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Isolation, characterization and identification of microorganisms from unorganized dairy sector wastewater and sludge samples and evaluation of their biodegradability

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ABSTRACT

In developing countries like India, the major part of the dairy sector is under the coverage area of unorganized sector, which lacks adequate treatment facility. In present investigation, the study was done to isolate most frequently occurring active strains adapted to the wastewater physical-chemical conditions and having good biodegradation potential. The 10 isolates were selected on the basis of their efficiency in reducing all the three pollution potential parameters i.e BOD, TSS and Oil and grease content. The identification of selected strains was done by 16 S rRNA sequencing. The maximum reduction in BOD_3 was shown by isolate no. 25 i.e 89.8% (90 mg/l). Isolate no. 4 and 25 were efficient in reducing the TSS content by 88.6%. Isolate no. 27 and 45 were more efficient in reducing the oil and grease content by 88.5% and 90% respectively.

1. Introduction

Dairy industries generate highly pollutant wastewater, characterized by high BOD (Biological Oxygen Demand), TSS (Total suspended solids) and Oil and grease content [1–3]. Wastewater with high organic load causes many ecological problems [4]. It shows adverse effects on both flora and fauna; its discharge to the land alters physical and chemical properties of the soil, thus reducing the fertility of land for crop production and its discharge to the water bodies may results in eutrophication, affecting the aquatic life and making water unfit for drinking [5–7]. Hence, the challenge for the safe disposal of the dairy wastewater cannot be ignored. Environmentalists and government are looking for cheap, efficient, effective and long lasting solutions for wastewater treatment and recycling. In developing countries like India, physico-chemical methods of waste water treatment are inevitably cost intensive and cannot be employed in all industries. Hence, in recent years, the biological treatment system has become popular and has helped in developing relatively efficient, low cost waste treatment systems [8]. In order to design an efficient biological waste water treatment it is important to know the microbiota composition of the wastewater and to identify the strains which metabolize organic compounds [9,10]. In India about 85% of the dairy sector is under the coverage of unorganized sector which lacks adequate treatment facility and management skills. Physico-chemical characteristics of the dairy wastewater generated by organized and unorganized sector exhibit huge variations. The wastewater generated by unorganized sector is rich in organic content. Its C:N ratio was calculated as 37.6 compared to the ratio of 11.9 of organized sector [11]. Variations in major pollution parameters of dairy effluent of both the sectors require appropriate treatment approaches for its safe disposal.

The present investigation was carried out to isolate the most frequently occurring and optimally performing microorganisms from dairy wastewater and sludge samples of unorganized sector and to test the bioremediation efficacy of the isolates by bioaugmenting them in dairy wastewater.

2. Material and methods

2.1. Sample collection

Five samples of dairy wastewater and five samples of dairy sludge were collected from unorganized dairy industry located in the districts of Patiala, Ludhiana, Shri Muktsar Sahib and Bathinda (Punjab, India) in dry plastic bottles which were rinsed with distilled water and then with dairy effluent. Physical properties like pH, temperature, odor, color were recorded at the site of sample collection. The pH was determined using a EI Deluxe pH meter - 101.

The sample was transferred to the laboratory immediately and stored at 4 °C to avoid any physical-chemical changes in the wastewater.

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Table 1
Colony and morphological characteristics of bacterial isolates.

