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Microbial Remediation of Dairy Industrial Wastewater Using Batch Mode Moving Bed Biofilm Reactor (MBBR)

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ABSTRACT

The purpose of the present study was to investigate the ability and efficiency of the biological treatment to reduce dairy wastewater pollutants by reaching acceptable limits for safe discharging using indigenous bacteria. Ten indigenous bacteria (DM1-DM10) were isolated from dairy wastewater effluent and screened for decontamination process for 7 days. Quality parameters including biochemical oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids (TSS), total dissolved solids (TDS), dissolved oxygen (DO) and total viable count of bacteria (TVCB) were determined before and after the bioassay and their removal efficiencies (REs) were calculated. The three most promising screened bacterial strains were molecularly identified and used as individual or mixed free-living cultures in a batch mode remediation assay. Results showed a general trend of increasing the REs of all parameters by all the tested bacteria with increasing the exposure time. Strains DM5 {*Bacillus Cereus* ATCC14579 (NR-114582.1)}, DM6 {(*Bacillus Aerius* 24 K (NR-118439.1)} and DM7 {*Bacillus cereus* ATCC14579 (NR-074540.1)} recorded the highest activity for removing the selective pollutants, while strains DM2, DM8 and DM10 recorded the lowest removal efficiency for the same parameters. Therefore, DM5, DM6 and DM7 were used as individual and mixed free-living cultures in a batch mode remediation process. Raw dairy wastewater contains a very high level of COD (7680 mg/l), BOD (2700 mg/l) and a high level of TSS (1923 mg/l) indicating high organic load and suspended particles. It also contained an intermediate level of TDS (1220 mg/l) and a low DO level (0.49 mg/l) due to high organic contents and high microbial oxidation demand. *Bacillus cereus* ATCC14579 (DM7) showed the highest removal efficiency of BOD (78.11), COD (88.66%) and TSS (70.10 %) from dairy wastewater. It also exhibited the highest increase in TDS (59.10 %) and DO (389.89%). However, the mixed culture showed the lowest removals for the included contaminants. Moreover, DM7 showed the highest biomass yield (growth stimulation) during the batch mode treatment bioassay where it possesses the highest ability to biodegrade and benefit from the organic pollutants in the dairy effluents for its growth among all tested strains. Results of the present study confirmed that DM7 {*Bacillus cereus* ATCC14579 (NR-074540.1)} is the most promising for either minimization or decontamination of pollution load (mostly organic) from the dairy wastewater.

INTRODUCTION

The dairy industry occupies a large compartment of the food industry. It is considered a water base industry because it consumes large quantities of water for its operations such as heating, cooling, disinfection, cleaning and washing so it is considered a fundamental cause of water pollution (Chatterjee *et al*., 2015; Tocchi *et al.,* 2012; Leena *et al.,* 2016).

Wastewater produced by this industry causes serious ecological problems (Chonde and Ravt, 2017; Daneshvar *et al*, 2018; Ghinea and leav, 2020). Raw dairy effluent is one of the most polluted industrial effluents because it is characterized by high BOD, COD, nutrients and organic matters (Leena *et al.,* 2016; Khan, 2019; Santos *et al.,* 2020). It is also containing phosphorous, suspended solids, some other elements (sodium, calcium, magnesium, cobalt and manganese) and some heavy metals such as nickel, zinc and copper. pH of dairy wastewater ranged from 4.0 to 11.0, while suspended solids vary between 500.0 to 80,000 mg/l (Shete and Shinkar, 2013).

Dairy wastes vary in quantity and quality according to the method and type of operation. Dairy wastewater contains detergents, milk solids, chemicals, disinfectants and lubricants (Mehrotra and Trivedi, 2016). Sources of dairy wastes are lubricants from equipment, dusts from coal, foaming, cleaning of operations tanks and manure (Mehrotra and Trivedi, 2016; Yonar *et al.,* 2018; Naji *et al*, 2015). These dairy pollutants can cause serious ecological problems if they are discharged without any treatment (Chonde *et al*., 2017; Daneshvar *et al*, 2018; Ghinea and leavh, 2020).

There are many technologies for industrial wastewater treatment. The conventional methods (physical and chemical treatment) are less effective than biological treatment because they need a large space, and high cost in addition to the problem of sludge elimination (Tompe and Wagh, 2017). The most common wastewater treatment techniques may consist of one or more physical, chemical and biological treatments (Khan, 2019).

Bioremediation is a biological process in which microorganisms can be used to remove contaminants from polluted water. It is an effective and costless method because it removes a large amount of toxic industrial contaminants which can be degraded by microorganisms. Many types of biological processes can be used for dairy wastewater treatment such as Sequencing Batch Reactor (SBR), Moving Bed Biofilm Reactor (MBBR), Rotating Biological Contactors (RBCs), Up-flow Anaerobic Sludge Blanket Bioreactor (UASB), Aerobic Lagoons, Activated Sludge (ASP) and Trickling Filters (TF) (Joshiba *et al.,* 2019). and Constructed Wetlands (CW) (Zhao *et al.,* 2020). Biological treatment was found to be the superior technique for dairy effluent treatment compared to chemical and physical processes.

Sequencing batch biofilm reactor (SBBR) and SBR could achieve 81.8 and 63.5% COD removal efficiency respectively from dairy effluent (Han *et al.,* 2020). In another study SBR achieved removal efficiency of 90.8, 86.5 and 78.5% for TSS, BOD and COD respectively (Joshiba *et al.,* 2019). AS process achieved high removal of COD, BOD, nitrogen, phosphorous and other nutrient compounds during dairy effluent treatment, while the application of RBC could remove 96, 80 and 79% of BOD, COD and TSS respectively at a rotational speed of 8 rpm.

MBBR is a hybrid process gathering advantages of both suspended (free-living) and the attached biological treatment processes. MBBR accounts for 3/5 of COD and ammonium removal in the mixed dairy wastewater (Rathnayake and Herath, 2020). Santos *et al*. (2020) used MBR in the dairy wastewater treatment and achieved 98% removal efficiency of COD after 8 hr. of treatment using a filling ratio of 20% for a lower COD concentration of 600-800 mg/l. Licata *et al.* (2021) applied the constructed wetland technique for the remediation of dairy wastewater in small and medium dairy

farms. It achieved high removal efficiency of 62, 76 and 50.7% for COD, BOD and nitrogen.

The major objective of the present study was to examine the reduction of contaminants generated by the dairy industry using bioremediation technology. Bioremediation was carried out using powerful indigenous bacterial species individually or in mixed cultures under optimum conditions.

MATERIALS AND METHODS

Collect of Samples:

Dairy wastewater samples were collected from the effluent discharge points of two manufacturing points for cheese and milk located at Borg El-Arab City, Alexandria Governorate for four successive seasons (Jan.-Dec. 2020). The physicochemical and microbiological characterization of the collected samples were examined to determine the pollution severity and calculate the parameters removal efficiency.

Microorganisms:

Ten indigenous bacteria were isolated from the mixed dairy wastewater. The ten bacterial species were investigated as individual or mixtures, free and fixed for their ability to remediate the dairy effluent.

