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BIOREMEDIATION OF DAIRY WASTE WATER FOR NITRATE REDUCTION

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ABSTRACT

The dairy industry being one of the growing industries in India which produces high amount of effluent per unit of production. It is one of those sectors which are the largest producer of waste water where it is directly allowed to flow in rivers or agricultural lands. This waste water has large amount of organic and inorganic load. This organic load is basically constituted of milk (raw material and dairy products), reflecting an effluent with high levels of chemical oxygen demand (COD), biochemical oxygen demand (BOD), oils, grease, nitrogen and phosphorus. Hence, degradation of these compounds from source itself is necessary which can be achieved by bioremediation. Bioremediation is an environmental clean-up technique that uses microorganisms for degrading recalcitrant chemicals by utilizing them as metabolic substrate. The present study was based at screening of effluent adapted bacteria from untreated dairy waste BOD, COD, nitrate and phosphate were estimated. It was observed that there was reduction of 85.5% in nitrate content by *Bacillus sp*.

KEYWORDS: Bioremediation, Dairy waste water, Microorganisms Nitrate, Pollution.

INTRODUCTION

Pollution is the introduction of contaminants into the natural environment that causes adverse change. The food industry consumes large volumes of waste water and concomitantly

generates substantial amount of effluent per unit production. The effluent is characterized by high Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD), organic and inorganic contents mainly nitrate and phosphate. Additionally, generally contain fats, nutrients, lactose, detergents, sanitizing agents as well as milk constituents such as casein, lactose, fat, inorganic salts. The wastewaters generated from milk processing can be separated into two groups—the first group comprises wastewater having high flow rates and the second comprises the effluents produced in small milk-transformation units [Castillo, S. et al 2007]. Nitrate and Phosphate are recognized as the major nutrients which are required by living organisms for their physiological processes. They are most commonly added as fertilizer to enhance the quality of soil. However they have emerged as most abundant pollutants in the world due to their excess usage. The traditional agricultural practices like dry farming with marginal irrigation, flood plain farming and random application of fertilizers are considered as diffused sources of nitrate and phosphate in soil and aquifers [DebRoy, S. et al 2012] in addition to biogeochemical cycling of nitrate, anthropogenic activities, uncontrolled land discharges of treated or raw domestic and industrial wastewaters, landfills, and animal wastes predominantly from animal farms [Galloway, J.N. et al 2008]. Several studies focused on the nitrates removal from wastewater in order to achieve an acceptable concentration in treated waters to be discharged into the environment. Complementary techniques are often required for obtaining a free nitrate stream in treatment plants, being the most common methods either physicochemical or biological and sometimes a combination of both [Bhatnagar and Sillanpa, 2008; Ghafari, S. et al 2008]. The biological denitrification is recommended for the removal of relatively low concentration of nitrogen components and it is operated by the so called denitrifying bacteria in anoxic conditions, where they use nitrates as electron acceptors during their respiratory process in the place of the oxygen. As demonstrated by the most recent literature, new bacterial strains are continuously isolated and tested for their $NO^{3}-$ removal abilities [Peng, Y. et al 2014, Sun, F.et al 2014, Van De Hende, S. 2014]. Depending on their characteristics, different bacteria are employed in different waste treatment facilities, with a preference towards those microorganisms capable of combined heterotrophic nitrification and aerobic denitrification. However, other characteristics are often desirable, for example bacteria with a marked resistance to high salinity, are generally employed in the treatment of polluted seawater [Zheng, S.Y. et al 2014] and strains isolated from critically polluted environments are used for the treatment of special industrial wastes, such as tannery wastewater [Kim, I.S. et al 2014].

Keeping the above fact in mind, the present study envisaged isolation of bacterial strains from treated dairy waste water to establish the bioefficacy potential for nitrate reduction.

MATERIALS AND METHODS

Sampling: The effluent samples (inlet/untreated/raw) from treatment plant of Jaipur dairy were collected in triplicates in pre-sterilized bottles in accordance with standard procedures [APHA, AWWA 2000] (Figure 1).

Figure 1: Sampling site (Inlet of Effluent Treatment Plant, Jaipur Dairy)

Screening of denitrifying bacteria from untreated dairy waste water

Indigenous bacterial strains were isolated from effluent by enrichment technique [DebRoy, S. et al 2012]. The samples were serially diluted and cultured onto Nitrate Agar. The media composition was as follows (g/l) (Table 1).