Culture No	Opacity	Size (Diameter 24 h of incubation) (mm)	Shape	Color	Margin	Elevation	Gram character	Shape of cell	Arrangement
1	Opaque	3	Irregular	White	Undulate	Raised	-ve	Rods	Pairs
2	Translucent	< 1	Round	White	Entire	Convex	+ve	Cocci	Pairs and Chains
3	Opaque	3	Irregular	Pale yellow	Filiform	Convex	+ve	Cocci	Chains
4	Opaque	2	Round	White	Undulate	Flat	-ve	Coccobacilli	Pairs
5	Transparent	< 1	Round	White	Entire	Flat	-ve	Rods	Chains
6	Transparent	3	Irregular	Golden yellow	Curled	Raised	-ve	Rods	Singly present
7	Translucent	2	Round	White	Entire	Flat	+ve	Rods	Present in group of 3
8	Transparent	< 3	Irregular	White	Undulate	Raised	+ve	Cocci	Singly present
9	Opaque	1	Round	White	Filamentous	Convex	+ve	Coccobacilli	Singly present
10	Transparent	1	Round	Pale white	Curled	Raised	+ve	Coccobacilli	Singly Present
11	Transparent	1	Round	White	Entire	Raised	+ve	Coccobacilli	Pairs
12	Opaque	2	Rhizoid	White	Undulate	Flat	-ve	Cocci	Pairs
13	Translucent	1	Irregular	White	Curled	Raised	-ve	Cocci	Chains
14	Opaque	1	Filamentous	White	Curled	Convex	+ve	Rods	Singly present
15	Transparent	< 3	Irregular	White	Undulate	Convex	+ve	Coccobacilli	Clusters
16	Opaque	< 3	Irregular	Golden yellow	Undulate	Raised	+ve	Coccobacilli	Pairs
17	Transparent	2	Rhizoid	White	Entire	Flat	-ve	Rods	Pairs
18	Opaque	< 3	Irregular	Grey	Undulate	Raised	-ve	Rods	Clusters
19	Opaque		Irregular	White	Entire	Raised	+ve	Coccobacilli	Singly presents
20	Opaque	1`	Round	White	Entire	Convex	+ve	Coccobacilli	Pairs
21	Transparent	1	Filamentous	Grey	Curled	Raised	+ve	Coccobacilli	Pairs
22	Opaque	2	Round	White	Filamentous	Raised	-ve	Cocci	Chains
23	Opaque	3	Rhizoid	Golden yellow	Undulate	Flat	-ve	Cocci	Pairs
24	Translucent	< 3	Irregular	White	Filamentous	Flat	-ve	Coccobacilli	Chains
25	Opaque	2	Round	White	Entire	Raised	+ve	Rods	Pairs
26	Transparent	< 3	Irregular	Pale White	Filamentous	Flat	+ve	Coccobacilli	Pairs
27	Opaque	< 3	Rhizoid	White	Entire	Raised	-ve	Rods	Chains
28	Transparent	< 3	Filamentous	White	Undulate	Flat	+ve	Coccobacilli	Pairs
29	Opaque	< 3	Filamentous	Pale white	Filamentous	Flat	-ve	Coccobacilli	Chains
30	Opaque	2	Rhizoid	White	Entire	Raised	-ve	Coccobacilli	Pairs and in Chains
31	Transparent	2	Round	White	Undulate	Flat	-ve	Rods	Singly and in pairs
32	Opaque	1	Irregular	White	Entire	Raised	+ve	Coccobacilli	Singly and in pairs
33	Opaque	< 3	Irregular	White	Undulate	Flat	+ve	Rods	Pairs
34	Opaque	2	Round	White	Entire	Raised	+ve	Rods	Pairs
35	Transparent	2	Transparent	Pale White	Udulate	Flat	-ve	Rods	Singly present
36	Opaque	< 3	Round	Pale White	Entire	Raised	-ve	Rods	Chains
37	Opaque	< 3	Irregular	White	Undulate	Flat	-ve	Rods	Singly present
38	Translucent	< 3	Irregular	White	Filamentous	Flat	+ve	Rods	Singly present
39	Opaque	2	Round	White	Entire	Raised	+ve	Coccobacilli	Pairs
40	Translucent	< 3	Irregular	White	Entire	Raised	+ve	Rods	Singly
41	Opaque	< 3	Irregular	White	Filiform	Raised	+ve	Rods	Singly
42	Opaque	< 3	Round	White	Filiform	Raised	+ve	Rods	Clusters
43	Opaque	< 3	Irregular	Pale White	Filiform	Raised	-ve	Rods	Clusters
44	Opaque	2	Round	White	Entire	Round	-ve	Rods	Clusters
45	Transparent	< 3	Round	Pale White	Entire	Round	-ve	Cocci	Pairs
46	Opaque	2	Irregular	White	Entire	Raised	+ve	Rods	Chains
47	Opaque	< 3	Irregular	White	Filamentous	Raised	+ve	Rods	Singly
48	Opaque	1	Round	White	Entire	Raised	+ve	Rods	Chains
49	Opaque	2	Irregular	White	Entire	Raised	+ve	Rods	Singly
50	Translucent	< 3	Irregular	White	Entire	Flat	+ve	Rods	Singly
51	Opaque	< 3	Irregular	White	Filamentous	Raised	+ve	Rods	Singly present and in Pairs
52	Opaque	< 3	Irregular	White	Filamentous	Raised	+ve	Coccobacilli	Singly present
53	Opaque	2	Round	Pale White	Filiform	Raised	+ve	Rods	Singly present
54	Transparent	< 3	Irregular	White	Undulate	Raised	+ve	Rods	Singly present
55	Transparent	< 3	Irregular	White	Undulate	Flat	+ve	Coccobacilli	Pairs
56	Opaque	1	Round	Pale White	Filamentous	Flat	+ve	Cocci	Singly present
57	Translucent	2	Irregular	White	Undulate	Raised	+ve	Coccobacilli	Pairs
58	Opaque	1	Round	White	Entire	Raised	+ve	Coccobacilli	Pairs
59	Opaque	< 3	Round	Pale White	Entire	Raised	+ve	Coccobacilli	Chains