Media and Culturing Conditions:

Dehydrated nutrient broth (NB) and nutrient agar (NA) that contain a great variety of nutritional requirements were supplied by OXOID and used during the present study. NB medium contained (g/l) Lab-Lemco Powder, 1.0; Yeast extract, 2.0 (contain vitamin B which is useful for saving growth factors); Peptone (hydrolyze protein), 5.0 and Sodium Chloride, 5.0. NA medium contained similar ingredients as NB plus Agar, 15 g/l. They were prepared by dissolving 13.0 and 28.0 g/l from NB and NA dehydrated media respectively. pH was adjusted to 7.4 and sterilized by autoclaving at 121ºC for 20 min. and freshly used for growth experiments as well as biodegradation assays.

Bacterial Isolation, Purification and Identification:

Indigenous heterotrophic bacterial colonies isolated from the mixed dairy wastewater were purified by streaking on NA agar plates and incubated at 37°C. Purification was performed either on one step or sometimes required culturing and re-culturing till obtaining pure isolates. The pure isolates were inoculated onto NA slants, incubated under the previously mentioned conditions and kept as a stock in the fridge for further investigations. Bacteria were subjected to identification using Gram staining (Colco, 2005) then followed by molecular characterization of the most promising isolates after the screening test.

Molecular Identification:

Total genomic DNA was extracted from 5 mL overnight NB culture of the purified isolates (Wood, 1983). PCR was performed in a light cycler Eppendorf PCR machine. A 1300 bp fragment was obtained by PCR amplification of the 16S rDNA gene (Tan *et al.*, 1999) using the primers:

F-start: 5'- AGAGTTTGATCMTGGCT CAG-3'

R-1387: 5'- CGGGCGGTGTGTAC

PCR mixture was composed of 100 ng of genomic DNA, 30 pmol of each primer, 200 µM of dNTPs, 1U of Taq polymerase and 10 µL of 10X PCR reaction buffer and the reaction volume was adjusted to 100 µL in 0.5 mL Eppendorf tube. The PCR amplification conditions were performed by an initial denaturation step at 94°C for 10 min followed by 30 denaturation cycles at 94°C for 1 min., annealing at 60°C for 1 min. and an extension at 72°C for 1 min. followed by a final extension step at 72°C for 10 min. Amplicons of 16S rDNA were purified using PCR purification kits (QIAGEN). Each of these purified products was sequenced by the chain terminator method (API model 3730xl, Bioneer, Germany) using the two corresponding PCR primers separately. The resulted DNA sequences were phylogenetically analyzed using the BLAST search program (Tan *et al.*, 1999). Multiple sequence alignment and molecular phylogeny were performed using MEGA 5.0 software (Wood, 1983).

Bioremediation Bioassays:

1. Screening of Bacterial Isolates:

The 10 pure isolated indigenous bacteria were screened for bioremediation of the mixed dairy effluent in order to select the most promising candidates. Bacterial isolates were individually inoculated in 1000 ml. flasks containing red stones acting as supporting media to form a biofilm which took 14 days to be maturated. Then 500 ml. of dairy wastewater was added to each flask. During the screening test, samples were drawn from the flasks at 24 hr. intervals to calculate the removal efficiency of the tested parameters.

2. Bioassays Using Free-Living Bacteria:

According to the preliminary screening experiment, **3** promising bacterial candidates {DM5, DM6 and DM7} were selected and employed as individuals and mixture fixed on red stone aggregates in a batch mode for remediation of dairy contaminated effluent. Bacterial inocula were cultured individually and as a mixture in 100 ml. NB medium and incubated at 35-37°C for 24 hr. then added individually to one-liter flasks containing sterilized red stone to support the biofilm formation. After biofilm maturation, 500 ml. dairy wastewater was individually added to flasks and incubated at room temperature. Effluent cultures (individual and mixed), as well as a control sample (one-liter un-seeded effluent), were incubated for 7 days where samples were aseptically drawn at 24 hr. intervals to measure the selected parameters.

Characterization of the Raw and Treated Industrial Effluent*:*

Industrial wastewater was characterized before and after the proposed treatment. Characterization of the wastewater included its pH, temperature, DO, TSS, TDS, BOD, COD and total viable count of bacteria (TVC), all of which were determined using the standard techniques described in Standard Methods for the Examination of Water and Wastewater (Rice *et al*., 2017). After treatment, the selected parameters were analyzed to determine their residual levels at each exposure time and their removal efficiency was calculated to determine the effectiveness of the remediation process according to the following equation:

Removal Efficiency (RE %) = C0 - RC/ C0 X 100

Where **C0**= Initial Concentration before Treatment (Zero Time);

RC= Residual Concentration after Treatment at each Exposure Time

1. TDS, pH and Temperature:

Temperature, pH and TDS were determined using Hanna HI9813-5 portable pH/EC/TDS/Temperature meter.

2. Total Suspended Solids (TSS):

A known volume of well-mixed sample was filtered through a weighed standard glass-fiber filter (47 mm. circles GF/C-Whitman, England) and the solids residue retained on the filter was dried at 105 ؛C for 1 hour, and then weighted using a digital balance (AS200.R2 RADWAG) till constant weight. The increase in weight of the filter represented the total suspended solids according to the following equation (Rice *et al*., 2017):

$$\textbf{Total Suspended Solids (mg/l)} = \frac{\textbf{(A-B) X 1000000}}{\textbf{Sample (ml)}}$$

Where **A** = weight of the filter plus the dried residue, and

B = weight of filter

3. Biochemical Oxygen Demand (BOD):

BOD was determined using Method 5210 B as described in the Standard Methods for Examination of Water and Wastewater (Rice *et al*., 2017). Using the following equation:

BODM5, mg/l = DM1-DM2 / P

Where **DM1 = DO** of the diluted sample immediately after preparation in mg/l,

DM2 = **DO** of the diluted sample after **5-** day incubation at **20**°C in mg/l,

P = Decimal volumetric fraction of sample (**300** ml).

4. Chemical Oxygen Demand (COD):

COD test was determined using Closed Reflux Colorimetric Method 5220 D using potassium dichromate as a chemical oxidant as described in the Standard Methods for Examination of Water and Wastewater (Rice *et al*., 2017). Samples vials were placed in a digesting unit (DRB 200 HACH COD Reactor) at 150 °C and refluxed for 2 hr. after which they were cooled to room temperature.

The color developed in the samples, as well as blank and standards, was measured as COD concentration at 620 nm using DR900 HACH VIS spectrophotometer.

5. Total Viable Count of Bacteria:

Samples were serially diluted, cultured in NA medium and incubated at 37°C for 24 hr. Colony forming units (CFUs) of the bacterial **TVC** were recorded and averages were calculated.

6. Statistical Analysis:

Statistical analysis was carried out using SPSS v 18 (Anova – one way) post hoc = Tuckey. It was used to determine the best isolated bacterial strain in bioremediation of dairy wastewater treatment. The mean, standard deviation and ***P***. value for each sample were calculated.

