

DAIRY WASTE WATER: A POWERHOUSE OF MICROBIAL FACTORIES FOR BIODEGRADATION

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ABSTRACT

We have proposed a prospective microbial study to screen for autochthonous bacterial and fungal isolates to investigate their biodegradation potential. Requisite samples were collected from Jaipur Dairy Effluent Treatment Plant (JDETP), Jaipur Dairy (Rajasthan Dairy Co-operative Dairy Federation Limited, Jaipur) in accordance with standard procedures. Through a culture-dependent approach, both bacterial and fungal isolates were screened by supplementing 0.1% w/v whey in the culture medium. Actively growing cells (O.D₆₀₀= 0.6) for bacterial cells and visible mycelia mesh (0.1% v/v) were harvested and spiked in freshly procured, sterilized, inlet samples. Incubation was carried out for 7 days under agitating conditions at 37°C and 25°C for bacteria and fungi, respectively. The biodegradation study was contrasted with abiotic and positive controls (p<0.05). Ubiquitous to dairy effluents, *Bacillus* sp. and *Mucor* sp. were screened. Enrichment was carried out in broth culture to ascertain logarithmic growth, quintessential for bio-augmentation study. Biodegradation studies revealed a significant reduction of 43.4-57% and 61.4-76% in BOD and COD for *Bacillus* sp. and 63-69% and 74-89% for *Mucor* sp., respectively, when contrasted with abiotic controls at regular intervals. These preliminary investigations provided an impetus about a probabilistic, cost-effective bioremediation model for dairy wastewater. Furthermore, this necessitates the exploration of microbial metabolomics profiles to unlock biochemical pathways of biodegradation.

Keywords: - *Bacillus* sp., Biochemical Oxygen Demand (BOD), Biodegradation, Chemical Oxygen Demand (COD), Jaipur Dairy Effluent Treatment Plant (JDETP), *Mucor* sp.

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INTRODUCTION

Water is paramount in any industrial manufacturing process owing to subsidiary unit processes and operations, the dairy industry is no exception. Concomitant with this fact, significant effluent volumes are being generated post-manufacturing. Proper treatment and disposal of effluents remain a daunting task, though.¹ Physico-chemical constituents of dairy effluent are primarily composed of milk-based ingredients comprising casein, lactose, inorganic salts, detergents and sanitizers²; hormones^{3,4}, high protein organic matter⁵; Fats, Oil and Grease (FOGs) which cause foul odors and clogging of pipes.^{6,7} These pollutants contribute to high Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) in dairy effluents, deeming it necessary to treat prior to its disposal because untreated effluents, when disposed of, are known to exert detrimental effects on the ecosystem, manifesting ecotoxicological implications.⁶

Management and disposal of effluents in a dairy industry are met by the *end of pipe* treatment approach utilizing an Effluent Treatment Plant (ETP) as a mandate by Central/State Pollution Control Board in the Indian context. The functionality of an ETP is essentially based on inlet and outlet parameters of pollution indicators like BOD, COD, Nitrate, Phosphate and Total Suspended Solids (TSS).⁸

Microbiological Interventions

Biological degradation of dairy effluents is attributed to the presence of indigenous micro-flora, which by virtue of their adaptability and *natural attenuation* property, remarkably degrades and mineralizes the

pollutants.⁹ This microbial feature has been explored as bioremediation which is based on bio-transformation of pollutants into a less toxic form and bio-mineralization, end products being water and CO₂.¹⁰ Bioremediation has gained wide acceptance for treating both domestic and industrial effluents with on-site applicability.¹¹