Table 1: Composition of Nitrate Agar for Screening of denitrifying bacteria

S.No	Constituent	Amount(g/l)
1	Beef extract	10
2	NaCl	05
3	Peptone	10
4	Agar	20
5	Potassium Nitrate	01
6	Distilled water	1000(ml)
7	pH	7

Strain Acclimatization and growth curve analysis

The screened isolates were re inoculated into Nitrate Broth with the following composition (Table 2) and incubated under agitating conditions at 37˚C for 24-48 hours until the O.D. $_{600}$ (0.6) had attained [Suizhou, R. et al 2006]. Simultaneously, negative biotic controls were also maintained in nitrate free medium.

Growth curve analysis

The actively growing strain($O.D_{660}$= 0.6) in nitrate broth was inoculated as monoculture 1% v/v in a 250 ml erlenmeyer flasks containing 100 ml of Nitrate Broth and were incubated at 37°C for 24 to 48 hours under agitated conditions (120 rpm). Negative biotic controls were also maintained which were devoid of amended nitrate. $O.D_{660}$ for both the set of experiments was monitored at regular intervals.

Denitrification Assay: Nitrate removal efficacy of screened isolates was conducted with a slight modification in method devised by Debroy, S. et al 2012. The isolates were inoculated (2%v/v) in Nitrate broth and incubated for24 h at 37°C in under agitating conditions at 120 rpm. Actively growing cells were harvested at 8609×g for 10min and Cell Free Extract (CFE) was taken for estimation of nitrate removal. To 40 µL CFE, was added 200 µL of Salicyalic acid (5% Salicylic acid in H_2SO_4).The tubes were incubated in dark for 10 min. The reaction was stopped by addition of 2 mL of 4N NaOH. Optical density of reaction mixture was measured after 20 minutes at 420 nm and compared with and O.D was then compared to the standard curve prepared with known concentrations of $NaNO_3$ (100- 1000 ppm) to determine the concentration of left over nitrate in medium [Cataldo, D.A. et al 1975].

RESULTS AND DISCUSSION

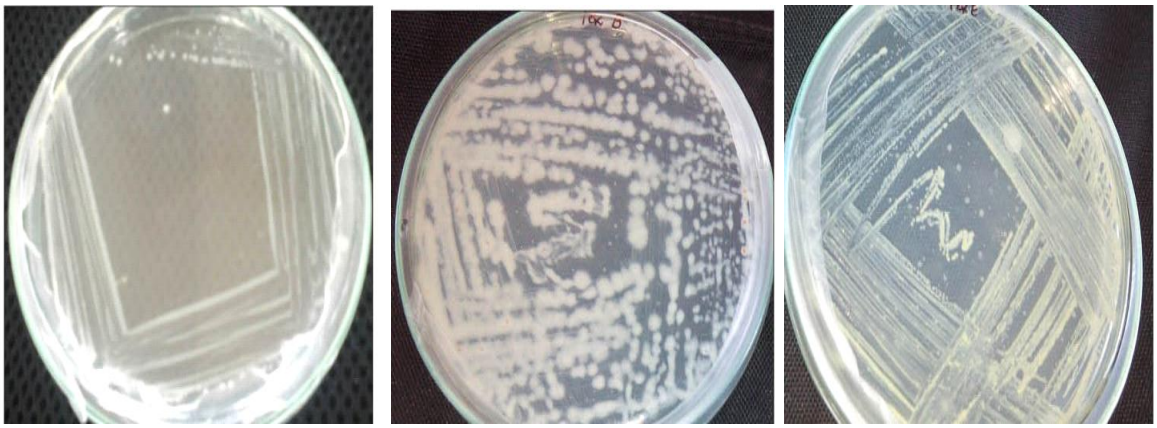
Screening of denitrifying bacteria from untreated dairy waste water

