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RESEARCH ARTICLE

EFFICIENT OF BACTERIAL ISOLATES IN BIO-TREATMENT DAIRY INDUSTRIES WASTEWATER

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ABSTRACT

Biodegradation has been proven as the most efficient, eco-friendly and cost-effective technique for the treatment of wastewater. The present investigation was planned to identify the bacterial strains which are effective for the biological degradation of dairy wastewater. Samples were collected from Arab dairy factory located at Sendbes Al-Qanater El-Khayria City, Qalubia, Egypt. Seven bacterial isolate were isolated and screening of protease producing bacteria on skim milk agar. Using 16S rDNA sequence analysis to identify the best metabolizing bacterium for different carbon and nitrogen compounds similar to those from the dairy effluent, the bacterium was identified as *Bacillus subtilis*. The biodegradation ability of the *Bacillus subtilis* was assessed, Inoculated with 10^7 cell concentration and with volume 10 % (v/v) showed a maximum chemical oxygen demand removal of 79 % in 15 days and significant decrease in biological oxygen demand (BOD).Two-stages low coast laboratory model was designed. The first coloum contain untreated dairy effluent and inoculated by 10 mL of identified bacterial culture. Activated carbon, small stones and sand layers were used as filtration media in the second coloum. Results represent the high ability of *Bacillus subtilis* in removal of BOD and COD demand in the dairy effluent with removal percent 82% and 85% respectively. Activated carbon, small stones and sand bed were added causing enhancement the quality of physicochemical parameters as total dissolved solids removal percent were 92 % and increased the removal efficiencies.

INTRODUCTION

Water is one of the most major components of the human environment and considered the key factor in human health. Man needs water for his survival and for many other purposes. Today, there is an increase worry about wastewater treatment for industries that have high rigorous regulation (Keffala *et al*., 2017). Rapid industrialization enhanced the productivity and also released toxic substances into the water bodies destroying the environment and causing health hazards for humans (Porwal *et al*., 2015). The food sector of all industrial activities using huge amounts of water and represent the biggest producers of wastewater effluents. In addition, during the biological treatment a greatamount of sludge were produce reported by (Ramjeawon, 2000). Among the food industries, the dairy industry is considered to be the most polluting one, because of its large water consumption and wastewater generation, which are the main source of pollution of this type of industry (Vourch *et al*., 2008). Also, (Ganapathy *et al*., 2011) reported that the high pollution load of dairy wastewater can cause serious environmental problems.

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The presence of high organic load in the dairy effluents make them the propagation place for mosquitoes carrying malaria and other dangerous diseases such as fever (Kumar and Desai, 2011). The wastewater of dairy effluent contain large quantities of milk components like casein, lactose, salt and detergents used during washing conducted by (Kolhe *et al*., 2009). In a study of Montuelle *et al*. (1992)about 15% of organic matter come from the dairy effluent more than the domestic wastewater and this can cause a significant hazard when it is released without appropriate treatment. Some chemicals are used to remove organic matter from wastewater in many industrial wastes, but these methods are more expensive than using biological removal method using bacterial isolates from the same testing area. Also, chemical methods sometimes cause greater pollution to the environment. Therefore, there is interest to use microbial culture for wastewater treatment. The selected microorganisms must have strong decomposition capacity and high toxicity resistance (Maghsoodi *et al*., 2007). Recently, bioremediation was used in biological treatment methods for dairy wastewater treatment. This method depends on using microorganisms or their enzymes to treatment the polluted wastewater, uncomplicated and eco-friendly (Janczukowicz *et al*., 2008). In this study two-stages low cost model was prepared to evaluate the efficiency of some bacterial strains isolated from dairy wastewater in degrading the organic nutrients found in dairy effluent also showing their effects on

total sugars content and COD removal improving the physicochemical quality of dairy wastewater.

MATERIALS AND METHODS

Sample collection: Samples from the effluent of Arab dairy factory located at Sendbes Al-Qanater El-Khayria City, Qalubia, Egyptwere collected in pre-sterilized containers and samples were stored at 4°C for the isolation of microorganism.