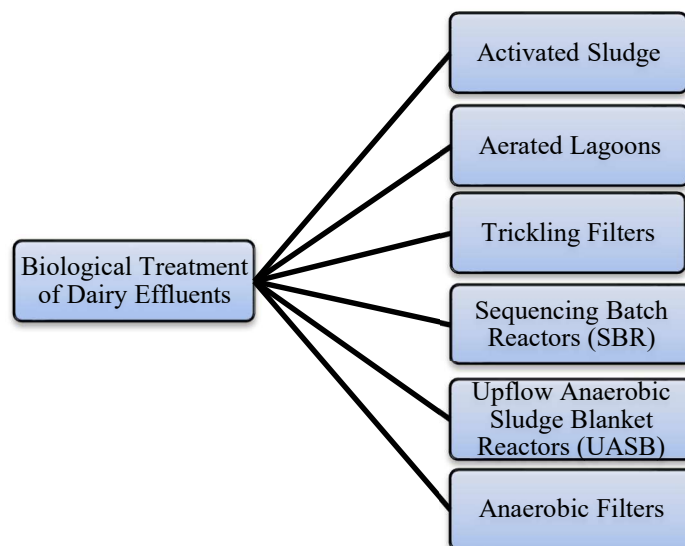


Fig.-1: Biological Treatment Options for Treating Dairy Effluents in an ETP

Additionally, factors like biochemical properties, the origin of contaminants and optimum metabolic activity also govern the overall process of bioremediation.¹² Minuscule size, high surface area-to-volume ratio and large contact interfaces with the external environment are promiscuous microbial traits that qualify them as prospective pollutant bio- indicators.¹³ On the contrary, the merely bio-efficacy potential of indigenous microbes does not suffice with biological treatment. Supplementing and seeding effluent systems with external microbes exhibiting high hydrolytic potential have reportedly enhanced the performance of ETPs, by a process known as bio-augmentation. The co-existence of bio-augmented microbes in conjunction with native micro-flora exerts a cumulative effect on accelerating biomass activity, growth efficiency, and production of enzymes responsible for transforming pollutants into lesser toxic forms. In addition, microbial consortia by virtue of their co-metabolism have been found to circumvent feedback regulation and catabolic repression.¹⁴ Microbes in consortium form are known to exhibit lucrative degradative capacities than mono cultures.¹⁵

Heterotrophic bacterial populations such as *Bacillus cereus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Escherichia coli*, *Streptococcus faecalis*, *Enterobacter* sp., yeasts belonging to genus *Candida* sp., *Saccharomyces* sp. and *Cryptococcus* sp. have been reported in dairy effluents.¹⁶

Considering the potential impact of micro-organisms indigenous to the dairy effluent system, a prospective bioremediation-based pilot study aimed to establish bio-efficacy of screened bacterial and fungal strains to reduce BOD and COD of dairy effluent, respectively, was hypothesized.

EXPERIMENTAL

The study was divided into different phases (Fig.-2).

Sample Collection

Jaipur Dairy Effluent Treatment Plant (JDETP) was selected to conduct this study. Requisite effluent samples (triplicates) were collected in pre-sterilized bottles from the inlet point referred to as Inlet Dairy Waste Water (IDWW) and stored on ice until transported to the laboratory followed by their refrigeration at 4°C until further analysis¹⁷(Fig.-3a to c).

Inlet Quality Assessment

On-site parameters like temperature, pH, odor and color were observed in accordance with standard procedures for BOD and COD, samples were collected for further analysis as specified earlier.¹⁷

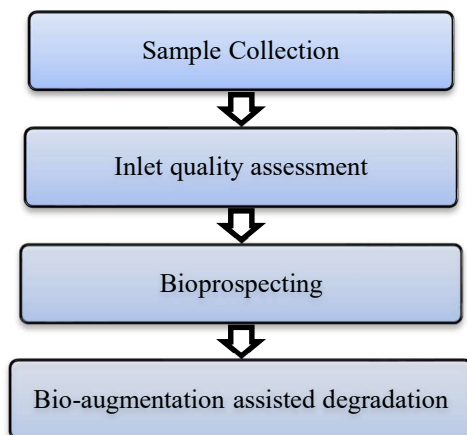


Fig.-2: Study Plan

(a): Sampling Point
(Inlet, ETP, Jaipur Dairy)(b): Sample Collection (Grab
Methodology, APHA, 2005)

(c): Samples in Triplicates

Fig.-3

Bio-prospecting

For bio-prospecting, two independent and studies in parallel were conducted for bacterial and fungal screening, respectively.