RESULTS

1. Screening of Bacterial Isolates*:*

Ten strains were screened for bioremediation of mixed dairy wastewater influent in order to select the most promising candidates. Liquid cultures (24 hr. old) were inoculated individually in 1000 ml. flasks containing red stones (supporting medium for bacterial fixation) and dairy wastewater. The test was performed for 7 days, where, treated samples were drawn at 24 hr. intervals. Removal efficiencies of the tested parameters were calculated and compared with the maximum permissible limits (MPLs) stated in the Egyptian Environmental Law (No 44/2000) for discharging of industrial effluents into the sanitary discharge network (Fig. 1 & Table 1). Results concluded the following points:

1. Raw dairy wastewater contained very high levels of COD (7905 mg/l) and BOD (3160 mg/l), and a high level of TSS (2030 mg/l) confirming high organic and suspended matter load. It also contained intermediate TDS (1630 mg/l) and a low DO level (0.56 mg/l) due to high consumption during microbial oxidation demand for organic content.
2. Isolate DM7 achieved the highest removal efficiency of COD and BOD (88.14 and 89.21% with RCs of 935 and 341 mg/l, after 7 and 5 days respectively).
3. The highest RE% of the TSS was recorded by isolate DM5 as (69.61%, 617 mg/l) after 7 days.
4. The highest TDS increase (59.08%, 2593 mg/l) was achieved by isolating DM6 after 6 days.
5. Isolates DM7, DM6 and DM5 showed the highest activity for selective removal of the tested pollutants during the treatment screening test of the dairy effluent. Therefore, they were selected to proceed with the following bioremediation assays of the contaminated dairy effluents.
6. Most importantly, the lowest recorded RCs of TSS, BOD and COD in the treated effluents achieved by DM7 was lower than their MPLs for the safe discharging into the general sanitary drainage network.

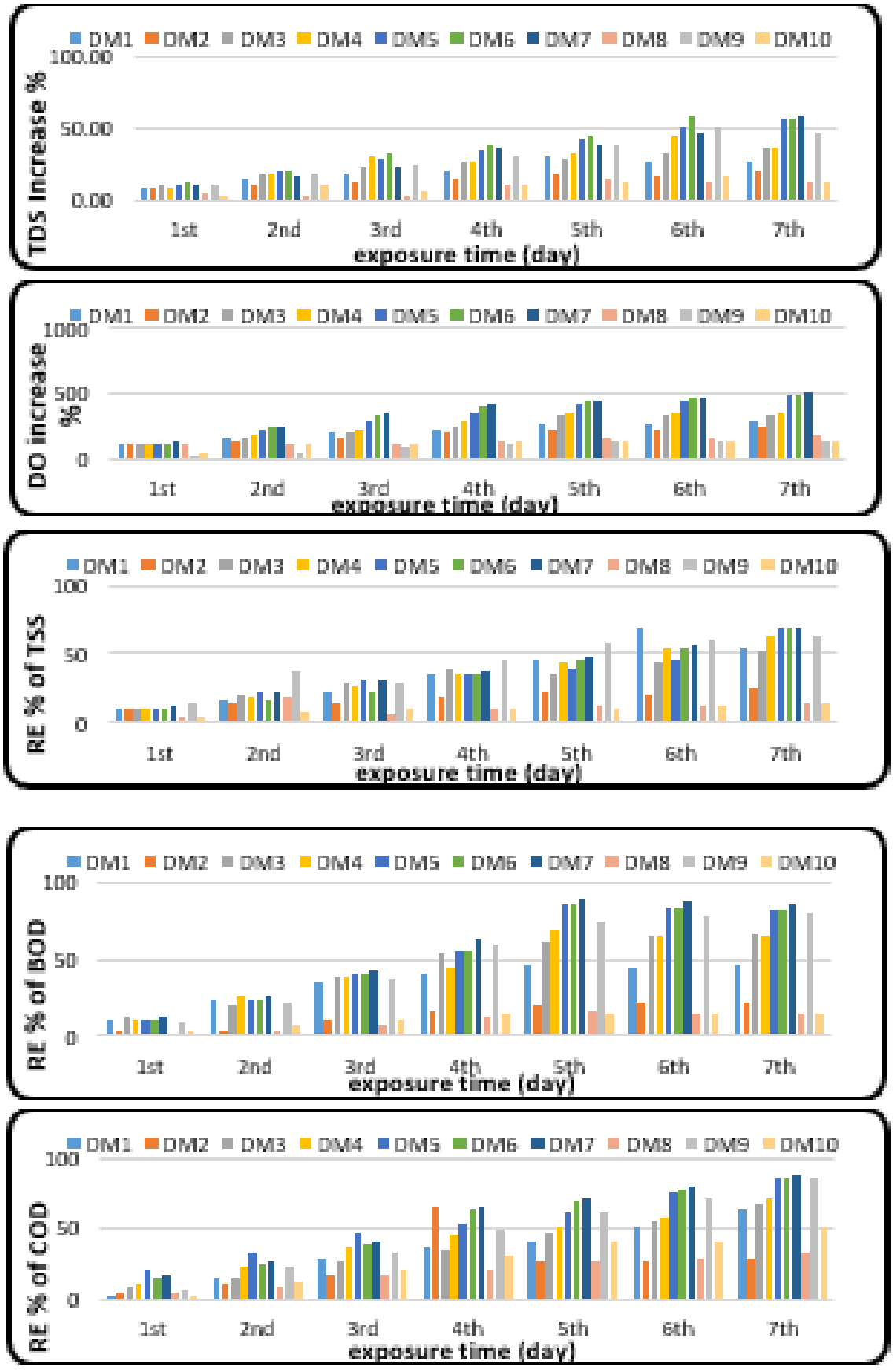

Fig. 1: Removal Efficiency/ Increase % of the Quality Parameters in the Treated Dairy Effluent Using Bacterial Isolates (DM1-DM10) at Different Exposure Times

Table 1: The Highest and lowest REs% of the different parameters.

Parameter	Raw Wastewater (mg/l)	%Highest RE, I	Lowest RE, A	MPL (mg/l)
DO	0.56	3.38 mg/l, 503.57 I%, Isolate DM7	1.37 mg/l, 144.64%, Isolate DM9	----
TDS	1630	2593 mg/l, 59.08%, Isolate DM6	1840 mg/l, 12.88%, Isolate DM8	----
TSS	2030	617 mg/l, 69.61%, Isolate DM5	1739 mg/l 14.33%, Isolate DM10	800
BOD	3160	341 mg/l, 89.21%, Isolate DM7	2670 mg/l, 15.51%, Isolate DM10	600
COD	7905	935 mg/l, 88.17%, Isolate DM7	5635 mg/l, 28.72%, Isolate DM2	1100

2. Molecular Identification of Bacterial Isolates:

Gram stain of the ten indigenous bacterial isolates showed that strains DM1, DM2, DM4, DM5, DM8 and DM9 are Gram Positive and strain DM3 is Gram Negative. On the other hand, strains DM6, DM7 and DM10 are Positive -Negative (Fig. 2). As mentioned before, the screening bioassay indicated that isolates DM5, DM6 and DM7 are the most promising in the batch bioassay, therefore, they were molecularly identified as *Bacillus Cereus* ATCC14579 (DM5), *Bacillus Aerius* 24 K (D 6) and *Bacillus cereus* ATCC 14579 (D 7) with 99.64, 99.36 and 98.96 % similarity (Table 2).