2.2. Analysis of the dairy wastewater and sludge samples

Parameters of dairy waste samples analyzed included pH, color, temperature. BOD_3, Oil and grease and TSS (Total suspended solids) which were carried out as per standard procedure. Total suspended solids were determined by the equation TSS = TS (Total solids – TDS (Total dissolved solids). The Oil and grease content was determined by partition gravimetric method. The BOD was analyzed by titrimetric method [12].

2.3. Isolation of most frequently occurring micro-organisms from dairy sludge

Appropriately dilute sludge samples were plated onto Nutrient Agar

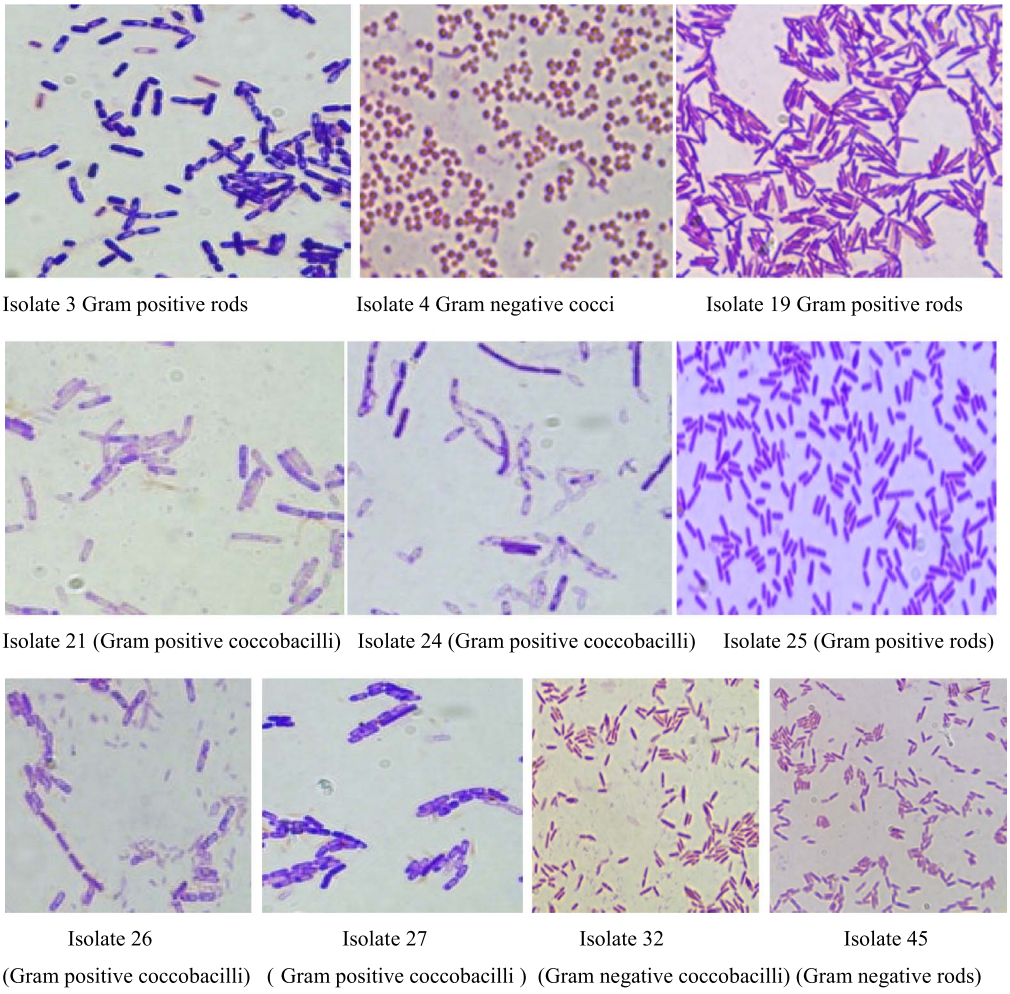

Fig. 1. Microscopic characteristics of the most efficient isolate.