Bacterial Screening

Nutrient Agar (NA) supplemented with dried whey (0.1%w/v) with the following composition (Table-1) was prepared with a modification of protocol devised.¹⁸ Serially diluted (up to 10^{-8}) IDWW samples were quadrant streaked and petri plates were incubated at 37°C for 24-48 hours. A loopful of bacterial inoculum was aseptically transferred into Nutrient Broth (NB) (250 ml) with the following composition (Table-2). pH of the medium of adjusted to 7 ± 0.2 and incubated as described earlier until a desired $\text{O.D.}_{600} = 0.6$ was attained.⁹

Fungal Screening

Potato Dextrose Agar (PDA) supplemented with dried whey powder (0.1%w/v) with the following composition (Table-3) was used for culture experiments. By serial dilution, IDWW samples were inoculated by spread plate technique, following which the plates were incubated at $20-25^{\circ}\text{C}$ for 8 days. To generate biomass, mycelial plugs (approximately 0.7 cm) from the edge of the colony were aseptically transferred into Potato Dextrose broth (PDB) with the following composition (Table-4). pH of the medium was adjusted to 6.0 ± 0.2 and was incubated for 8 days at $20-25^{\circ}\text{C}$ until a thick scum was developed.¹⁹

Table-1: Composition of Nutrient Agar (NA)(Gms/Litre)

S.No.	Component	Quantity (gms)
1	Beef Extract	3
2	Peptone	5
3	NaCl	5
4	Whey	0.1
5	Agar	15
6	Distilled Water	1000
7	Final pH	7± 0.2

Table-2: Composition of Nutrient Broth (NB)(Gms/Litre)

S.No.	Component	Quantity (gms)
1	Beef Extract	3
2	Peptone	5
3	NaCl	5
4	Whey	0.1
5	Distilled Water	1000
6	Final pH	7± 0.2

Table-3: Composition of Potato Dextrose Agar (PDA) (Gms/Litre)

S.No.	Component	Quantity (gms)
1	Beef Extract	3
2	Peptone	5
3	NaCl	5
4	Whey	0.1
5	Agar	15
6	Distilled Water	1000
7	Final pH	6.0 ± 0.2

Table-4: Composition of Potato Dextrose Broth (PDB)(Gms/Litre)

S.No.	Component	Quantity (gms)
1	Beef Extract	3
2	Peptone	5
3	NaCl	5
4	Whey	0.1
5	Distilled Water	1000
6	Final pH	6.0 ± 0.2

Bio-augmentation Assisted Biodegradation

The study was planned in accordance with our similar study conducted on bioremediation of textile effluents which was necessarily based on pre-sterilizing real effluent prior to seeding with desired strains as depicted below^{15,20} (Fig.-4).