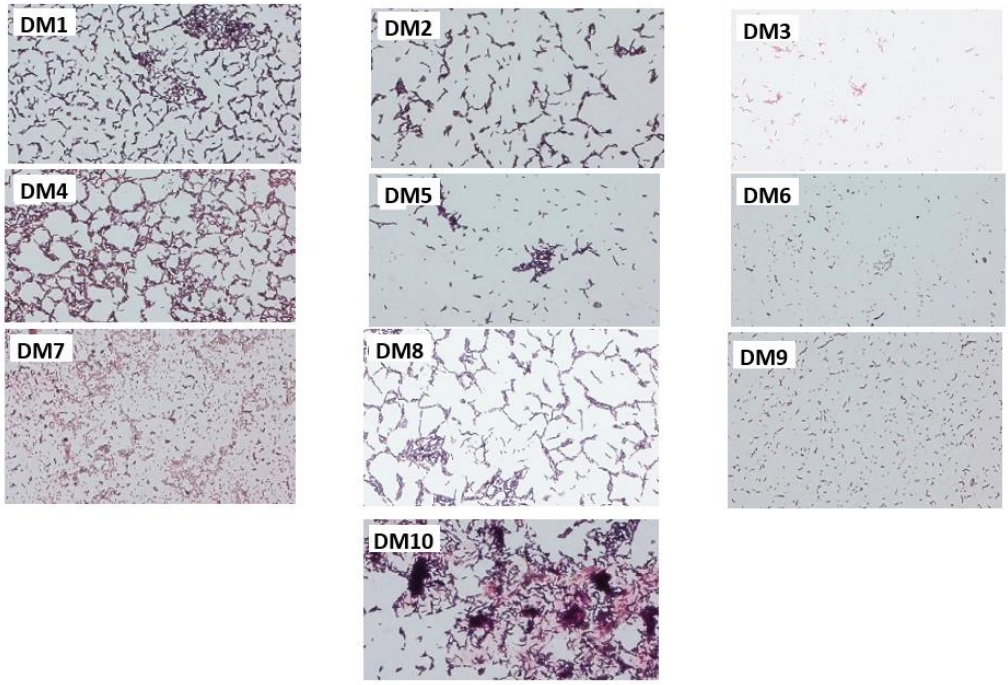

Fig. 2: Gram stain of the ten bacterial strains

Table 2: Similarity % of the most active isolates with their closest neighbors

Code	Strain	Similarity %	Accesion No.
DM5	*Bacillus Cereus* ATCC14579	99.64	NR-074540.1
DM6	*Bacillus Aerius* 24 K	99.36	NR-118439.1
DM7	*Bacillus cereus* ATCC 14579	98.96	NR-074540.1

They were deposited with the accession number NR-074540.1, NR-118439.1 and NR-074540.1 and their phylogenetic relationships and the most closely related bacterial species are illustrated in Figure 3.

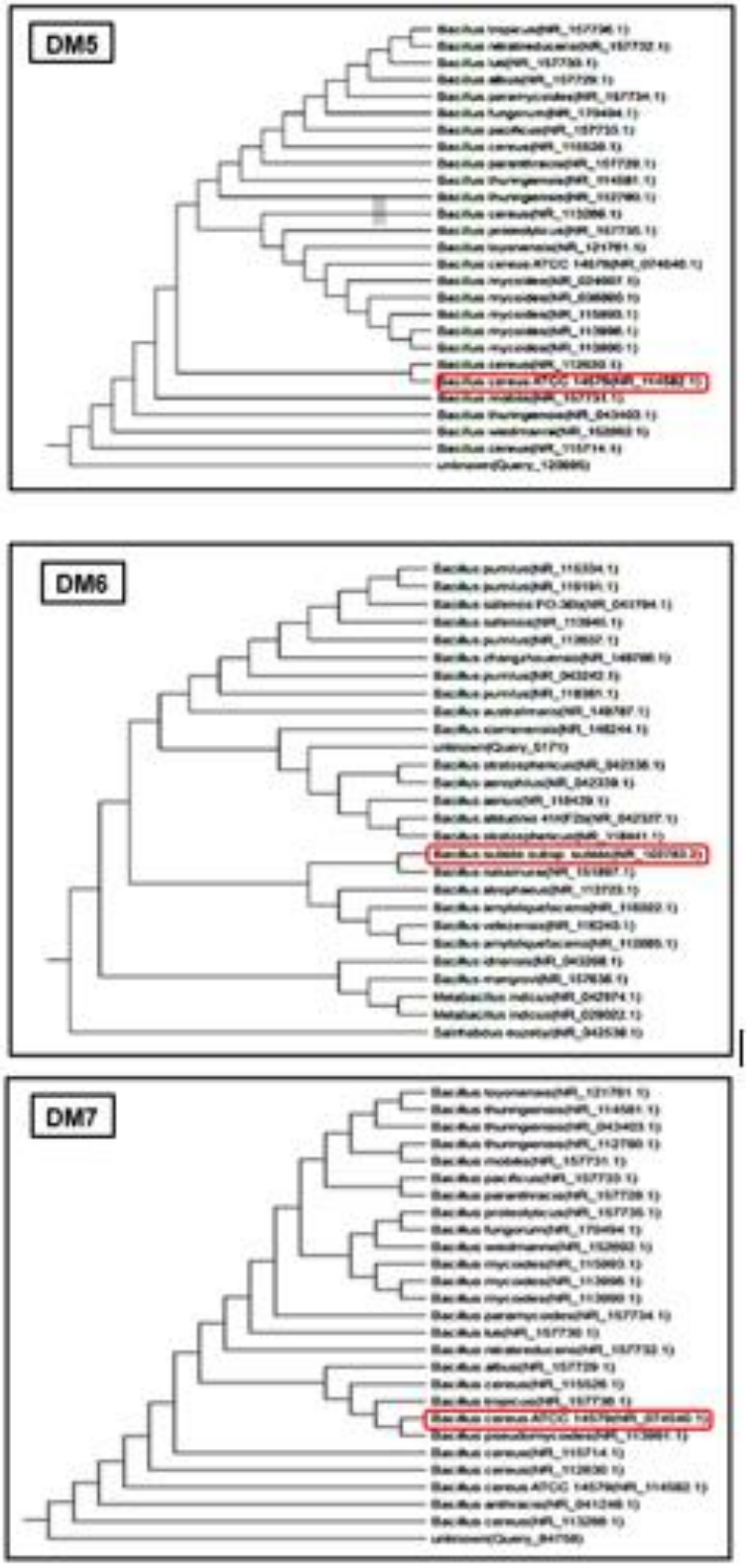

Fig. 3: Phylogenetic Relationships of the Tested Strains (DM5, DM6 and DM7) and the Most Closely Related Bacterial Species