A total of 3 bacterial isolates (NP1, NP 2 and NP 3) were obtained from treated dairy waste water when screened for presence of nitrate reduction. The ability to indigenous microbes uptake nitrate is concomitant with its adaptability under conditions of excessive nitrate (Vigliotta, G. et al 2010). Complementary techniques are often required for obtaining a free nitrate stream in treatment plants, being the most common methods either physicochemical or biological and sometimes a combination of both [Bhatnagar and Sillanpaa, 2011; Ghafari, S. et al.2008]. Based on different biochemical tests, the isolates were identified as *Bacillus sp., Streptococcus sp., and Escherichia sp.* (Table 2). Figure 3 depicts pure culture of screened isolates. It has been stated that filamentous bacteria also play a crucial role in Biological

Nutrient Removal (BNR) (Machnika,A. et al 2005, Sarioglu, M. et al 2005). Very appropriately, denitrifiers have been classified as true denitrifiers, sequential denitrifiers, nitrate respirers and non-denitrifiers [Drysdale, A.G. et al 1999]. Likewise, Azospira *sp.*, OGA-24 isolated from polluted river influxed with nitrate has been reported to reduce nitrate significantly [Rossi, F. et al 2015].

Table 2: Biochemical properties of screened bacterial isolates

S. No.	Characteristic	NP1	NP2	NP3
1	Gram Stain	+ve cocci	-ve rod	-ve rod
2	Lactose	A	-	-
3	Dextrose	A	+	A
4	Sucrose	A	+	A
5	H_2S production	-	+	-
6	NO3 reduction	+	+	+
7	Indole production	-	+	-
8	MR Reaction	+	+	-
9	VP Reaction	±	+	±
10	Citrate utilisation	-	+	-
11	Urease	-	+	-
12	Catalase	+	+	+
13	Oxidase	-	-	-
14	Gelatin	+	+	-
15	Starch	-	+	+
16	Lipid	+	-	-
17	Strain	*Streptococcus sp.*	*Bacillus sp.*	*Escherichia sp.*



Streptococcus sp. ***Bacillus sp.*** ***Escherichia sp***

Figure 3: Pure culture of Bacterial isolates.

Strain acclimatization and growth curve analysis

When the strains were grown in presence of nitrate, the utilisation of nitrate reflected a fastidious approach in uptake of nutrients (nitrate in this case) [DebRoy, S. et al 2012]. A

significant increase ($p<0.05$) was observed with respect to negative biotic control. Acclimatization of nitrate has been attributed to low concentration of nitrogen components and it is operated by the so called denitrifying bacteria in anoxic conditions, where they use nitrates as electron acceptors during their respiratory process in the place of the oxygen. Nitrates are efficiently removed when an external organic carbon source, generally methanol, ethanol or acetic acid, is ammended [Kapoor and Vir Raghavan, 1994]. Figure 4 represents growth of bacterial isolates in nitrate broth. Growth curve analysis is represented in Figure 5.

Strains inoculated in Nitrate broth **Strains post inoculation**

Figure 4: Growth of bacterial isolates in Nitrate Broth.

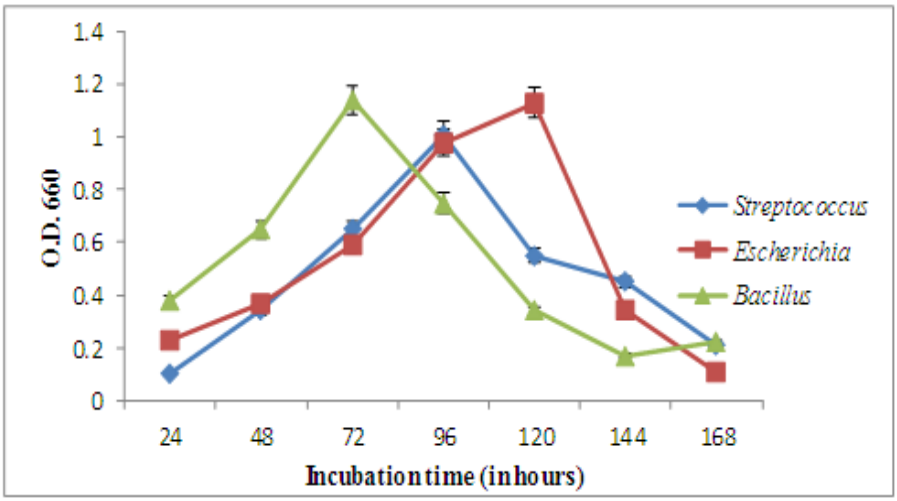


Figure 5: Growth profile of bacterial isolates.