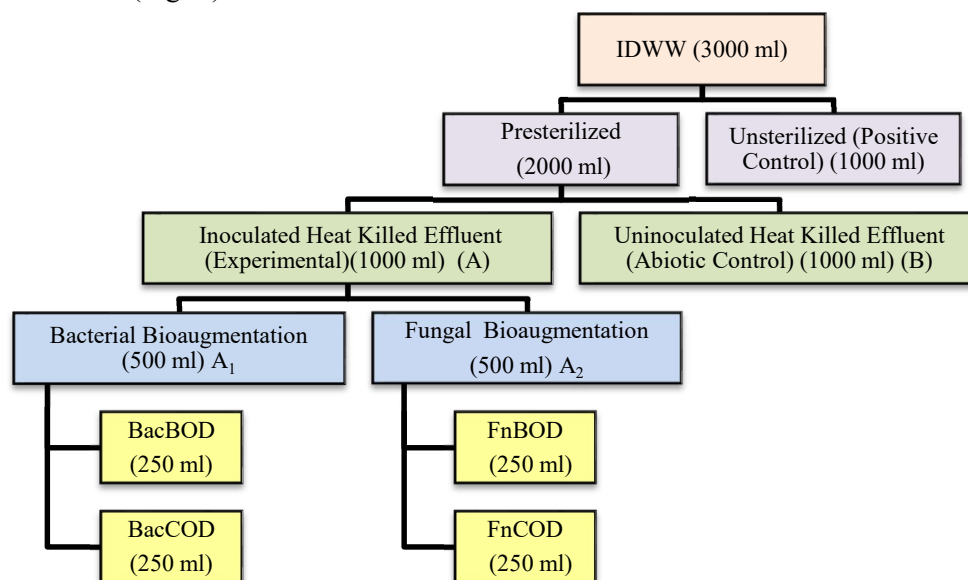


Fig.-4: Schematic representation of proposed microbial study

Briefly, 3000 ml of IDWW was used for the microcosm study. 2000ml IDWW was pre-sterilized and dispensed into two Erlenmeyer flasks (1000 ml each) labeled as Experimental (A) and Control (B). Set A was further sub-divided into bacterial and fungal bio-augmentation (500 ml each) labeled as A₁ and A₂, respectively. Furthermore, A₁ was divided into two fractions (250 ml each) BacBOD and BacCOD. Likewise, A₂ was divided into two fractions (250 ml each) FnBOD and FnCOD, respectively. Both abiotic and positive controls were run in parallel to experimental group A₁ and A₂, respectively. To all the experimental sets, 0.1% (w/v) actively growing cells (bacterial and fungal) as monocultures were

aseptically transferred for biodegradation to take place and incubated for 48-72 hours under static conditions at 37° C for BacBOD and BacCOD and at 20-25°C for FnBOD and FnCOD respectively. Following 24 hours incubation, aliquots of 100 ml (FnBOD and FnCOD) were aseptically withdrawn on each alternate day and spinned at 5000 rpm for 15 minutes to remove fungal biomass.¹⁸ Following the same, BOD and COD were evaluated in Cell-Free Extract (CFE) and compared with abiotic controls (Experimental set up B) and positive control (IDWW: Experimental set up C). In a similar manner, 100 ml aliquots of BacBOD and BacCOD were aseptically withdrawn and centrifuged at 10,000 rpm for 15 minutes to remove bacterial biomass. The same procedure was adopted for abiotic controls and BOD and COD were evaluated respectively.¹⁷

RESULTS AND DISCUSSION

Sample Collection

JDETP was chosen to conduct this study in continuum with our previous studies for different parameters like phosphate, nitrate, and phosphate accumulating micro-organisms and performance evaluation across inlet and outlet streams.^{8, 9, 20-22} JDETP functions on *end of pipe treatment* strategy adopted for treating and discharging effluents. This strategy has been accepted widely to check and control effluent discharges from ETPs and waste emissions at large.¹⁷ Promiscuous technological approaches like resource recovery and recycling strategy innovative technologies for treating effluents have been devised.¹⁹ Structured operation and control have been put forward with a minimal integrated approach between ETPs and sewer systems.²⁰

Inlet Quality Assessment

The samples were collected in the month of January 2018. Following were the observations (Fig.-5, Table-5).

Table-5: Inlet Quality Parameters		
S.No.	Parameter	Characteristic
1	Sample Type	Grab
2	Sampling Date	17.01.2018
3	Time of Collection	14:20 hours
4	Color	Pale white
5	Temperature	22° C±2° C
6	pH	5.6±0.4
7	Turbidity	Present
8	Consistency	Translucent
9	Organic matter	Particulate
10	Flocs	Pellicular



Fig.-5: Assessment of Site Parameters of IDWW

Color

The color of IDWW was milky white in accordance with findings.¹ It has been reported that removal of color across inlet and outlet is attributed to the decomposition of organic load by vigorous algal activity.²¹

Temperature

The temperature of IDWW was 22° C±2° C A variation in dairy effluent samples ranging from 10-35° C has been reported.²² This is attributed to seasonal, physicochemical and biological changes occurring in effluents.²³

pH

pH of the sample was found to be slightly acidic 5.6 ± 0.4 , with findings similar to those reported.¹¹ Acidic pH is attributed to by-products of lactose breakdown into lactic acid.²⁴ It has been reported that pH of untreated waters can follow a trend ranging from 4.7-12.2^{22,24} highlighted the significance of excessive use of cheese in the dairy sector as a governing factor for acidic pH.

BOD

BOD is a paramount indicator that assesses the quality of wastewaters. BOD of IDWW was found to be 560.7 ± 7.8 mg/L, reportedly lower than the findings reported by¹ (1010 ± 18.45). The presence of cleaning agents in effluents is known to contribute to high values of BOD.²⁵

COD

COD is considered a pollution indicator that reflects the oxidizable organic load of effluents. COD of IDWW was found to be 1890.8 ± 10.3 mg/L, reportedly lower findings for raw dairy effluent reported by (2398 ± 16.98).¹

