ANTIBIOTIC RESISTANCE PROFILING OF DAIRY WASTEWATER DEGRADING NATIVE EFFICIENT MICROBIAL ISOLATES

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ABSTRACT

Objective: The objective of this study is to make sure biotreatment process used for treatment of dairy wastewater (DWW) is safe for human and its surrounding environment; microbes were evaluated for their antibiotic resistance profile against commonly prescribed antibiotics.

Methods: Microbes were isolated using spread plating and streaking method and used to treat DWW. Reduction in organic load in DWW was determined by comparing physicochemical parameters (PCP) of DWW before and after treatment process. After selection of efficient microbial isolates, they were evaluated for their antibiotic resistance profile using antibiotic disc diffusion method.

Results: In this work, 53 microbes were isolated from DWW, and these microbial isolates were screened for DWW degradation capacity by analyzing PCP. Four microbial isolates E3, E5, E11 (bacterial isolates) and F5 (fungal isolate) showed highest reduction in chemical oxygen demand (COD), biological oxygen demand (BOD), and dissolved oxygen (DO) respectively. After 72 hrs of antibiotic susceptibility testing, E3 strain had shown 100%, E5 90%, E11 70%, and F5 80% susceptibility to antibiotics.

Conclusion: The present study concluded that four microbial isolates had the potential of reducing the organic load of DWW along with lessor or negligible adverse effect on human or its surrounding environment and they appear to be most promising strains for treatment of DWW.

Keywords: Dairy wastewater, Biological treatment, Antibiotic resistance, Microbial isolates.

INTRODUCTION

For the two successive years 2015-2016, India faced drought. India’s agriculture sector employ’s 60% of the population and therefore drought mainly affects agriculturist or farmers. During this period, 330 million people of middle India were affected by it. To overcome this major problem, there is an urgent need to save water. Thus, treated wastewater should be reused for irrigation purposes and to maintain underground water table.

Dairy industry is one of the major food industries in India. The dairy sector in India, on an average has been reported to generate 0.2-1.0 l of wastewater per liter of the milk processed [1]. However, careless use of wastewater can cause health problems to farmers, and there have been instances where farmers using untreated sewage showed the prevalence of diarrheal and skin diseases and infections. It is always better to treat sewage before irrigation as runoff from sewage can pollute streams, water bodies, and groundwater. About 73,000 ha of peri-urban agriculture in India use wastewater for irrigation. Treated wastewater should be made available for non-potable urban uses such as flushing, cleaning, gardening, and for industries.

Biological treatment using trickling filters and activated sludge process are efficient in complete removal of organic load from dairy wastewater (DWW) but are non-economical because more chemical consumption and large area are required for treatment process [2]. To enable dairy industry to contribute in water conservation, an efficient and cost-effective treatment technology has to be developed [3]. Many studies on the degradation of DWW with microbes have been reported. Among the wastewater parameters, biological oxygen demand (BOD) and chemical oxygen demand (COD) are widely used as a primary indicator to gauge water pollution.

Work on getting efficient and more economical treatment process to treat DWW are still going on worldwide. In the previous studies, an optimization model for microbial mediated degradation of oil [4], diesel [5], endosulfan [6], and textile dye [7] has been reported. Today, at global level healthcare is facing the problem of the emergence of multi-resistant bacteria thus the threat of epidemics and pandemics [8] and became a major global health problem [9,10]. Hence, the present study was designed to isolate native DWW degrading microbes under optimized conditions and treatment of DWW under aerobic and anaerobic conditions and determination of antimicrobial resistance profile of efficient strains by disc diffusion method.

METHODS

Sample collection
Fresh untreated DWW was obtained from effluent treatment plant of dairy industry Verka, Mohali, Punjab, India in two different seasons of summer (August, May) and winter (November, February). Composite sampling was performed according to standard procedures from American Public Health Association (APHA, 1989) [11].