3. Bioremediation Assays Using Moving Bed Biofilm Reactor in a Batch Mode:

The three selected isolates (DM5, DM6 and DM7) and their mixed culture were investigated for their treatability strength towards decontamination of dairy wastewater as a moving bed biofilm reactor (free-living and fixed cultures) in a batch mode bioassay. In addition to the bacterial cultures, a control (raw uninoculated wastewater) was tested under the same conditions. The bioassay was carried out for 7 days at room temperature where samples were collected every 24 hr. Quality parameters (DO, TDS, TSS, BOD and COD) were determined in the raw and treated wastewater and their REs were calculated to determine the most efficient culture for removing the contaminants (Table 3). The following summarizes the achieved results:

3.1. Dissolved Oxygen Levels (DO):

Results revealed a very low DO level (0.49 mg/l) in the raw dairy wastewater at the starting point (zero time) indicating high pollution strength and high consumption of the DO during the decomposition of organic matter (Fig. 4 and Table 3). Results revealed the following points:

1. Due to aeration (stirring) there was a general trend of increasing DO level with time during the test by all strains either seeded or not.
2. The highest increase in DO level (2.4 mg/l, 389 %) was achieved by isolating DM7 after 7 days followed by the control culture (2.1 mg/l, 328.57%), DM5 (1.94 mg/l, 295.92%), DM6 (1.27 mg/l, 159.18%) and finally the mixed culture the lowest DO level increase (1.15 mg/l, 134.69%).

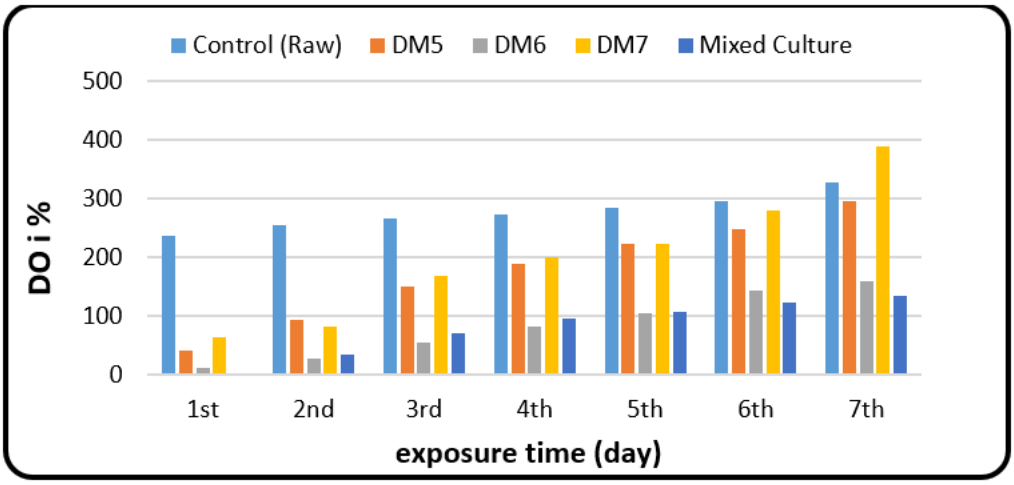

Fig. 4: Increase % in Dissolved Oxygen (DO) Levels in the Treated Dairy Effluent Using Bacterial Isolates at Different Exposure Times during the Batch Bioassay

Table 3: Residual Concentrations (mg/l) and I or RE% of Quality Parameters in the Raw and Treated Dairy Effluent during the Batch Bioassay**.**

Exposure Time (Days)	Bacterial Isolates									
	Control		DM5		DM6		DM7		Mixed Culture	
	Raw Wastewater DO: 0.49 mg/l									
	DO	I%	DO	I%	DO	I%	DO	I%	DO	I%
1	1.65	236.73	0.69	40.82	0.55	12.24	0.8	63.27	0.5	2.04
2	1.74	255.10	0.95	93.88	0.63	28.57	0.89	81.63	0.66	34.69
3	1.8	267.35	1.23	151.02	0.76	55.10	1.32	169.39	0.84	71.43
4	1.83	273.47	1.42	189.80	0.89	81.63	1.47	200.00	0.96	95.92
5	1.89	285.71	1.59	224.49	1.01	106.12	1.59	224.49	1.02	108.16
6	1.94	295.92	1.71	248.98	1.19	142.86	1.86	279.59	1.1	124.49
7	2.1	328.57	1.94	295.92	1.27	159.18	2.4	389.80	1.15	134.69
TDS	Raw Wastewater TDS: 1220 mg/l									
	RC	I%	RC	I%	RC	I%	RC	I%	RC	I%
1	1266	3.77	1244	1.97	1320	8.20	1301	6.64	1235	1.23
2	1290	5.74	1326	8.69	1469	20.41	1450	18.85	1392	14.10
3	1320	8.20	1477	21.07	1556	27.54	1490	22.13	1397	14.51
4	1489	22.05	1611	32.05	1633	33.85	1520	24.59	1412	15.74
5	1387	13.69	1696	39.02	2043	67.46	1662	36.23	1557	27.62
6	1401	14.84	1834	50.33	1782	46.07	1736	42.30	1675	37.30
7	1433	17.46	1795	47.13	1900	55.74	1941	59.10	1719	40.90
I%: Increase%										
TSS	Raw Wastewater TSS: 1923 mg/l									
	RC	RE %	RC	RE %	RC	RE %	RC	RE %	RC	RE %
1	1894	1.51	1744	9.31	1749	9.05	1741	9.46	1819	5.41
2	1742	9.41	1528	20.54	1478	23.14	1563	18.72	1892	1.61
3	1675	12.90	1363	29.12	1640	14.72	1394	27.51	1740	9.52
4	1652	14.09	1384	28.03	1246	35.21	1246	35.21	1590	17.32
5	1596	17.00	997	48.15	969	49.61	575	70.10	1479	23.09
6	1530	20.44	846	56.01	828	56.94	933	51.48	1349	29.85
7	1483	22.88	842	56.21	561	70.83	1065	44.62	1161	39.63
BOD	Raw Wastewater BOD: 2700 mg/l									
	RC	RE %	RC	RE %	RC	RE %	RC	RE %	RC	RE %
1	2467	8.63	2411	10.70	2397	11.22	2338	13.41	2438	9.70
2	2176	19.41	2165	19.81	2090	22.59	2034	24.67	2342	13.26
3	1887	30.11	2063	23.59	1698	37.11	1641	39.22	2292	15.11
4	1560	42.22	1717	36.41	1455	46.11	1347	50.11	1595	40.93
5	1296	52.00	1574	41.70	2557	5.30	899	66.70	1973	26.93
6	1118	58.59	1390	48.52	863	68.04	591	78.11	1963	27.30
7	1207	55.30	836	69.04	664	75.41	718	73.41	1660	38.52
COD	Raw Wastewater COD: 7680 mg/l									
	RC	RE %	RC	RE %	RC	RE %	RC	RE %	RC	RE %
1	6927	9.80	6665	13.22	6251	18.61	6289	18.11	6819	11.21
2	6288	18.13	5604	27.03	4984	35.10	5537	27.90	5473	28.74
3	5744	25.21	4618	39.87	4362	43.20	4620	39.84	5126	33.26
4	5176	32.60	2591	66.26	3164	58.80	2698	64.87	4605	40.04
5	4216	45.10	2063	73.14	2780	63.80	1966	74.40	3678	52.11
6	3563	53.61	1597	79.21	1728	77.50	1348	82.45	2800	63.54
7	2978	61.22	940	87.76	1013	86.81	871	88.66	2357	69.31