plates which were then incubated at 37 °C for 24 h. After incubation, the pure cultures of the most frequently encountered isolates were prepared and used in the study. Each of the isolates was observed for the colony characters like size, shape, color, margin, elevation and opacity and also morphological characters like Gram reaction, shape and arrangement of cells. Total of 59 isolates were obtained and they were designated as 1, 2, 3.....59.

2.4. Bioaugmentation of dairy wastewater using isolates

To obtain greater cell biomass pure culture of isolates were inoculated in Nutrient Broth incubated at 37ºC for 24 h. Centrifugation was done at 5000 rpm. 0.1% (w/v) wet weight basis of inoculum was used in 100 ml of dairy waste water and incubated for 3 days in BOD incubator at 27 °C. Control was run simultaneously without bioaugmentation. The ability of the isolates to reduce BOD_3, TSS and Oil & grease was examined.

2.5. Identification of bacterial isolates

Extraction of DNA from bacterial isolates was done as per the protocol described by Atashpaz et al. [13]. A single colony was inoculated in nutrient broth and was grown for 24 h at 37 °C. From the 5 ml of culture, the cells were harvested. 800 μL of lysing buffer (2% CTAB, 100 mM Tris-HCl, 1.4 M NaCl, 1% PVP, 20 mM Na_2EDTA and 0.2% LiCl) was added to the sample and incubated at 65 °C (30 min for Gram negative bacteria; 2 h for Gram positive bacteria). The sample was centrifuged at 10000 rpm for 5 min at 4 °C. After the extraction of supernatant an equal volume of chloroform – isoamilalcohol (24:1 v/v) was added to it and was centrifuged at 12000 rpm for 8 min at 4 °C. The DNA was extracted from the aqueous layer by adding cold (−20 °C) isopropanol. The dried DNA pellet was dissolved in 50 μL of 1X TE buffer. The quality and intactness of the extracted DNA was checked by running on 1% agarose gel which contain 1 μg/ml ethidium bromide. The A_{260}/A_{280} absorbance ratio was used to determine undesired contaminations.

2.5.1. PCR amplification and sequencing of 16 S rRNA gene

PCR amplification and sequencing of the extracted DNA samples was done by Yaazh Genomics, Tamil Nadu. Amplification of 16 S rRNA universal primers gene fragment was done by using MJ Research Peltier Thermal Cycler.

The universal primers (Forward primer 27 F AGAGTTTGATCMTGGCTCAG and Reverse primer 1492 R TACGGYTACCTTGTTACGACTT) were used.

1 μL of template DNA was added in 20 μL of PCR reaction solution. The PCR reaction was performed with the following conditions: Initial denaturation was done at 94 °C for 2 min, followed by 35 amplification cycles at 94 °C for 45 s, annealing temperature of primers was 55 °C for 60 s, and extension at 72 °C for 60 s. Final extension was done at 72 °C for 10 min. The resulting PCR products were purified using Montage PCR Clean up kit (Millipore) and sequenced using ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

Table 2
Bioremediation efficacy of dairy wastewater.