Procurement of chemicals
All the chemicals and analytical reagent grade were commercially procured from HIMEDIA®, LobaChemie®, SRL® (Sisco Research Laboratories) and Qualigen® and used as received without further modification. Double-distilled water was filtered through a Millipore membrane filter before being used.

Analysis of physicochemical parameters (PCP) of DWW
Season wise collected fresh untreated DWW samples were analyzed to determine their PCP. The analysis was done according to APHA, 1989. PCP such as salinity, conductivity, and total dissolved solids (TDS) was...
determined using “water quality analyzer” kit (Electronics India make) with resolution value for salinity 0.01 ppm, conductivity 0.01 ms, and TDS 0.01 ppm. While organic load was determined by analyzing BOD, dissolved oxygen (DO), and COD using titr meter methods [12,13].

Isolation of the microbes

Microbes were isolated from the dairy effluent (DE) samples collected in different season by Koch method based on the serial dilution and spreading on nutrient agar media (composition gl⁻¹: Peptic digest of animal tissue [5.0], sodium chloride [5.0], beef extract [1.5], yeast extract [1.5], agar [15.0]) and PDA (composition gl⁻¹: Potatoes, infusion form [200.0], dextrose [20.0], agar [15.0]). From the individual colonies resulted on the media, bacterial and fungal pure cultures were obtained by inoculation in test tubes with sloping medium surface at 37±1°C, 48 hrs and 25±1°C, 72 hrs, respectively [3].

Inoculum preparation

To study the biodegradation efficiency of the bacterial isolates, each bacterial isolate with 0.1 ml suspension with cell density 3.1×10⁶CFUml⁻¹ was inoculated in 100 ml nutrient broth (composition gl⁻¹: Peptic digest of animal tissue [5.0], sodium chloride [5.0], beef extract [1.5], yeast extract [1.5]). Similarly, 0.1 ml suspension of fungal isolate was inoculated in 100 ml potato dextrose broth (composition gl⁻¹: Potatoes, infusion form [200.0], dextrose [20.0]). The flask was kept on rotary shaker at 150 rpm for 3 days at 25°C. The flask was kept on rotary shaker at 150 rpm for 24-72 hrs at 37/25°C. Actively growing microbial culture of each isolate was centrifuged at 10,000 rpm for 10 minutes to get wet pellet of each isolate and it was washed thrice with sterile distilled water. The pellet was resuspended in sterile deionized water till turbidity reaches at or above that of McFarland 0.5 standard [3].

Screening of DWW degrading bacterial isolates

Season wise isolated microbial isolates were further screened for their DWW degradation efficiency to reduce PCP. The reduction potential of selected isolates was collectively analyzed for the treatment of DWW recollected in the month of May. Individual bacterial isolate with 2.5% inoculum concentration was inoculated in 250 ml Erlenmeyer flask containing 100 ml of DWW at pH 7 ± 2°C for 24 hrs on a rotary shaker at 150 rpm and 2.5% concentration of individual fungal isolate inoculated in 100 ml DWW at pH 6 for 72 hrs. After 24 hrs (bacteria) and 72 hrs (fungi), samples were taken from each flask, and physicochemical analysis was done. Percentage reduction in COD, BOD, DO, TDS, salinity, and electrical conductivity was calculated and the bacterial isolates exhibiting more than 45% reduction ability and fungal isolate having more than 25% reduction ability were selected for further optimization experimentation.

The percentage reduction was calculated by:

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\text{% Reduction} = \frac{\text{Initialvalue}-\text{finalvalue}}{\text{Initialvalue}} \times 100
\]

Optimized treatment of DWW by native microbial isolates under aerobic and anaerobic conditions

To achieve the highest reduction rate of the organic load by the selected isolates of different microbial media parameters were optimized. Preliminary studies were conducted to select the standard conditions such as inoculum concentration, incubation period, pH, and temperature. All the experiments were conducted in triplicate. The reduction was determined by taking aliquots after optimum incubation period, and PCP were analyzed before and after treatment process [14]. After optimization DWW was treated aerobically and anaerobically under optimized conditions.