Denitrification Assay: The nitrate removal from the medium is the primary step for the reduction of nitrate though after removal the bacteria may use the nitrate by assimilatory or dissimilatory pathway [DebRoy, S. et al. 2012]. The percent removal of nitrate when monitored in Cell Free Extract (CFE) of bacterial isolates ranged from a minimum of 65% for

Streptococcus sp. to a maximum of 85.58% for *Bacillus sp.* when contrasted with negative biotic control which was found to be 7.05% (Figure 6 and Table 3).

Facultative aerobes that can utilise nitrate instead of oxygen as a final electron acceptor are responsible for denitrification [Ramothakong, T.R. 2006].

The rate of denitrification depends on the nature and concentration of the carbonaceous matter undergoing degradation. Most investigations agree that denitrification is a zero-order reaction with respect to nitrate being reduced to very low nitrate concentration levels [Martin, A.M. 1991]. Microalgal-bacterial systems in harmony are utilised for removal of nitrogen containing compounds from waste water has been utilised in recent past [Hurse and Konnor, 1999]. Reduction of nitrite has been attributed to sequential nitrifiers [Ramdhani, N. 2005]. Ramothokang, T.R. 2006 has reported the presence of true denitrifiers, the total diversity being only 11%.

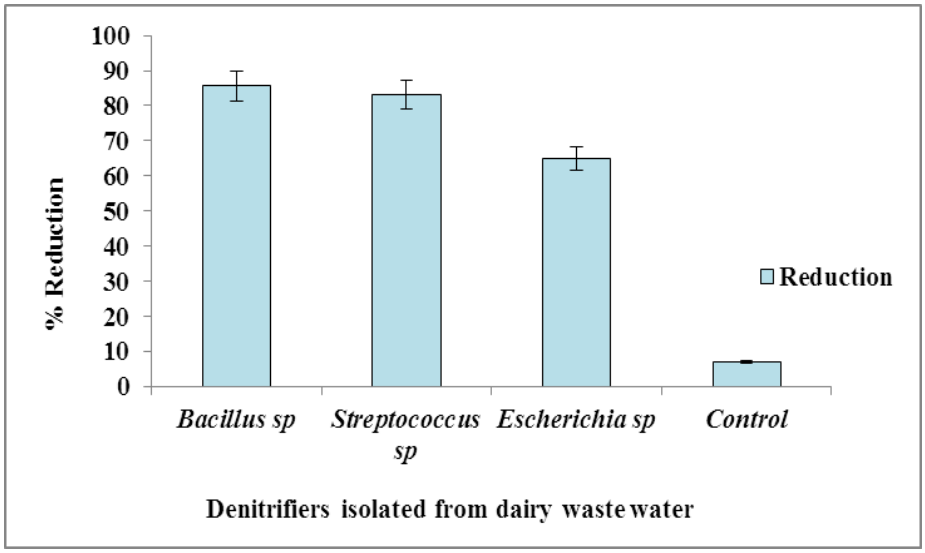


Figure 6: Percent reduction of nitrate by screened bacterial isolates.

Table 3: Initial and final nitrate concentration after denitrification activity.

STRAIN	PARAMETERS		
	Initial nitrate concentration (mg/l)	**Final nitrate concentration(mg/l)**	**Percentage(%) reduction in nitrate**
Bacillus sp (NP 1)	3.4mg/l	0.49mg/l	85.58%
Streptococcus sp (NP 2)	3.4 mg/l	0.56 mg/l	83.52%
Escherichia sp (NP 3)	3.4 mg/l	1.19mg/l	65%

CONCLUSION

In this study we report the isolation of 3 bacterial strains with *Bacillus sp*., being most promising with potential for nitrate removal. Dairy industry being an agro based industry comprises of different inhouse unit operations release effluents which significantly contains nitrates higher than the permissible standards. The bacterial isolates could be utilized for bioremediation of nitrate contaminated sites leading to environmental protection. Future insights into diversity based analysis leading to metagenomics may prove to be substantial in bioprospecting based studies. Also, enzymatic mechanisms should be explored which play a plausible role in bioremoval or denitrification of nitrate containing waste waters.

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