TVBC	Growth of Bacterial Cultures								
	Control	DM5		DM6		DM7		Mixed Culture	
	CFU/ml	CFU/ml	S/I %	CFU/ml	S/I %	CFU/ml	S/I %	CFU/ml	S/I %
1	7.0×10^6	8.0×10^6	14.29	9.2×10^6	31.43	9.0×10^6	-------	9.0×10^6	28.57
3	4.0×10^6	17.0×10^6	325.0	5.1×10^6	27.5	14.0×10^6	375.0	14.0×10^6	250.0
5	3.1×10^6	8.0×10^6	158.1	7.0×10^6	125.81	2.3×10^6	577.42	2.3×10^6	- 25.81
7	3.0×10^6	1.6×10^6	- 46.67	3.2×10^6	6.67	1.9×10^6	------	1.9×10^6	- 36.67

S/I%: Stimulation/ Inhibition % in the Bacterial Growth, +ve Sign indicates stimulation and -ve Sign indicates inhibition

3.2. Total Solids (TDS & TSS):

Raw dairy wastewater recorded 1220 mg/l TDS and 1923 mg/l TSS at the starting point indicating high turbidity and pollution strength. TDS increased regularly in all the tested cultures with time reaching the highest residues at the last exposure day due to the breaking down of complex pollutants into simply dissolved salts. On the other hand, the removal efficiency of TSS increased in all the tested isolates with time reaching the highest RE% at the last exposure day except isolate DM6 (Table 3 and Fig. 5 A & B).

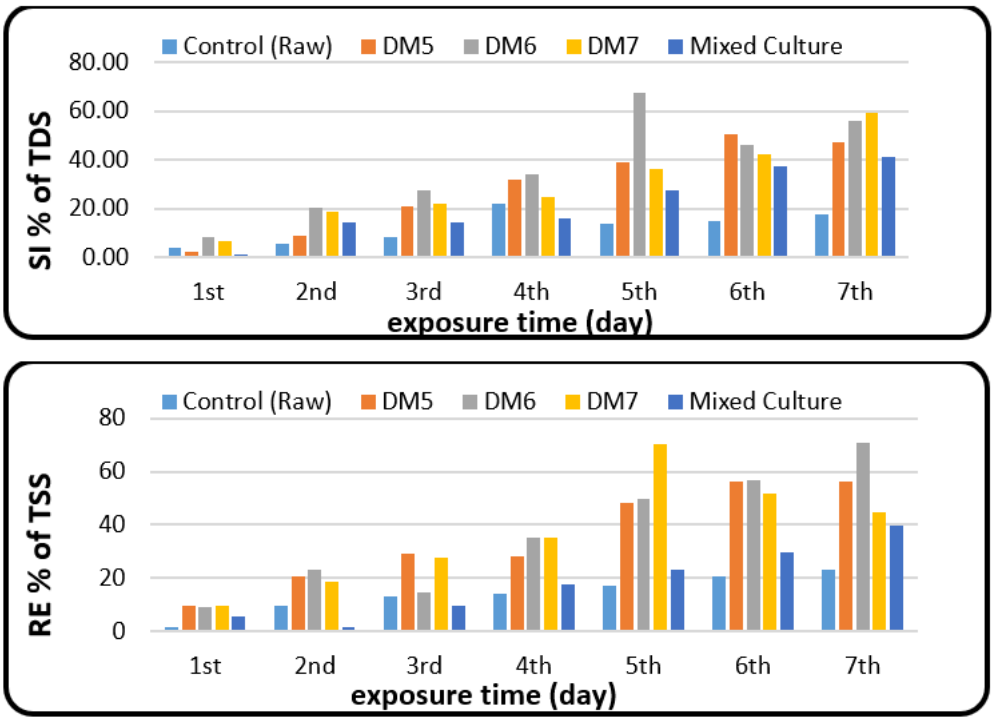

Fig. 5: Increase % of TDS (A) and Removal Efficiency % of TSS (B) in the Treated Dairy Effluent Using Bacterial Isolates at Different Exposure Times during the Batch Bioassay

3.3. Organic Matter Removal (BOD & COD):

Raw dairy wastewater is characterized by the very high BOD and COD levels (2700 & 7680 mg/l respectively) indicating high organic load and pollution strength (Table 3 and Figs. 6 A & B). Results revealed the following points:

1. There was a general trend of increasing the removal efficiency of BOD and COD by all the tested isolates with time reaching the highest RE% at the 7th exposure day with few exceptions.
2. The highest BOD removals were achieved by isolates DM7 (78.11%, 591 mg/l) and DM6 (75.41%, 664 mg/l) after and 6 and 7 days respectively. In contrast, the mixed culture (DM5, DM6 and DM7) achieved the lowest BOD removal (40.93 %, 1660 mg/l). The control culture achieved BOD removal of 58.59 % (1118 mg/l) which is lower than the tested individual cultures but higher than the mixed culture (Fig. 6A).
3. The highest COD removals were achieved after 7 exposure day by isolates DM7 (88.66%, 871 mg/l), DM5 (87.76 %, 940 mg/l), DM6 (86.8%, 1013 mg/l) and finally the mixed culture with the lowest COD removal (69.31 % and 2357 mg/l). The control culture achieved COD removal of 61.22 % (2978 mg/l) which is lower than the tested cultures (Fig. 6B).

4. The lowest BOD residue in the treated effluent was recorded at 591 mg/l by DM7 which is lower than its MPL (600 mg/l) for safe discharging into the general sanitary drainage network. the lowest COD residue in the treated effluent recorded 871 mg/l achieved by DM7 which is lower than its MPL (1100 mg/l) for safe discharging into the general sanitary drainage network.

3.4. Total Viable Count of Bacteria (TVCB):

Results of TVC of the tested indigenous bacterial isolates during the batch treatment bioassay (Table 3) revealed that isolate DM7 inoculum had the highest growth stimulation density (577.42 %, 21.0 x 10^6 CFU/ml) after 5 days. This was followed by isolate DM5 (325.0 %, 17.0 x 10^6 CFU/ml) after 3 days, the mixed culture (250.0 %, 14 × 10^6 CFU/ml) after 3 days and finally DM6 (125.81 %, 7.0 x 10^6 CFU/ml) after 5 days. The only growth inhibition was recorded by DM5 (- 46.67 %, 1.6 x 10^6 CFU/ml) after 7 days and the mixed culture (- 25.81 to - 36.67 %) after 5 and 7 days respectively.