Determination of antibiotic resistance pattern of DWW degrading microbial isolates

The antibiotic resistance was determined by standard agar disc diffusion method on nutrient agar using commercial discs (Hi-Media, Mumbai, India). 100 µl of fresh bacterial and fungal cultures were spread on nutrient agar plates. Antibiotic discs were placed on the plate, within 15 minutes of the application of the discs, the plates were inverted and incubated at 37°C for bacteria and 28°C for fungi. The plates were examined after 24 hrs of incubation for bacteria and after 96 hrs of incubation for fungi, and the diameters of the zones of complete inhibition to the nearest whole millimeter were measured. The zone diameter for individual antimicrobial agents was then translated into sensitive and resistant categories [15].

RESULTS AND DISCUSSION

Isolation and screening of DWW degrading bacteria

Microbes were isolated from wastewater samples collected in different seasons by spread plating and streaking technique. From winter season (November and February), 21 bacteria and 7 fungi were isolated while from summer season (May and August) 18 bacteria and 6 were isolated. All the bacterial and fungal isolates were isolated for their COD, BOD, DO, TDS, salinity and electrical conductivity reduction efficiency. Out of 39 bacterial isolates, 14 bacterial strains showed significant degradation of effluent in terms of percentage reduction in PCP while out of 13 fungal isolates 6 fungal isolates were efficient in reducing PCP.

These 14 bacterial isolates and 6 fungal isolates were collectively reseed to treat fresh DWW collected in the month of May and to confirm their reduction potential. All the bacterial and fungal isolates were isolated for their COD, BOD, DO, TDS, salinity and electrical conductivity reduction efficiency. Out of 14 bacterial isolates, 5 isolates reduced more than 45% COD while 6 and 9 bacteria reduced more than 50% BOD and DO respectively after 24 hrs. These isolates showed a different range of reduction potential for COD (16-58%), BOD (35-55%), DO (41-57%), TDS (22-61%), conductivity (5-56%), and salinity (28-62%) as compared to the initial value of sample (taken as control). It is evident from the results (Table 1) that the COD, BOD, and other parameters of effluent after biotreatment were significantly reduced by E3, E5, E6, E9, and E11. These 14 bacterial isolates showed more than 45% reduction in COD, BOD, DO. Percentage reduction in COD, BOD, DO of these isolates were E3 (58, 54, 57), E11 (48, 54, 52), E6 (47, 54, 54), E5 (47, 49, 53), and E9 (45, 55, 55). The strain E14 showed minimum reduction potential of PCP. Significant reduction in PCP was observed after bacteria-mediated

| PCP          | November | February | May | August |
|--------------|----------|----------|-----|--------|
| COD mg l⁻¹   | E₁       | E₂       | E₃  | E₄     |
|              | 33       | 42       | 58  | 47     |
|              | 47       | 47       | 23  | 33     |
|              | 18       | 45       | 44  | 48     |
|              | 16       | 34       | 18  | 17     |
| BOD mg l⁻¹   | E₁       | E₂       | E₃  | E₄     |
|              | 50       | 51       | 54  | 59     |
|              | 54       | 54       | 51  | 41     |
|              | 35       | 55       | 50  | 53     |
|              | 38       | 51       | 36  | 35     |
| DO mg l⁻¹    | E₁       | E₂       | E₃  | E₄     |
|              | 52       | 54       | 57  | 53     |
|              | 54       | 54       | 51  | 41     |
|              | 41       | 55       | 50  | 53     |
|              | 41       | 48       | 44  | 42     |
| TDS (ppt)    | E₁       | E₂       | E₃  | E₄     |
|              | 22       | 61       | 40  | 47     |
|              | 42       | 42       | 49  | 41     |
|              | 36       | 44       | 55  | 39     |
|              | 32       | 32       | 36  | 55     |
| Conductivity | E₁       | E₂       | E₃  | E₄     |
| mS           | 57       | 55       | 53  | 41     |
|              | 43       | 41       | 30  | 24     |
| Salinity     | E₁       | E₂       | E₃  | E₄     |
| ppt          | 50       | 52       | 62  | 50     |
|              | 55       | 55       | 39  | 56     |
|              | 56       | 56       | 50  | 50     |
|              | 28       | 48       | 39  | 38     |

PCP: Physicochemical parameters, COD: Chemical oxygen demand, BOD: Biological oxygen demand, TDS: Total dissolved solids, DO: Dissolved oxygen, ppt: Precipitate, mS: Siemens per meter
treatment could be associated with consumption of organic material by bacteria as a food source.