Isolate no	pH	BOD (% Reduction)	TSS (%Reduction)	Oil and grease (% Reduction)
1	7.50	69.70 (155 ± 17 mg/l)	43.09 (233.3 ± 14 mg/l	57.08 (103 ± 1./7 mg/l)
2	7.40	52.40 (300 ± 3.0 mg/l)	39.02 (250 ± 0 mg/l)	52.70 (113.3 ± 11 mg/l)
3	7.50	84.10 (100 ± 173 mg/l)	73.58 (108.3 ± 25 mg/l)	75.00 (125 ± 10 mg/l)
4	7.50	82.9 (180 ± 0 mg/l)	81.24 (100 ± 5.7 mg/l)	82.50 (41.9 ± 0.9 mg/l)
5	7.20	87.30 (80 ± 17.3 mg/l)	68.85 (127.7 ± 25.5 mg/l)	76.40 (56.6 ± 5.7 mg/l)
6	7.30	70.00 (170 ± 10 mg/l)	57.30 (175 ± 25 mg/l)	79.16 (50 ± 10 mg/l)
7	7.20	34.90 (410 ± 10 mg/l)	51.20 (200 ± 10 mg/l)	45.80 (130 ± 26.5 mg/l)
8	8.20	57.60 (233.3 ± 14 mg/l)	69.70 (166.6 19.2 mg/l)	25.90 (333.30 57 mg/l)
9	9.20	68.57 (330 ± 8.6 mg/l)	49.90 (266.7 ± 25 mg/l)	74.40 (55.8 ± 11.5 mg/l)
10	9.20	72.00 (290 ± 8.7 mg/l)	24.90 (400 ± 2.0 mg/l)	50.00 (250 ± 11.5 mg/l)
11	9.50	65.00 (175 ± 8.6 mg/l)	55.60 (200 ± 0 mg/l)	46.70 (116.6 ± 28.9 mg/l)
12	9.70	47.40 (265 ± 8.5 mg/l)	25.90 (333.3 ± 11.6 mg/l)	32.90 (146.6 ± 5.8 mg/l)
13	9.20	65.71 (360 ± 5 mg/l)	43.74 (300 ± 20.2 mg/l)	53.34 (233 ± 10 mg/l)
15	8.00	66.70(116.3 19 mg/l)	57.60 (190 10.5 mg/l)	55.60 (195 10.7 mg/l)
16	8.43	60.90 (160 ± 17.4 mg/l)	70.40 (133.3 ± 28.8 mg/l)	76.20 (83.3 ± 6.4 mg/l)
18	8.12	62.20 (155 ± 8.0 mg/l)	64.80 (158.3 ± 7.5 mg/l)	80.00 (70 ± 10 mg/l)
19	8.29	82.90 (70 ± 17.3 mg/l)	77.80 (100 ± 25 mg/l)	79.10 (73.3 ± 11.5 mg/l)
20	7.58	63.50 (108.3 ± 10.4 mg/l)	51.90 (216.6 ± 5 mg/l)	71.40 (100 ± 2.5 mg/l)
21	8.21	60.90 (160 ± 22.9 mg/l)	61.10 (175 ± 25.2 mg/l)	66.70 (116.6 ± 10.4 mg/l)
22	3.95	38.90 (540 ± 0 mg/l)	51.90 (433.3 ± 5.7 mg/l)	24.90 (250 ± 5 mg/l)
23	4.07	61.00 (250 ± 5 mg)	87.10 (116.3 ± 3.5 mg/l)	69.90 (100 ± 5.7 mg/l)
24	4.70	84.2 (70 ± 10 mg/l)	75.60 (220 ± 36.4 mg/l)	77.80 (100 ± 0 mg/l)
25	3.97	89.80 (90 ± 0 mg/l)	88.60 (100 ± 5.6 mg/l)	83.90 (84.90 ± 6.9 mg/l)
26	3.96	85.30 (130 ± 17.3 mg/l)	82.10 (161.1 ± 34.7 mg/l)	84.90 (40 ± 11.5 mg/l)
27	4.72	85.40 (60 ± 0 mg/l)	77.6 (123.3 ± 25.2 mg/l)	88.50 (40 ± 0 mg/l)
28	4.49	70.70 (120 ± 5.2 mg/l)	68.20 (175 ± 18.0 mg/l)	71.40 (100 ± 5.7 mg/l)
29	4.55	70.70 (120 ± 0 mg/l)	69.70 (166.6 ± 19.2 mg/l)	59.10 (143.3 ± 5.8 mg/l)
30	4.63	70.70 (120 ± 17.4 mg/l)	72.70 (150 ± 10 mg/l)	67.60 (113.3 ± 7.6 mg/l)
31	4.59	30.40 (285 ± 15 mg/l)	57.60 (233.3 ± 28.8 mg/l)	59.10 (143.3 ± mg/l)
32	4.76	87.80 (50 ± 8.0 mg/l)	81.80 (100 ± 0 mg/l)	85.70 (50 ± 10 mg/l)
33	3.95	38.90 (540 ± 0 mg/l)	51.90 (216.6 ± 5 mg/l)	24.90 (250 ± 5 mg/l)