Bacterial Screening

The most predominant bacterial isolate based on its efficacy to utilize whey as fastidious approach belonged to *Bacillus* sp. (Fig.-6a). Biochemical attributes of *Bacillus* sp. are represented in Table-6.⁸ A loopful of the pure culture obtained was transferred into 250 ml NB and growth was observed until the early exponential phase ($O.D_{600}=0.6$) was attained. Actively growing cells (0.1% v/v) were withdrawn, washed with physiological saline (0.9% NaCl v/v) as recommended with 0.5% peptone followed by re-suspending in fresh NB (100ml) and lyophilized for further studies²⁶ (Fig.-6b). Culturable fraction of nitrifying bacterial and fungal diversity of dairy sewage sludge by plate method has been reported⁷. Through metagenomics, 152 bacterial clones were found to be associated with five phyla: Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria, and Synergistetes in dairy wastewater storage ponds.²⁷

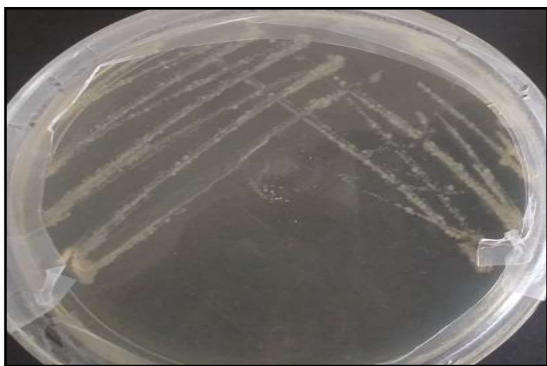


Fig.-6a: Pure Culture of *Bacillus* sp.



Fig.-6b: Re suspended *Bacillus* sp. in NB

Fungal Screening

Based on qualitative screening, the most promising fungal isolate was characterized as *Mucor* sp. by its morphological (Fig.-7a), Broth culture (Fig.-7b) and microscopic characteristics (Fig.-7c and d). In a similar study conducted, fungal isolates belonging to *Alternaria* sp., *Fusarium* sp. and *Aspergillus* sp. were explored for their potential to degrade raw dairy effluent.¹⁰ The role of yeast in aerobic biodegradation of raw dairy effluents leading to a reduction in pollution indicators like BOD, Oil & Grease, turbidity and COD has been explored.^{1,10} Microbes inhabiting contaminated sites exhibit an excellent property of gratuitous or fortuitous metabolism, which indicates co-metabolic activities utilizing xenobiotic compounds and contaminants as their energy source leading to catalytic degradation.²⁸

Bio-augmentation assisted Degradation

For this objective to ascertain an experimental ecosystem (microcosm) was corroborated for biodegradation of contaminants in IDWW by observing reduction in BOD and COD as inspired by our previous study conducted on textile effluents.^{20,21}

Bacterial Biodegradation

Experimental set A1 comprised of actively growing *Bacillus* sp. (0.1%w/v) in two sets BacBOD and BacCOD (250 ml heat-killed effluent) each. At regular intervals of 24 hours, both BOD and COD were monitored in CFEs and contrasted with abiotic and positive control, respectively. A significant reduction of $43.4-57 \pm 2.5\%$ (Fig.-8a) and $61.4-76 \pm 1.7\%$ (Fig.-8b) in BOD and COD was observed in simulated effluent when contrasted with abiotic and positive control with $6.7-14.3 \pm 0.6\%$ for COD and $5.2-12.4 \pm 0.3\%$ for BOD respectively; being non-significant attributed to abiotic losses. % Reduction was calculated with respect to raw IDWW¹⁰ exemplified the effect of aeration and filtration on reduction in BOD of dairy effluent from 74.2% to 78.7%.



Fig.-7a: Pure Culture of *Mucor* sp.



Fig.-7b: Broth Culture of *Mucor* sp.