Enrichement: Ten ml ofthe collected dairy wastewater sample was inoculated to milk broth was prepared as specific enrichment cultural media, which contained: peptone=50 g/l, yeast extract=3 g/l, milk solid or fresh milk=10 ml/l into 250 ml Erlenmeyer flasks and incubating at 35C for 24h (Maghsoodi *et al*., 2007).Continuous enrichment was carried out by inoculating 1 ml into a new 90 ml of milk broth until good growth of bacteria was observed.

Screening of protease producing bacteria: Isolation of protease producing bacteria by taking a loop ful of the enrichment and plated on milk agar plates and incubated at 35 °C for 24 h. Purified bacterial colonies were transferred on nutrient agar plates then preserved on nutrient agar slant at 4°C. First, proteolytic activity for isolate was tested on skimmed milk agar plates which made out of nutrient agar and 1% skimmed milk powder. Bacterial strain showing the highest zone of inhibition defined as proteolytic active bacteria then selected for further study (Moharam *et al*., 2019).

The biodegradation essay of isolated bacterial colonies: Bacterial colonies preserved on nutrient agar were tested for metabolizing different carbon and nitrogen compounds similar to those from the dairy effluent. Carbohydrates utilization tests for different sugars like lactose, glucose, and starch were examined, besides nitrogen metabolites were determined using casein. The selected isolate were inoculated into basal agar medium supplemented with different carbon sources then incubated at 30ºC for 24 hours using media described by (Palela *et al*., 2008) as follows: NaCl - 0.5%; $NH_4H_2PO_4$ - 0.1%; $MgSO_4$ - 0.02%; K_2HPO_4 - 0.1% and Agar - 2.0%. Supplemented with Carbon sources as 1% glucose, 1% lactose, 1% starch; Nitrogen source like 1% casein. The ability of bacterial isolates for biodegradation potential of different carbon compounds was determined as inhibition zones (mm) around the colonies for positive test of lactose, glucose, and starch and casein hydrolysis. All experiments were performed in triplicates and the present data represent average values.

Molecular identification

DNA extraction and PCR amplification: The most potent bacterial isolate in the biodegradation of study was culture in nutrient broth (Difco) with the following composition (g/liter): (5.0g peptone; 3.0g Beef extract and 1.0L distilled water) then using centrifuge about 15 minat 5000 rpm. DNA extraction was carried out using Kit (PN 4346298). Amplification of extracted DNA by PCR using forward primer: (5-TTGCTCCCTGATGTTAGCGG-3), and reverse primer: (5-TGCGGAAGATTCCCTACTGC-3) with the thermal cycler; initial denaturation at 94 °C for 5 min, denaturation for 60 s at 94 °C, annealing at 55 °C for 1 min and extension for 2 min at 72 °C followed by final extension for 10 min at 72 °C. These cycles were repeated for 30 times.

S rDNA sequencing and data analysis: PCR products was used for sequencing analysis using genetic analyzer ABI 3100. Multiple alignments of the sequences were performed and phylogenetic tree was constructed using (ver. 1.7) of the CLUSTAL program (Thompson *et al*., 1994).Software (MEGA) analysis for Molecular Evolutionary genetics were used with version 3.0 for carry out the Neighbor joining (NJ) of the Phylogenetic relationships among isolate with other international isolates registered in NCBI site (Tamura *et al*., 2007).

Purification of crude enzyme affecting on natural proteins: Exponentially growing cultures (10^6 cells/ ml) of the selected bacterial isolates in Nutrient broth (Difco) tubes and then incubated at 30 °C for 24 h. Inoculating1 ml of 24hrs old culture suspensions into Erlenmeyer flask contain 100 ml media of the following composition (Glucose - 0.10 gm, Peptone - 0.15gm, $MgSO_4$ -0.25gm, KH_2PO_4 - 0.1gm, $FeSO_4$ - 0.004gm, Distilled water - 100ml) (Chakrabarti *et al*., 2018). The flask was incubated for 72 hrs at 35°C, then centrifugate for 10 minutes at 15,000 rpm. The supernatant (crude enzyme) was preserved for further studies. Ammonium sulfate was gradually added slowly to the supernatant with a final concentration (w\v) of 20% and constant stirring was continued for an overnight at 4ºC.The precipitate was collected by centrifugation at 10000 rpm for 20 min. The pellet was dissolved in 10 ml of phosphate buffer solution (pH 7.2). An experiment for digestion of natural proteins were evaluated by inoculating the crude enzyme into sterile test tubes filled with 7 ml of milk then incubated for 24 hrs at 37°C.The tubes were checked for milk clotting after 24 hrs(Chakrabarti *et al*., 2018).