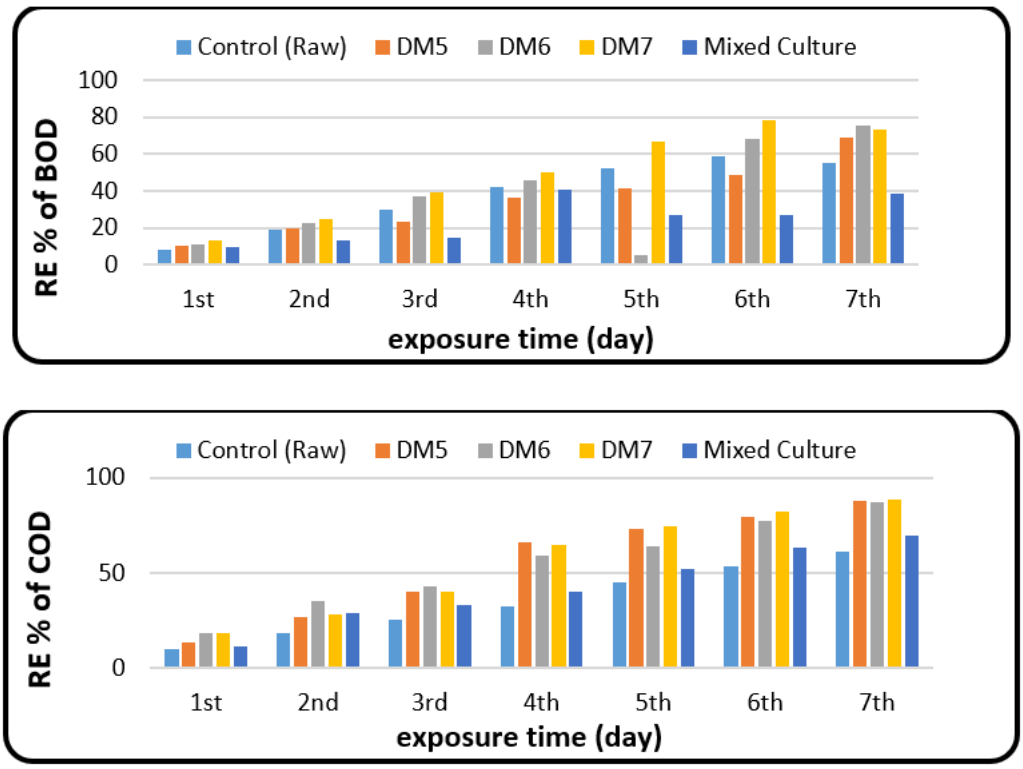

Fig. 6: Removal Efficiency % of BOD (A) and COD (B) in the Treated Dairy Effluent Using Bacterial Isolates at Different Exposure Times during the Batch Bioassay

DISCUSSION

Among the ten indigenous bacterial isolates from dairy wastewater, three isolates showed high activity in the breakdown and removal of the contaminants. The tested dairy effluent was a mixture of waters from cheese and milk production lines located at Borg El-Arab, Alexandria Governorate. Molecular characterization identified the 3 most active bacterial isolates (DM5, DM6 & DM7) as *Bacillus cereus* ATCC14579 (NR-114582.1), *Bacillus aerius* 24 K (NR-118439.1) and *Bacillus cereus* ATCC14579 (NR-074540.1) respectively. Results proved that *Bacillus cereus* ATCC14579 (strain DM7) is the most capable and exhibited the highest removal efficiency of the included pollutants in the highly contaminated dairy wastewater. On the other hand, the mixed culture (DM5, DM6 and DM7) showed less efficiency in remediation of dairy effluent which may be attributed to the competition or antagonistic

effect among them, since bacteria compete with each other for space and resources. Resources of nutrition are a vocal point of bacterial competition. In contrast with the present study, some researchers stated that bacterial mixture could achieve the best results in the bioremediation of wastewater compared to individual strains (Keffala *et al*., 2017) where individual bacteria cultures recorded 71.6% COD removal compared to the mixed culture which achieved 75.8% after 25 days treatment periods.

Strains DM5 and DM7 belong to *Bacillus cereus*, a rod-shaped, gram-positive bacterium forming motile endospores (Gharib *et al*., 2020). It may be an aerobic or facultative anaerobic pathogenic bacterium (Parihar, 2014) that can cause food spoilage and many health problems (Rajkovic *et al.*, 2008; Bottone , 2010; Logan., 2012; Keita *et al.*, 2013; LO *et al.*, 2015; Liu *et al.*, 2020). Its endospores are resistant to many factors such as (radiation, low pH values, dryness, heat, disinfectants, desiccation and cleaning processes (Parihar, 2014; Jessberger *et al*., 2020). *Bacillus cereus* is widely prevalent in the environment. It can be found in soil, air, water, dust, ground, surfaces of plants and disintegrated materials (Eglesoz,s., 2014). It may exist in various dairy products and food.

Strain DM6 belongs to *Bacillus aerius* and is a gram-positive and motile rod-shaped bacterium forming irregular white colonies on nutrient agar at 37 ؛C and pH 6-10. It can be found in polluted water causing many health problems (Shivaji *et al.*, 2006; Dunlap, 2015). Its growth depends on the presence of oxygen (Shafi *et al*.., 2017). Many *Bacillus species* were applied in various agricultural, pharmaceutical, medical, food and industrial utilizations because of their production of enzymes, antibiotics and other substances (Pereyra *et al*., 2018; Hamiche *et al.*, 2019; Asmani *et al*., 2020).

Dairy wastewater applied in the present study is categorized as strongly polluted wastewater including very high levels of examined pollutants that demand effective treatment to reduce contamination and discharge it safely. Dairy wastewater contains high levels of organic compounds, detergents, minerals, proteins and a broad range of pH values (Wu *et al.*, 2013; Kushwaha *et al.*, 2010; Chakchouk *et al.*, 2017; Masi *et al.*, 2016; Rott *et al.*, 2017; Akratos *et al.*, 2018; Amini *et al.*, 2019; Kauv, 2021). It was reported that dairy wastewater contains 1400-50.000 mg/l BOD, 2000-90.000 mg/l COD and 70-800 mg/l TSS (Licata *et al*., 2021).

Raw dairy wastewater was subjected to treatment in a batch experiment using free and fixed individual and mixed bacteria (MBBR) that was time and species dependent and accordingly resulted in varying levels of contaminants REs. The Egyptian Environmental Law (No 44/2000) stated MPLs of the different water contaminants in the industrial effluents for their safe discharge into the sanitary drainage network. These limits are used to reduce ecological disturbances and protect the aquatic and soil environment from discharges.

Dairy effluent contains high organic matter, which is responsible for the rapid depletion of the DO level. Hence dairy effluent had low DO levels (0.1: 0.5 mg/l). When organic matter is consumed by the aerobic bacteria and oxidized (combined with oxygen), dissolving oxygen is reduced in the wastewater. Low DO levels produce many side effects in water and decrease the water quality.

Results revealed TDS increases with increasing exposure time due to high organic matters in dairy wastewater and activities of examined bacteria. TSD in raw wastewater recorded 1220 mg/l at zero time reaching the highest level (2043 mg/l, 67.46%) by DM6 due to degradation of organic materials into soluble salts which is opposite to results obtained by other workers under their operation conditions (Alwasify *et al.*, 2017) where 79.1% and 77.3% *TDS* removals were achieved by bacterial and fungal isolates respectively.

