing unit.

Salinity

Salinity values noted of final dairy effluent was 1.4 ± 0.24 in month of January and 1.3 ± 0.40 in July' 12.

Conductivity

Electrical conductivity of water is also an important parameter for determining the water quality. It is a measure of concentrations of ionized substance in the water which is directly proportional to the water's capacity for carrying electrical current. Conductivity of dairy effluents noted in year 2012 was 3.5 ± 0.36 mS and 3 ± 0.29 mS in months of January and July respectively for treated dairy effluent after ETP. No standards have been specified for conductivity and TDS in General Indian Standards for Discharge of Environmental Pollutants (IS: 10500).

pH

pH (Hydrogen ion concentration) is a measure of activity of the hydrogen ion. It is used to find the acidity and basicity of the sample. pH less than < 7 are said to be acidic and greater than >7 are said to be basic. pH values noted in year 2012 for treated dairy effluent were 6.8 ± 0.64 and 6 ± 0.69 in months of January and July. (Table 1). Other studies conducted by (Kolhe and Pawar, 2011) also found pH of dairy effluents within 6 – 9.5 range, which was quite similar to the values obtained in present study. Alkaline pH of dairy effluent was also observed by (Medhat and Usama, 2004; Monroy, *et al.,* 1995; Khojare, *et al.,* 2002; Gaikar, *et al.,* 2010; Hancock, 1973). Presence of nutrients, use of alkaline cleaning agents and high organic load in dairy industry lead to its alkaline pH.

Biochemical Oxygen Demand (BOD)

It is defined as amount of oxygen required to break down organic matter by aerobic microbial organism in water bodies. It is most important parameter which define the strength of industrial wastewater to create pollution. In the present study, the BOD of treated effluent range between 320 ± 26.76 in Jan'12 to 355 ± 78.99 in July'12. BOD recorded high in July (rainy season) due to dissolution of various solids in wastewater. Milk constituents such as lactose, casein, fat, inorganic salts and detergents and sanitizers used for washing also increases BOD. Low value of BOD is comparatively in winter months may be due to lesser quantity of suspended solids, total solids in water as well as to the quantitative number of microbial population. (Avasan and Rao, 2001).

Chemical Oxygen Demand (COD)

The oxygen required for chemical oxidation of organic matter with the help of strong chemical oxidant. The waste is measure in terms of equality of oxygen required for oxidation of organic matter to produce CO_2 and water. It is a fact that all organic compounds with a few exceptions can be oxidizing agents under the acidic condition. COD test is useful to pinpoint toxic condition and presence of biological resistant substances. In the present study the value of treated effluent range from 954 ± 86.18 Jan'12 and 982 ± 67.57 July'12. (Trivedi, *et al.*, 1986; Shaikh, *et al.*, 2009) observed COD value of textile industry ranges from 300 ppm to 2400 ppm.

Similar, high BOD_5 570 mg/l and COD 1486.8 mg/l loads were also reported by (Vishakha, *et al.*, 2013; Welch, 1980; http://en.wikipedia.org/wiki/Conventional_pollutant)for dairy industry of Vijaynagar, Maharashtra. High values of BOD_5 and COD obtained in present study are in accordance with earlier studies. Emmanuel (2002) recorded a mean BOD_5 value as high as 603 mg/l and mean COD value as high as 1223 mg/l. The discharge of wastewater to the environment without any treatment plays significant risk for public health and environmental pollution.

Chloride

Chloride content in the present study noted was 230 ± 0.2 mg/l in Jan'12 and 241 ± 1 mg/l in July'12. (Kolhe, *et al.*, 2008) observed untreated effluent chloride 205 mg/lit and the treated effluent was 170-180 mg/lit of sugar mill.

Sulphate

The Environmental Protection agency (EPA) classified sulphate under secondary maximum contaminant level (SMCL) standards. The SMCL for sulphate in drinking water is 25 mg/l or (ppm). Sulphate concentration in dairy industrial wastewater after ETP treatment noted was 81 ± 0.2 mg/l in Jan'12 and 74 ± 1 mg/l in July'12 which is much lesser than standards of EPA. (Kolhe, *et al.*, 2008) observed the sugar mill effluent was having sulphate of untreated effluent is 660 mg/l and treated effluent showed 220 mg/l which is much higher to as observed in the present study.

Oil and grease

The oil and grease content of domestic and certain industrial waste water and of sludge's is an important in handling and treatment industry and prompting their ultimate disposal. If present in excessive amount, they can interfere with an aerobic and anaerobic biological process and lead to decrease in waste water treatment efficiency. If improperly discharged it can create humus and can decrease soils fertility. A knowledge of quantity of oil and grease present in effluent can prove to be helpful for designing and properly handling wastewater system. In the present study oil and grease of treated effluent was 1.9 ± 0.1 mg/l in January and 2.3 ± 0.2 mg/l in July'12. (Trivedi, *et al.*, 1986) reported oil and grease in textile industry effluent varies from 230 to 1897 mg/l.