Biodegradation experiment using dairy wastewater effluent as a medium: Biodegradation experiments were carried out in vitro using flasks (500 ml) including 200 ml of the dairy wastewater samples. First, the selected bacteria were adjusted to be (10^5, 10^6 and 10^7) and added in the Erlenmeyer flasks with a concentration of 1% (v/v) and incubated at 30°C for 15 days. Second, three inoculums of the selected bacterial cells (1, 5 and 10 % (v/v)) were examined and incubated at 30°C for 15 days.COD values for the set of experiments were measured at different intervals (0, 3, 6, 9, 12 and 15 days) of incubation at 30 C°.

Low coast laboratory model design: A laboratory two-stage low cost model was set up was a modified model suggested by Porwal *et al*., (2015) for treatment of dairy wastewater, photo (3). Two plastic bottles were reused where the bottom of the bottles were cut to create two columns then washed with alcohol and then with sterile distilled water. In the first coloum, 10 ml of bacterial isolate was added to the effluent for 48 h at room temperature. In the second coloum, activated charcoal, small stones and sand bed were used that following the microbial treatment in first coloum. Treated effluent was permit to flow from the firstcoloumto the second. Testing the treated effluent for different physicochemical parameters in laboratory was carried out.

Physicochemical- Characterization of dairy wastewater samples before and after treatment: Characteristics of dairy wastewater effluent were analyzed before and after treatment as recommended by American Public Health Association (APHA, 2012).The following tests were carried out: pH,

colour, total dissolved solids (TDS), electrical conductivity (EC), Turbidity, Chlorides, oli and grease (O and Gs), chemical oxygen demand (COD) and biochemical oxygen demand (BOD).

RESULTS AND DISCUSSION

Proteolytic active bacteria: After a good growth of bacteria were observed in the enrichment, 1ml were plated on milk agar platesled to the isolation of seven morphologically different bacterial isolates. Isolate codes from S1 to S7).Proteolytic activity test for the seven isolated strains were performed on nutrient agar supplemented with skim milk where S2 and S3 strains showed maximum hydrolytic zone (diameter 10 and 6 mm) respectively and was selected for further study. Figure (1) showed the zone of hydrolysis that produced by the proteolytic bacteria. Photo (2) shows the proteolytic activity observed with the potent bacterial isolates on milk agar.

Identification of *Bacillus subtilis* isolate: Carry out the molecular identification for the highest proteolytic bacterial isolate (S2) with 16S rDNA sequence analysis. The identified strain is about 500 bp long then the sequence product was compared with other bacterial strains in NCBI data base using MEGA program and the strain was identified as *Bacillus subtilis* with similarity of 95%. Strain has been recorded at NCBI with accession number MK811379. Using the neighbor joining method for mapping the Phylogenetic tree. A comparison between different eight partial sequences for *Bacillus subtilis* strains from the website of the DNA databank of United States (NCBI) were originated and eight groups were created. The highest symmetric were observed between *Bacillus subtilis* MK811379 and NC030964 with similarity 99%. Separated groups appeared as shown in Fig. 2 between *Bacillus subtilis* MK811379 and strains with accession no. MF438515 and KR637545 with the lowest similarity (78%).

Degradation of natural proteins: After incubation for 24 hrs the test tubes that filled with milk and inoculated with crud enzyme were found to be clotted. (Sims and Wander, 2002) reported that bacteria secrete proteases enzyme can hydrolyze the peptide bonds in proteins and then breaked down into amino acids .

The biodegradation efficiency experiment using *Bacillus subtilis*: The ability of *Bacillus subtilis* - the most potent bacterial strain during the study to reduce the chemical oxygen demand (COD) using dairy wastewater effluent as a medium representing their biodegradativeefficiency summarized in table 1 and 2) illustrated by fig (3 and 4).