Raw dairy effluent recorded a high TSS level (1923 mg/l) indicating high contamination with organic matters reaching the highest RE of 70.83% (561.5 mg/l) by

DM6) after 7 days which is lower than its MPL (800 mg/l) confirming its high ability to breakdown organic matters. This is even better than and supported by other workers where TSS residue levels reached up to 630 mg/l (Poweral *et al*., 2014) and 700.3 mg/l (Ali *et al*., 2021) and remarkably higher than TSS removal of 26% (Ranasinghe *et al*., 2016).

Raw dairy wastewater is characterized by a very high COD level (7680 mg/l) at start time which is 6.9 - fold its MPL (1100 mg/l) attributed to the presence of fats, lactose, casein, nutrients and salts (Kolhe *et al*., 2009). DM7 recorded the highest RE% (88.66%) of COD level (871 mg/l) which is much below its MPL. These results are consistent with those of (El-Sesy, M. and Mustafa, M.,2020) where a reduction of BOD and BOD by 80 and 79% respectively was reported after bacterial treatment. Compared to other workers where other bacterial species were tested such as *Sentrophomonas* (Mazzucotelli *et al*., 2014), *Pseudomonas* and *Bacillus* (Zhao *et al*., 2009), *Licheniformis* NW16, *Aeromonas hydrophilia* NS17 and *Paenibacillus* NW9 (Sonune *et al*., 2015) which achieved 42.86 and 82.76% BOD and COD removal, the present selection exhibited superior ability to biodegrade organic matter dairy wastewater under ambient conditions without agitation or any modification.

The lowest RE% of COD obtained by the control sample (unseeded wastewater) showed the lowest removal (61.22%, 2978 mg/l). This was supported by Sonune *et al*. (2015) who reported that bacterial species in the control sample has no significant effect on the reduction of BOD and COD.

CONCLUSION

Raw dairy wastewater contains a very high level of COD (7680 mg/l), BOD (2700 mg/l) and a high level of TSS (1923 mg/l) indicating high organic load and suspended particles. It also contained an intermediate level of TDS (1220 mg/l) and a very low DO level (0.49 mg/l) due to high organic contents and high microbial oxidation demand.

The results of the present study concluded the following points:

1. *Bacillus cereus* ATCC14579 (DM7) achieved the highest removal efficiency of BOD (78.11), COD (88.66%) and TSS (70.10 %), the highest TDS increase (59.10 %) and DO increase (389.89%).
2. DM7 showed the highest biomass yield (growth stimulation) during the batch mode treatment bioassay confirming that it is the most active bacterial isolate with the highest ability to biodegrade and benefit from the organic pollutants in the dairy effluents for its growth.
3. The mixed culture showed lower removals for the included contaminants compared to the DM7 and the other individual isolates.
4. As expected also, the control (unseeded wastewater) showed the lowest activity towards the dairy effluent's contaminants confirming the superiority of the tested bacteria especially DM7 for the biodegradation and removal of such contaminants.
5. In conclusion, isolate DM7 proved to be the most promising for either minimization or decontamination of pollution load (mostly organic) from the dairy wastewater.

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ARABIC SUMMARY

المعالجه الميكروبيه لمياه صرف مصانع الالبان بنظام المعالجه غير المستمره باستخدام البيوفيلم الحيوى المتحرك

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3-قسم الميكروبيولوجى جامعه طنطا

الغرض من هذه الدراسة هو التحقق من قدره وكفاءه المعالجه البيولوجيه لتقليل ملوثات مياه الالبان العادمه الى حدود مقبوله من مياه الصرف الصحى من أجل التصريف الامن باستخدام البكتيريا المعزولة. تم عزل عشر سلالات بكتيرية (DM1-DM10) وفحصها لعمليه ازاله الملوثات لمده 7 ايام .تم قياس معايير الجودة متضمنه طلب الاوكسجين الحيوي (BOD) و طلب الاكسجين الكيميائي (COD) و المواد الصلبة الكلية (TSS) والمواد الصلبة العالقة (TDS) والاوكسجين المذاب (DO) والعدد الكلى للبكتيريا (TVCB) قبل وبعد المقايسة الحيوية وتم حساب كفاءه (REs) ازالتهم .تم تعريف السلالات البكتيريه الثلاثه بواسطه Molecular Method واستخدامها كعزلات فرديه أو فى مزارع مختلطه فى اختبار المعالجه (الوضع الدفعى).

أظهرت النتائج اتجاها عاما لزيادة كفاءه ازاله الملوثات بواسطة كل البكتيريا المختبرة مع زيادة وقت التجربة. سجلت السلالات {(Bacillus Cereus ATCC14579 (NR-114582.1} DM5 و {(Bacillus Aerius} DM6 {(NR-118439.1) 24 K} و {(Bacillus cereus ATCC14579 (NR-074540.1} DM7 أعلى نشاط لا زاله الملوثات المحددة. بينما السلالات DM2 و DM8 و DM10 سجلت أقل كفاءه ازاله لنفس الملوثات. لهذا تم استخدام السلالات DM5 و DM6 و DM7 كعزلات فردية ومزارع مختلطه في عمليه معالجه الوضع الدفعي .تحتوى مياه الصرف الصحي للألبان على مستوى عالي جدا من طلب الاوكسجين الكيميائي (7680ملغم /لتر) و طلب الاوكسجين الحيوي (2700 ملغم / لتر) و مستوى عالي من المواد الصلبة الكلية (1923 ملغم / لتر) مما يشير الى وجود حمل عضوي ومواد عالقة .كما أنها تحتوى على مستوى متوسط من المواد الصلبة الذائبة (1220 ملم / لتر)ومستوى منخفض من الاوكسجين المذاب (0.49 ملغم / لتر) نتيجة المحتوى العضوي العالي والاكسدة الميكروبية.

حققت السلالة DM7 أعلى كفاءه ازاله للأوكسجين الحيوي (78.11%) والاكسجين الكيميائي (88.66%) والمواد الصلبة الكلية (70.10%) من مياه الصرف الصحي للألبان. وسجلت أيضًا أعلى زيادة فى نسبه المواد الصلبة الذائبة (59.10%) والاكسجين الذائب (389.89%). بينما أظهرت المزرعة المختلطة أقل كفاءه ازاله لكل الملوثات المتضمنة. علاوة على ذلك أظهرت السلالة DM7 أعلى انتاج للكتلة الحيوية (تحفيز النمو) حيث أنها تمتلك أعلى قدره على التحلل البيولوجي والاستفادة من المواد الملوثة العضوية في مخلفات الالبان لنموها بين جميع السلالات المختبرة. أكدت نتائج الدراسة الحالية أن السلالة {(Bacillus cereus ATCC14579 (NR-074540.1} DM7 هي أفضل سلاله لتقليل أو ازاله التلوث (العضوي في الغالب) من مياه الصرف الصحي لمنتجات الالبان .