Only 2 fungal isolates F4 and F5 were able to reduced COD above 10% after 72 hrs of treatment. These isolates showed a different range of reduction potential for COD (11-80%), BOD (2-20%), TDS (43-75%), conductivity (39-47%), and salinity (22-49%) as compared to the initial value of sample (taken as control). It is evident from the results (Table 2) that the COD, BOD, and other parameters of effluent after biotreatment were significantly reduced by F5.

**Optimized treatment of DWW by native microbial isolates under aerobic and aerobic conditions**

E3, E5, and E9 were isolated from DWW samples of winter season while E11 was isolated from summer season. It was observed that at native DWW bacterial population of winter season was more efficient DWW degrader as compared to summer season bacterial population. Efficient fungal isolate F5 was isolated from summer season dairy sample. Maximum percentage reduction in COD, BOD, DO, and conductivity were achieved by E3 of winter season followed by E11 of summer season and E5 of winter season. Along with fungal isolate F5, these three bacterial isolates were found to be the most efficient in biotreatment of DWW.

Under aerobic conditions maximum percentage reduction in PCP such as COD, BOD, DO, TDS, conductivity and salinity were achieved with E3 [66, 64, 66, 19, 60, 53], E5 [58, 64, 64, 34, 52, 52], E9 [47, 59, 58, 12, 41, 42], E11 [62, 65, 66, 52, 22, 53], and F5 [72, 62, 64, 42, 29, 42]. On the basis of percentage reduction in PCP shown by microbial isolates E3, E5, E11, and F5 were considered more efficient in degrading DWW.

Under anaerobic conditions maximum percentage reduction in PCP such as COD, BOD, DO, TDS, conductivity and salinity was achieved with E3 (78, 75, 77, 52, 69, 61), E5 (72, 72, 52, 49, 72, 62), E9 (34, 44, 44, 9, 74, 35), E11 (76, 75, 75, 44, 68, 54), and F5 (84, 70, 75, 50, 44, 54). On the basis of percentage reduction in PCP shown by microbial isolates E3, E5, E11, and F5 were considered more efficient in degrading DWW.

As shown in Tables 3 and 4, it is clear that microbial isolates performs better under anaerobic conditions. Anaerobic process is cost-effective approach as compared to aerobic process in which air should be continuously supplied from outside to bioreactor.

**Determination of antibiotic resistance pattern of DWW degrading bacterial and fungal isolates**

Four efficient degrading strains were selected on the basis of their PCP reducing capability. These selected strains were further evaluated for their antibiotic resistance pattern to make sure they are human-friendly or will have to take some measures while working with them. To get ensure about the fungus used for biodegradation is harmless to the environment these bacteria were evaluated for their resistance and susceptible pattern against ten commonly prescribed clinically significant antibiotics, namely, tetracycline (TC), cefotaxime (CT), amikacin (AK), cefixime (CF), ampicillin (AP), penicillin (PC), gentamycin (GM), cefaclor (CF), erythromycin (ER), and chloramphenicol (CH) using antibiotic disc diffusion method. To get ensure about the fungi used for biodegradation is harmless to the environment these fungi were evaluated for their resistance and susceptible pattern against five commonly prescribed clinically significant antibiotics, namely, voriconazole (VC), oleandomycin (OM), fluconazole (FZ), terbinafine (TF), and itraconazole (IZ) (Tables 5a and b, 6).