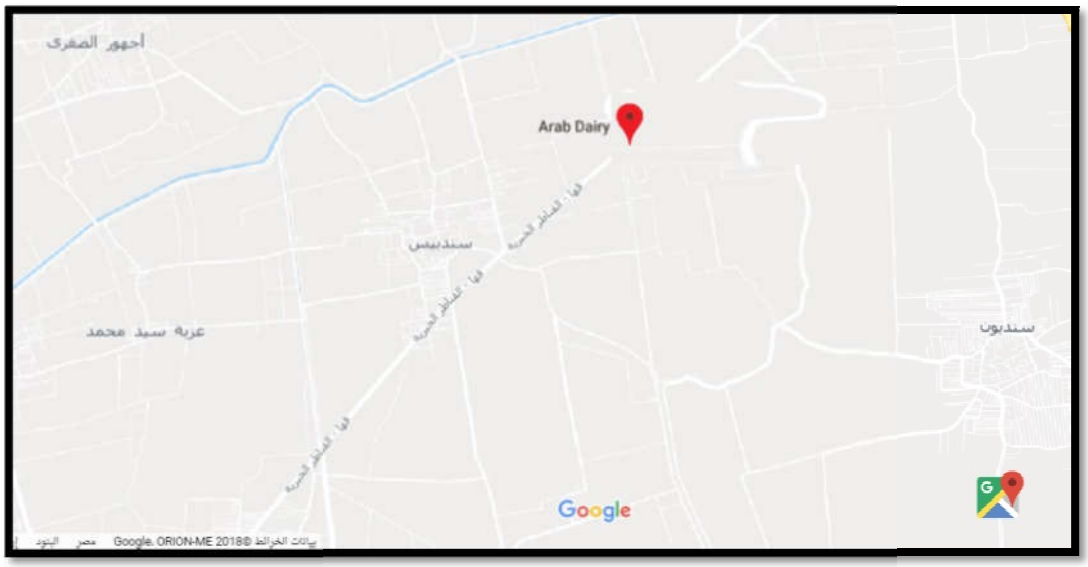
Physicochemical- Characterization of dairy wastewater samples before and after treatment: The characteristics of the dairy wastewater were described in table (3). Results showing that the pH value of the untreated dairy wastewater effluent was slightly acidic. Where BOD and COD values were very high values.

DISCUSSION

Seven bacterial isolates were isolated form Arab dairy waste water effluent factory on milk agar plates and purified by transferring on nutrient agar plates.

Skimmed milk agar plates were used for testing the proteolytic activity of the isolates and the isolate with code S2 have the highest proteolytic activity followed by isolate with code S3 with maximum hydrolytic zone (diameter 10 and 6 mm) respectively. The biodegradation essay showed the potential of the potent bacterial isolates to degread organic compounds, as found in the dairy industry wastewaters like lactose, glucose, starch and casein. The microorganisms already present in the dairy effluent can be used as a means for bioremediation (Porwal *et al*., 2015). The protelase bacteria isolated from dairy effluent become effectively lyse the milk proteins like casein and other carbohydrates conducted by (Chandran *et al*., 2014). 16S rDNA sequence analysis was carried out for S2 where showing the highest proteolytic bacterial isolate during the study. The identified strain is *Bacillus subtilis* with similarity of 95%at NCBI with accession number MK811379 and compared with other bacterial strains in NCBI data base using MEGA program. Using the neighbor joining method for mappe the Phylogenetic tree. Seven clusters were generated after comparison 16S rDNA sequences for *Bacillus subtilis* strains with different seven strains in NCBI. *Bacillus subtilis*MK811379 and NC030964 showing the highest symmetric with similarity 99%.Where *Bacillus subtilis* MK811379 and strains with accession no. MF438515 and KR637545appeared as a separate groups as it was represented the lowest similarity (78%).