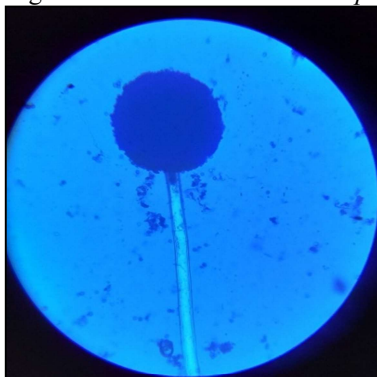


Fig.-7c: Fruiting Body of *Mucor* sp.
(40 X Magnification)

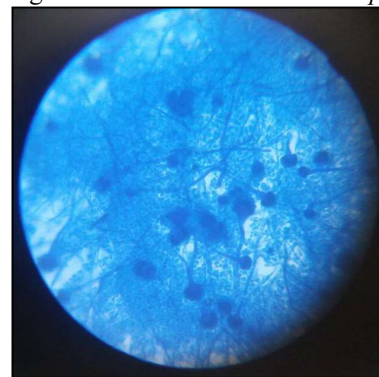


Fig.-7d: Fruiting Body of *Mucor* sp.
(10 X Magnification)

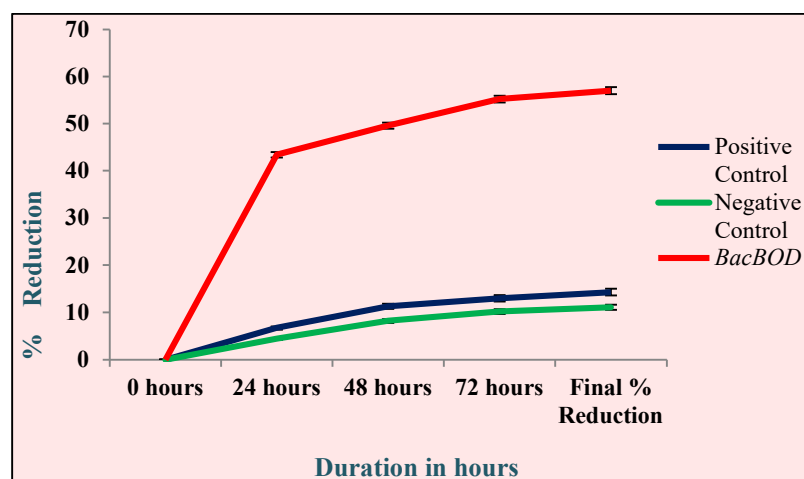


Fig.-8a: *Bacillus* sp. mediated biodegradation of IDWW for reduction in BOD with respect to un-inoculated control ($p < 0.05$)

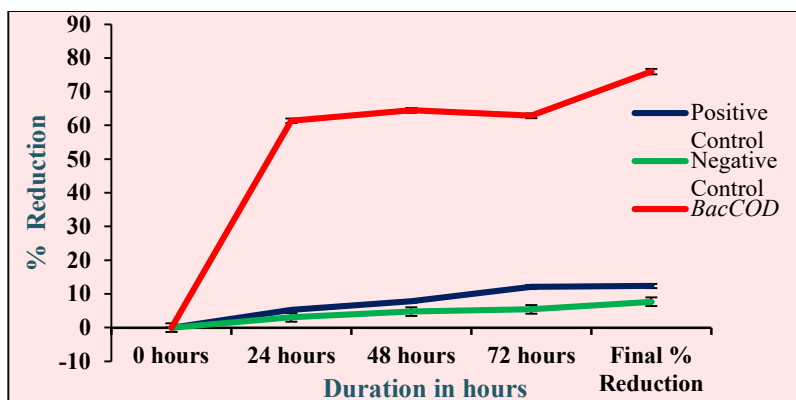


Fig.-8b: *Bacillus* sp. mediated biodegradation of IDWW for reduction in COD with respect to un-inoculated control ($p < 0.05$)

Fungal Biodegradation

Experimental set A₂ comprised of actively growing *Mucor* sp. (0.1%w/v) in two sets FnBOD and FnCOD (250 ml heat-killed effluent), each. At regular intervals of 24 hours, both BOD and COD were monitored in CFEs and contrasted with the abiotic and positive control, respectively. A significant reduction of 63-69 $\pm 1.6\%$ (Fig.-9a) and 74-89 $\pm 3.3\%$ (Fig.-9b) in BOD and COD was observed in simulated effluent when contrasted with abiotic and positive control with 11.4-13.9 $\pm 0.4\%$ for BOD and 9.7-15.4 $\pm 0.7\%$ for COD respectively; being non-significant attributed to abiotic losses.