Data revealed that all isolates show the variable sensitivities against the different antibiotics used in the study. After 24 hrs of antibiotic susceptibility testing E3 strain had shown 70%, E5 60%, E11 50% and F5 0% susceptibility to antibiotics. After 48 hrs of antibiotic susceptibility testing, E3 strain had shown 80%, E5 70%, E11 50%, and F5 0% susceptibility to antibiotics. After 72 hrs of antibiotic susceptibility testing, E3 strain had shown 80%, E5 70%, E11 50%, and F5 0% susceptibility to antibiotics.

**Table 2: Percentage reduction in PCP of dairy wastewater after treatment with selected degrading fungal**

| PCP | November | February | May | August |
|-----|----------|----------|-----|--------|
| COD mg/l | 1 | 7 | 6 | 13 | 13 | 7 |
| BOD mg/l | 2 | 10 | 9 | 15 | 20 | 7 |
| TDS (ppt) | 75 | 73 | 73 | 50 | 43 | 57 |
| Conductivity (mS) | 44 | 43 | 45 | 44 | 39 | 44 |
| Salinity (ppt) | 48 | 41 | 39 | 26 | 22 | 37 |

PCP: Physicochemical parameters, COD: Chemical oxygen demand, BOD: Biological oxygen demand, DO: Dissolved oxygen, TDS: Total dissolved solids, ppt: Precipitate, mS: Siemens per meter

**Table 3: Percentage reduction of PCP of dairy wastewater by selected efficient degrading isolates at respective optimized media parameters under aerobic conditions**

| Bacterial isolates | Optimized media parameters | Incubation period | pH | Temperature (°C) | COD | BOD | DO | TDS | Conductivity | Salinity |
|--------------------|---------------------------|------------------|----|----------------|-----|-----|----|-----|--------------|---------|
| E3                 | 2.5                       | 48 hrs           | 7  | 37             | 66  | 64  | 66 | 19  | 60           | 53      |
| E5                 | 3.0                       | 48 hrs           | 4  | 50             | 58  | 64  | 64 | 32  | 52           | 52      |
| E9                 | 2.5                       | 48 hrs           | 7  | 37             | 47  | 59  | 58 | 12  | 41           | 42      |
| E11                | 3.0                       | 48 hrs           | 4  | 15             | 62  | 65  | 66 | 52  | 22           | 53      |
| F5                 | 2.5                       | 6 days           | 7  | 30             | 72  | 62  | 64 | 42  | 29           | 42      |

**Table 4: Percentage reduction of PCP of dairy wastewater by selected efficient degrading isolates at respective optimized media parameters under anaerobic conditions**

| Bacterial isolates | Optimized media parameters | Incubation period | pH | Temperature(°C) | COD | BOD | DO | TDS | Conductivity | Salinity |
|--------------------|---------------------------|------------------|----|----------------|-----|-----|----|-----|--------------|---------|
| E3                 | 2.5                       | 48 hrs           | 7  | 37             | 78  | 75  | 77 | 32  | 69           | 61      |
| E5                 | 3.0                       | 48 hrs           | 4  | 50             | 70  | 72  | 72 | 48  | 72           | 62      |
| E9                 | 2.5                       | 48 hrs           | 7  | 37             | 34  | 44  | 44 | 9   | 34           | 35      |
| E11                | 3.0                       | 48 hrs           | 4  | 15             | 76  | 72  | 75 | 44  | 68           | 64      |
| F5                 | 2.5                       | 6 days           | 7  | 30             | 84  | 70  | 75 | 50  | 44           | 54      |

PCP: Physicochemical parameters, COD: Chemical oxygen demand, BOD: Biological oxygen demand, DO: Dissolved oxygen, TDS: Total dissolved solids
The prevalence of antibiotic resistance among biodegrading microbial isolates from DE and determined their resistance patterns. The study aimed to predict future emergence and guide the development of strategies to counteract this resistance before their application in biodegradation process. Hence, our research work opens scope for sustainable reuse of huge amount of wastewater generated by dairy industry.

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