According to (Goldwyn *et al*., 2013)*Bacillus sp* is one of the predominant bacteria found in dairy waste water (Chandran *et al*.,2014)reported the ability to isolate of protease enzyme from *Bacillus subtilis* by using the skim milk agar. Among the various bacterial strains *Bacillus sp*. was found to be the major group producing protease discussed by (Ferrero *et al*., 1996). Also, (Krishnaveni *et al*., 2012) proved that the dairy effluent can be used as the isolation source for protease bacteria such as *Bacillussubtilis*. Dairy waste water effluent samples were collected from Arab dairy factory and were found to contain high COD, TDS and oil and grease values that became unacceptable according to the permissible value of Egyptian Governmental Law (92 /2013). An experiment was prepared using dairy wastewater effluent as a medium to evaluate the biodegradtive efficiency of *Bacillus subtilis* the most potent bacterial isolate during the study. Biodegradtion performance of *Bacillus subtilis* has been known to be greatly influenced by various environmental conditions. For the enhancement of biodegradtion rate and to design an affordable treatment technology for dairy effluent, the bacterial cell concentration and inoculum size were studied. COD removal rate was examined at (3, 6, 9, 12 and 15 days) using different bacterial cell concentration. The highest COD removal was achieved with the highest bacterial cell concentration (10^7) after 15 days of incubation. The relationship between inoculum size and COD removal yield was examined and the maximum removal rate of about 80% for 10 % (v/v) for 15 days of incubation. Our results of reduction in COD were corroborated with the finding of Keffal *et al*. (2017) who reported that the isolated bacteria strain have the ability to decrease the COD value above 71.6 % and adding 5% (v/v) of inoculum were more better than 10% (v/v) in the biodegradaing. While (Saxena *et al*. 2015) revealed that the bacterial strains like *Pseudomonas sp*., *Staphylococcus sp.* and *Bacillus sp.* having high potential to biodegrade thedairy wastewater and alsocan reduce COD values above 76% using 1% inoculums for about 20 days.



Map 1. Location map of Arab dairy factoryat Sendbes Al-Qanater El-Khayria City, Qalubia. (By google maps)

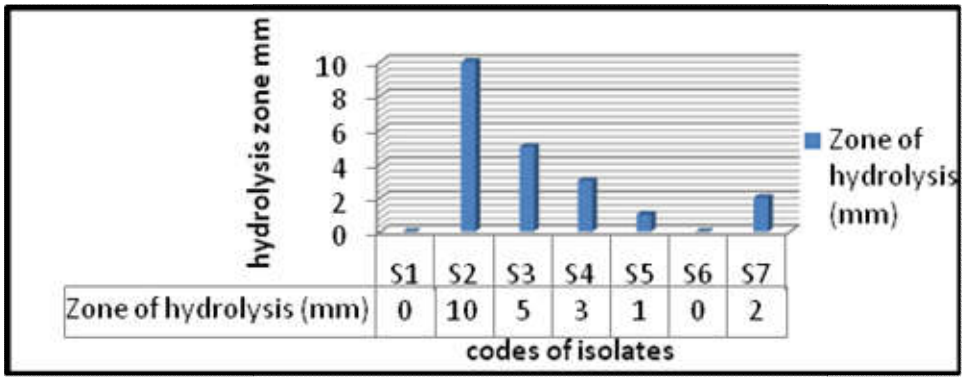


Figure 1. Zone of hydrolysis by proteolysis bacteria

Photo 2. Proteolysis activity of the potent bacterial isolates (S2) on skim milk agar

glucose	lactose	starch	casein
			

Plate 1. Different carbon and nitrogen compounds utilization using the potent bacterial isolates

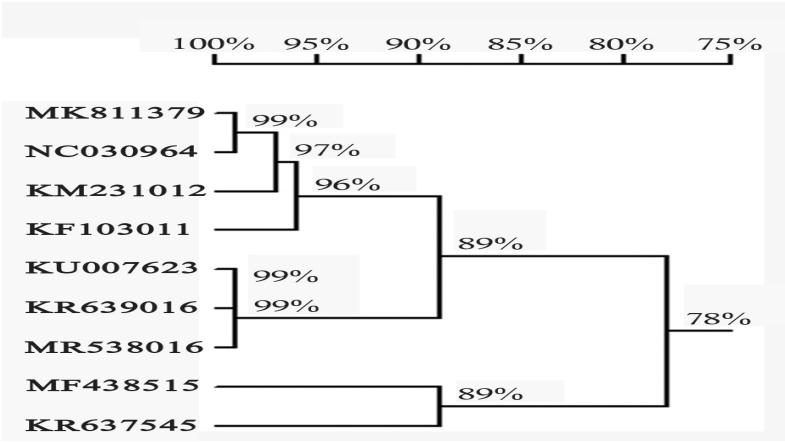


Fig. 2. Phylogenetic tree of Bacillus subtilis MK811379

Photo (3): clotting of natural milk using proteolysis bacteria

Table 1. COD values of treated dairy wastewater samples using different bacterial cells concentrations