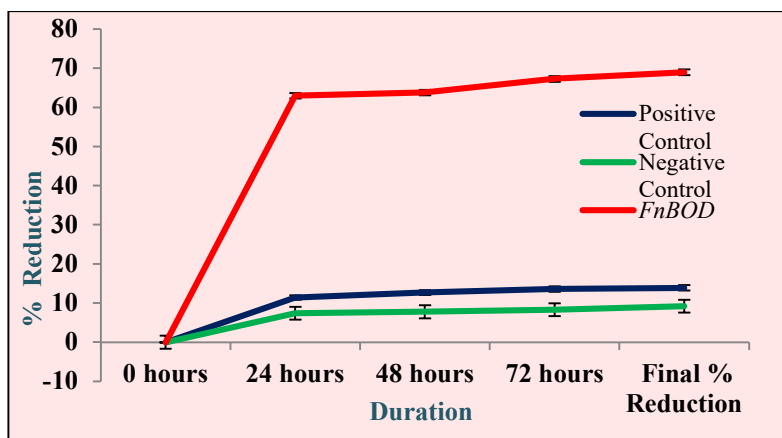


Fig.-9a: *Mucor* sp. mediated biodegradation of IDWW for reduction in BOD with respect to un-inoculated control ($p < 0.05$)

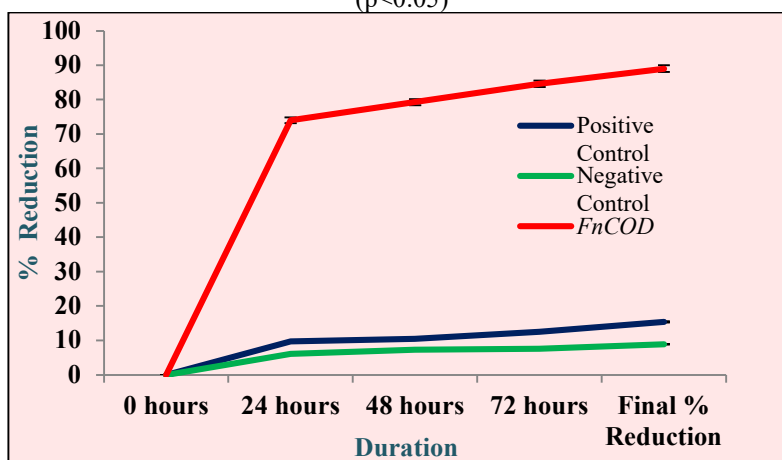


Fig.-9b: *Mucor* sp. mediated biodegradation of IDWW for reduction in COD with respect to un-inoculated control ($p < 0.05$)

Percentage reduction was calculated with respect to raw IDWW. The role of yeast in addressing the pollution load of dairy wastewater has been explored.¹ Bioefficacy of *Alternaria* sp., *Fusarium* sp. and *Aspergillus* sp. to lower download of organic and inorganic pollutants in dairy wastewater has been validated¹⁰. Reduction in pollution load is attributed to vigorous microbial activity and factors contributing to high COD are milk, cream and whey. Reduction in BOD has been correlated with a significant reduction in coliform population.²⁹

CONCLUSION

The present *microbial prospective study* was conducted to validate the bioefficacy of microbes native to dairy effluent; to reduce the level of pollution indicators. BOD and COD are the major *determinants* of our study. Inspired by our previous study on textile effluents, a microcosm (*In situ* bioremediation) approach based on bioaugmentation was adopted. Heat-killed effluent was inoculated with monocultures of *Bacillus* sp. and *Mucor* sp. To negate the abiotic losses, both positive and negative controls were contrasted with the experimental group. *Mucor* sp. assisted reduction in both BOD (69%) and COD (89%) was found to be statistically significant ($p < 0.05$). Likewise, significant bio-reduction was noteworthy, as exemplified by *Bacillus* sp. to be 57% for COD and 76% for BOD when contrasted with respective control experiments. With this pilot-scale study, we report *mycoremediation* as a promiscuous tool for minimizing the load of major pollution determinants contributing to potential eco-toxicological implications. Until recently, much attention has been given to bacterial-assisted bioremediation studies. Slime molds and yeasts are believed to take up nutrients more vigorously from effluents attributed to their fastidious growth and natural attenuation property. Furthermore, we instigate further exploratory studies to unlock enzymatic pathways responsible for biodegradation. Additionally, we also urge to conduct metagenomics-based investigations to understand microbial ecology and community dynamics of effluent systems which remains sparsely understood.

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