Calculation of data : Ames mutagenicity assay

"Two fold rule" is the most common method for evaluation of data from mutagenicity assay. According to this rule positive response is stated when spontaneous reversion rate doubles in one or two chemical concentrations. (Mortelmans and Zeiger, 2000). The rule states that if the test compound doubles or more than doubles which leads to spontaneous frequency and then the compound is considered to be significantly mutagenic. For this analysis mutant strains of S. typhimurium (TA 98 and TA 100) is used. This haploid strains of bacteria already contain particular mutation in the gene encoding and enzyme used to synthesize "histidine" amino acid. Such bacteria require histidine to make many of their proteins and will die in the absence of histidine.

Spontaneous revertants

Spontaneous mutations (those that occur by chance, not by chemical treatment) will appear as colonies on the control petri plates.

Induced revertants

If the test chemical is mutagenic it changes strains into histidine independent and now induced revertants will grow on petri plates.

Ames assay results are expressed by calculating mutagenicity ratio and analysing dose response curves. Here, Mutagenecity ratio is known as average induced revertants on test plates (spontaneous revertants plus induced revertants) to average spontaneous revertants on negative control plates (spontaneous revertants).

$$Mutagenecity\ ratio : \frac{Spontaneous\ revertants\ plus\ induced\ revertants}{Spontaneous\ revertants}$$

According to two fold rule, if mutagenicity ratio calculated greater than 2 ; Sample can be stated as mutagenic
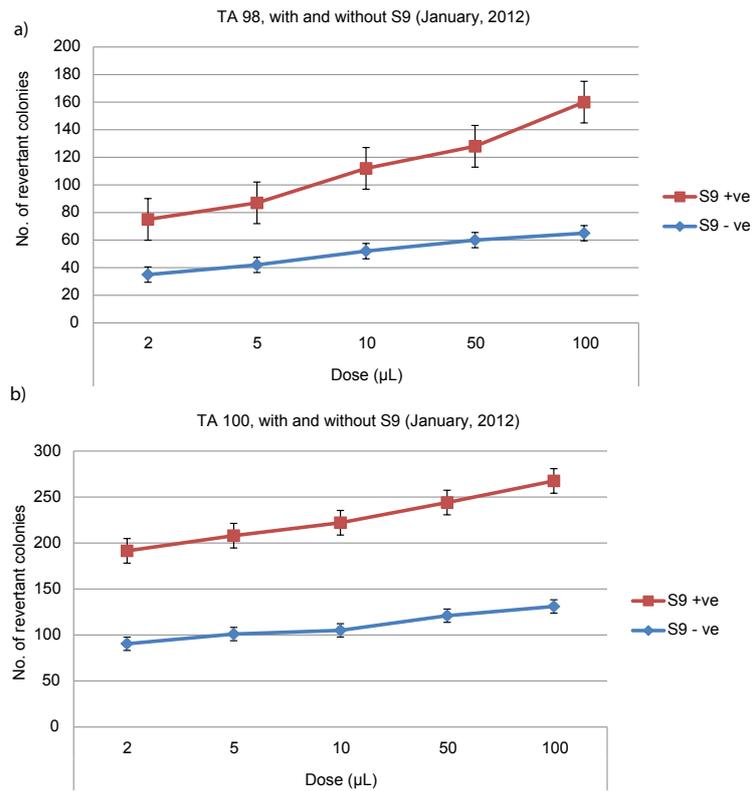
Genotoxicity of dairy waste was studied using

Fig. 1 (a and b) Concentration-response curves for dairy industry wastewaters with strains TA98 and TA100 in absence and presence of metabolic activation (January, 2012).
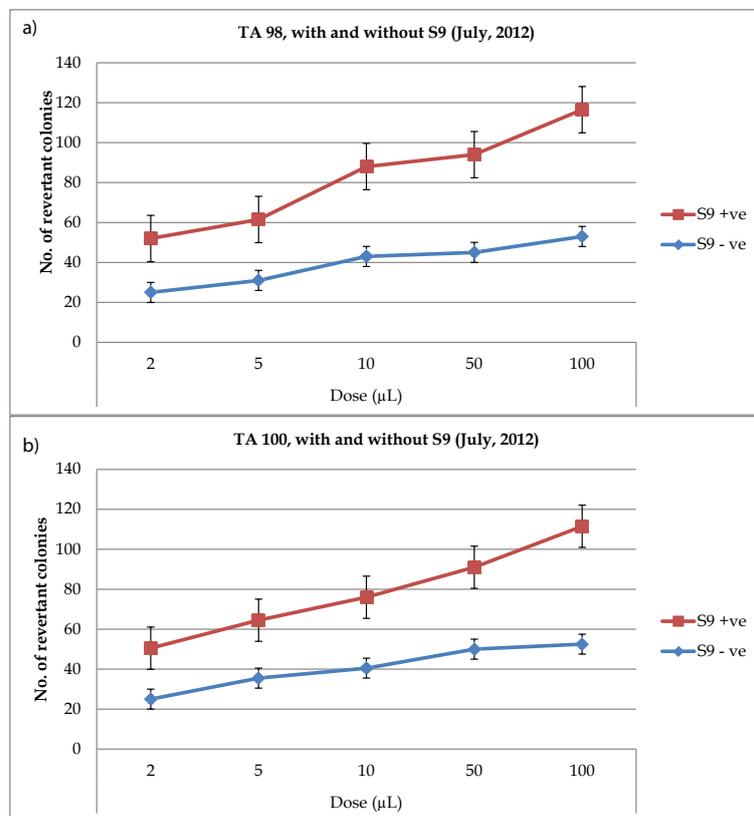

Fig. 2 (a and b) Concentration-response curves for Dairy Industry wastewaters with strains TA98 and TA100 in absence and presence of metabolic activation (July, 2012).