Bacterial cells concentrations	Time in Days					
	0d	3d	6d	9d	12d	15d
10^5	3700	3000	2700	2300	2100	2000
10^6	3700	2800	2400	2000	1950	1850
10^7	3700	2600	2000	1800	1600	1500

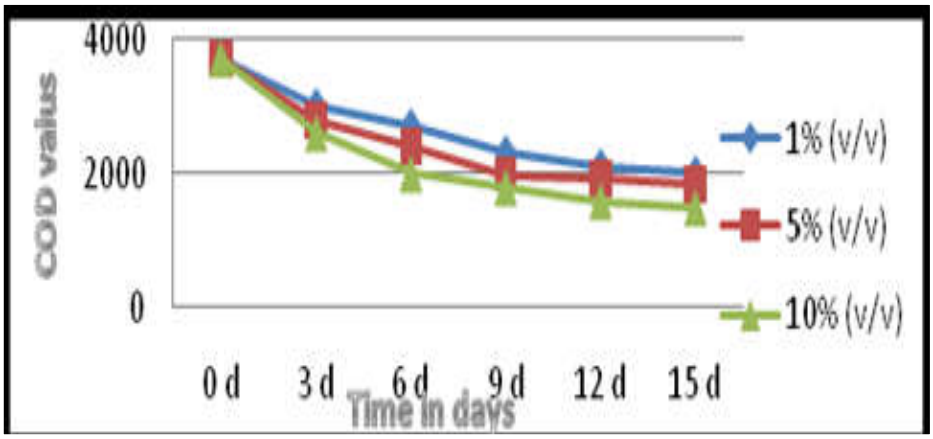


Table 2. COD values of treated dairy wastewater samples using different inoculums of the selected bacterial cells

Inoculums	Time in Days					
	0d	3d	6d	9d	12d	15d
1% (v/v)	3700	2600	2000	1800	1600	1500
5% (v/v)	3700	2000	1600	1100	1000	980
10% (v/v)	3700	1100	900	790	780	770

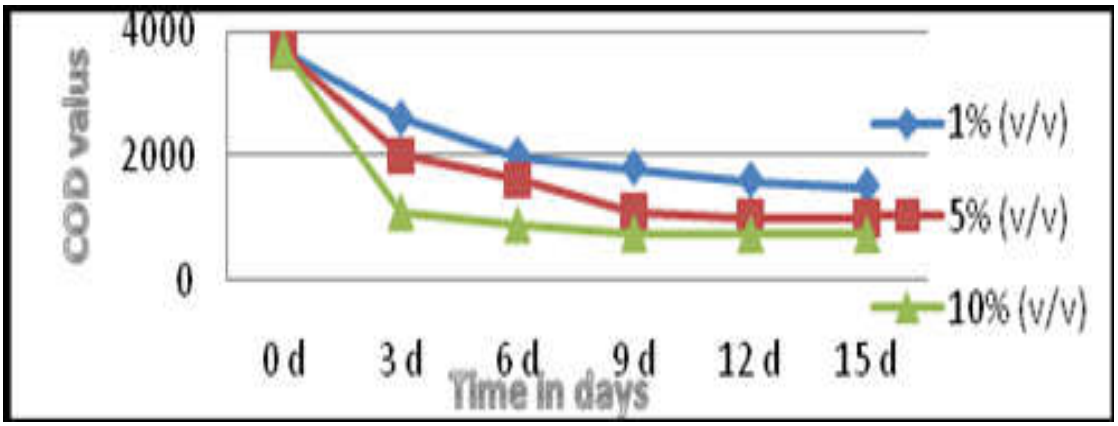


Figure 4. COD values of treated dairy wastewater samples using different bacterial cells concentrations

Photo 4. Low coast laboratory model setup

Table 3. Removal % of some Physico-chemical characterization for the Treated Dairy Effluent using a laboratory two-stage low cost model

Physico-chemical Parameters	unites	Before treatment	After bacterial stage	After filteration stage	Overall Removal %	Law (92)
pH		6.03	6.7	7.3		6.5-8.5
EC	Ms/cm	3.57	1.56	0.285	92%	-
Turbidity	(NTU)	2.230	1.449	0.115	95%	
TDS	(mg/l)	2321	1000	182	92%	1000
Chlorides	(mg/l)	220	55	28	87%	-
BOD	(mg/l)	3000	600	520	82%	-
COD	(mg/l	3700	775	555	85%	<50
O&Gs	(mg/l)	190	75	15	92%	

In a study by Pria *et al*. (2014) the COD values was decreased up to 81.7% using *Streptomyces indiaensis*. Also, (Sreemoyee and Priti, 2013) had reported reduction in COD values of dairy wastewater of about 67.1% and 48.3% using *Neisseria sp.* and *Citrobactersp*, respectively. The physicochemical parameters are considered as one the most important principles in the identification of the quality and type of water for any aquatic ecosystem. In this study, the colour of dairy wastewater was milky and become clear after the filtration treatment stage. The clearance of colour may be due to the breakdown of organic materials by bacterial isolates and completed by activated carbon and sand bed as a filter media led to clearing colour and removing suspended particles this is in accordance with (Verma and Madamwar, 2002).PH value of raw dairy wastewater was slightly acidic (6.03). The acidic pH was explained in a study by (Kolev Slavov, 2017) and attributed that to the breakdown of lactose into lactic acid. (Bako *et al*., 2008) reported that the dangerous effects of the acidic dairy effluent will apper on the water bodies and the soil. pH value moved slightly from acidic towards neutrality after bacterial treatment then become neutral after filtration stage this may be refer to the strength of bacteria to collect acids after biodegradation (Noorjahan and Jamuna, 2012).

The soluble salts content is one of the most important water quality parameters. Salinity is expressed as total dissolved salts (TDS in mg/l) which are related to the electrical conductivity (EC in ms/cm). EC value removal percent was 92% after filtration stage. (Porwal *et al*., 2015) discussed in a study that bacteria may be consuming ions for their growth and activities then enhanced by filtration stage, where ions absorbed on the activated carbon layer and this reflecting the reduction of EC. High values of TDS (2321 mg/l) may be due to high level of organic and inorganic compounds in raw dairy wastewater. Removal percent of TDS was 56 % after bacterial stage then addingthe activated carbon and sand bed increased the removal capacity of TDS up to 92 %.Shruthi *et al*., (2012) reported reduction of TDS up to 68.8% using Pseudomonas sp. Turbidity is a serious factor for public water supplies, during the study the turbidity removal efficiency for bacterial stage was 35% and the increase in removal efficiencies was significantly observed after using the filtration stage to reach about 95 %. Increasing in turbidity removal % after using filtration stage (charcoal and sand bed)may be due to the absorbance of suspended particulates in dairy effluent and the turbidity decreased up to 88.3% using a consortium of marine species was discussed by (Cosa and Okoh, 2014).

The reduction in BOD occurred during the bacterial stage was 80% and there is no high variation in removal percent after the filtration stage since the overall removal percent of BOD was 82%. The highest removal of BOD during bacterial stage may be attributed to usage oxygen by bacteria during utilization essential organic materials for growth reported by (Al-Wasify *et al*., 2017). A study by Das and Santra (2010) a significant reduction of BOD about 69.6% using bacterial isolates was reported. In this study high concentration of COD was recorded from the dairy effluent and this may be attributed to the presence of fats, nutrients, casein, lactose and salts (Kolhe *et al*., 2009). From results, reduction in COD values was recorded 79 % for bacterial stage this due to the consumption of nutrients and the dissolved materials by bacterial strains for their growth then reduction percentage become 85% after filtration. Similar findings were obtained by(Hur *et al*., 2010) as well, reported that BOD and COD value were decreased up to 86% and 84% respectively after the bacterial treatment. The powerful of bacterial strain in degradation dairy wastewater appear by determining the O and G values and the settlement effectiveness of physicochemical substrate (charcool and sand bed) during the filtration stage. Oil and grease (O and Gs) removal values in the present study was 60 % after using bacterial strain then removal present increased after using sand filter and activated carbon to 92%. (Al-Wasify *et al*., 2017) reported that removal percent of O and Gs depending on the ability of some bacteria in the degradation and recorded that removal percentage was about 44.5% for bacterial stage.

Conclusion

The present study clarify that bacterial strains isolated from the dairy effluents have high level of protease activity. Treatment of dairy effluent using a combination between two steps becomes highly effective in removal of BOD and COD. Where, activated carbon and sand bed enhance the ability of bacterial isolates to remove variety of compounds improving the physicochemical quality of dairy wastewaters effluent.

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