Table 2. Mutagenicity ratio* of *Salmonella* TA98 and TA 100 in Ames test on dairy industrial waste effluent sample Jan' 12 and July' 12.

Sample site	Dose (µL)	Mutagenicity Ratio TA98		Mutagenicity Ratio TA100	
		-S9	+S9	-S9	+S9
Jaipur Dairy Industrial Waste Effluent after ETP treatment	2	-	-	-	-
	5	-	-	-	-
	10	-	-	-	-
	50	-	-	-	-
	100	-	-	-	-

Salmonella typhimurium mutagenicity assay. At lowest concentration (2 µl), DaS final (effluent after ETP) samples collected during Jan 2012 produced 25-26 induced revertant colonies with strain TA 98 and 91-93 induced revertant colonies with strain TA 100 without metabolic activation. Increasing the sample concentration further resulted in increased number of induced revertants regularly. At 100 µl concentration, number of induced revertants with strain TA 98 was counted to be 50-51 and with TA 100 it was 130-132 in the absence of hepatic S9 fraction (Fig. 1 and 2).

For July 2012 samples, number of induced revertants at 100 µl were found to be 70-72 and 120-122 with TA 98 and TA 100 respectively, in absence of S9 (Fig. 1 and 2). There is increase in number of revertant colonies on addition of S9 mix with both strains for samples collected in 2012. Upon adding S9 mix, there is slight increase in number of induced revertants. At lowest concentration 2 µl count of induced revertants was 55 - 59 of treated final effluent collected in Jan 2012, 59-61 in July 2012 with TA 98 while number of induced revertant increases to 191 - 195 and 180 - 191 in January and July 2012 with TA 100. Further, increasing the concentration, more revertants were induced to grow January and July 2012, effluents show similar profile with TA 98 and TA 100 samples (Table 2). Mutagenicity ratio is less than 2.

The results obtained with the Ames assay, having mutagenicity ratio less than 2 indicate that discharged wastewaters after ETP treatment is non toxic (Table 2). With TA98 and TA100 the dairy effluent sample after ETP showed negative mutagenicity. The MR of less than 2 was obtained at all the sample doses of 2 µl to 100 µl (Table 2). Addition of hepatic fraction further increased the number of revertants in all cases, indicating that mammalian enzymes can convert some of the pro- mutagenic compounds into active mutagenic metabolites. But the increment was not able to increase mutagenicity ratio more than 2 hence, samples was noted non – genotoxic.

Similar toxicity analysis studies was done using onion bulb (Allium cepa L.) root growth inhibition

and chromosome aberration assay. (Fiskesjö, 1993, 1997; Rank, 2003) evaluated genotoxicity of treated and untreated dairy effluents and observed similar results to the present study. The onion bulbs when exposed to raw effluents showed greater root inhibition than those exposed to treated effluent (Olorunfemi, *et al.*, 2012).

CONCLUSION

The present study was confined to one dairy of Jaipur but this can give us a brief idea about physico – chemical scenario of waste water generated from different dairy plants. Study also emphasize on wastewater generated after ETP treatment i.e., final waste. Genotoxicity of final wastewater was also checked by Ames mutagenicity assay. Mutagenicity ratio of dairy waste water after ETP was less than 2 hence, dairy waste is nontoxic as being food industry no such harmful chemicals is used in dairy which can lead to toxicity. Ames test was basically designed to detect chemically induced mutagenesis (Ames, *et al.*, 1975). Over the years, values of this test have been recognized by the scientific community and by Government agencies and Corporations (Report, 1983; Dearfield, *et al.*, 1991; Auletta, *et al.*, 1988; Kirkland, 1993; Mortelmans and Zeiger, 2000). It is used worldwide as an initial screen to mutagenic potential of chemicals, drugs, complex mixtures, etc. because there is a high predictive value for rodent carcinogenicity, when a mutagenic response is obtained (McCann, *et al.*, 1975; Mohn, 1981; Ashby and Tennant, 1988; Zeiger, *et al.*, 1990; Ashby, *et al.*, 1991). This study shows the usefulness of combining two basic parameters physicochemical analysis and genotoxic analysis to bring about better understanding of the toxicity of industrial effluent pollutants and their influence on human health and plant life. All physico – chemical parameters except COD and BOD meets up the limits of State pollution board. Hence, watery effluent discharged can be used for gardening purposes while alternative bioremediation method should be researched for oily and greasy sludge discharged.

ACKNOWLEDGEMENTS

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