34	3.96	66.70 (116.3 ± 10 mg/l)	51.90 (433.3 ± 5.7 mg/l)	54.90 (150 ± 0 mg/l)
35	7.90	84.50 (135 ± 15 mg/l)	82.8 (150 ± 0.5 mg/l)	84.5 (85 ± 5.7 mg/l)
36	8.02	74.02 (226 ± 17 mg/l)	76.2 (166.7 ± 5 mg/l)	77.36 (125 6 ± 25 mg/l)
37	7.96	75.90 (210 ± 0 mg/l)	80.09 (133.3 ± 3.5 mg/l)	72.70 (150 ± 10 mg/l)
38	7.00	32.20 (590 ± 5.8 mg/l)	42.90 (400 ± 7.6 mg/l)	45.50 (300 ± 7.0 mg/l)
39	8.00	84.50 (135 ± 15.1 mg/l)	78.60 (150 ± 50 mg/l)	72.70 (150 ± 50 mg/l)
40	6.69	27.70 (810 ± 25.2 mg/l)	60.00 (200 ± 11.5 mg/l)	73.30 (133.3 ± 28.8 mg/l)
41	7.00	47.30 (590 ± 17.4 mg/l)	40.00 (300 ± 10 mg/l)	40.00 (300 ± 10 mg/l)
42	7.05	27.70 (810 ± 30 mg/l)	46.70 (266.7 ± 57.7 mg/l)	40.00 (300 ± 11.5 mg/l)
43	7.15	22.30 (870 ± 15.2 mg/l)	50.00 (250 ± 50 mg/l)	60.00 (200 ± 10 mg/l)
44	7.20	59.80 (450 ± 30 mg/l)	60.00 (200 ± 0 mg/l)	80.00 (100 ± 0 mg/l)
45	7.34	82.60 (350 ± 25.1 mg/l)	81.80 (133.3 ± 57.7 mg/l)	90.00 (100 ± 10 mg/l)
46	7.36	42.80 (1150 ± 45.8 mg/l)	45.50 (400 ± 0 mg/l)	36.40 (350 ± 50 mg/l)
47	7.17	68.20 (640 ± 17.3 mg/l)	63.60 (266.7 ± 30 mg/l)	81.80 (100 ± 0 mg/l)
48	7.72	49.30 (730 ± 45.8 mg/l)	21.00 (500 ± 10 mg/l)	52.40 (333.3 ± 50 mg/l)
49	7.68	50.00 (720 ± 0 mg/l)	47.40 (333.3 ± 0.5 mg/l)	57.10 (300 ± 0 mg/l)
50	7.76	40.30 (860 ± 45.8 mg/l)	63.20 (233.3 ± 50 mg/l)	71.40 (200 ± 35 mg/l)
51	7.70	34.70 (940 ± 45.8 mg/l)	21.00 (500 ± 0 mg/l)	50.00 (350 ± 0.5 mg/l)
52	7.77	56.90 (620 ± 0.45 mg/l)	60.50 (250 ± 0.5 mg/l)	85.70 (100 ± 0 mg/l)
53	7.70	50.70 (710 ± 0.17 mg/l)	71.00 (183.3 mg/l)	78.60 (150 ± 50 mg/l)
54	7.40	16.50 (1260 ± 60 mg/l)	68.80 (250 ± 50 mg/l)	71.40 (200 ± 28.9 mg/l)
55	7.20	27.80 (1090 ± 23.1 mg/l)	18.70 (650 ± 50 mg/l)	42.80 (400 ± 28.9 mg/l)
56	7.34	25.20 (1130 ± 25.1 mg/l)	31.30 (550 ± 57.7 mg/l)	57.10 (300 ± 10 mg/l)
57	7.20	27.80 (1090 ± 17.3 mg/l)	37.50 (500 ± 0 mg/l)	57.10 (300 ± 50 mg/l)
58	7.35	33.10 (1010 ± 23.1 mg/l)	62.50 (300 ± 50 mg/l)	71.40 (200 ± 28.6 mg/l)
59	7.30	32.50 (1020 ± 30 mg/l)	50.00 (400 ± 0 mg/l)	57.10 (300 ± 28.3 mg/l)

2.5.2. Bioinformatics protocol

The 16 S rRNA sequence was compared using NCBI blast similarity search tool. For multiple alignments of sequences, MUSCLE 3.7 program was used [14]. Further, the program Gblocks 0.91b was used to cured the poorly aligned regions (removes alignment noise) [15]. Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 was used for substitution model. The program Tree Dyn 198.3 was used for tree rendering [16].

3. Results and discussion

The physical parameters like temperature, pH, odor and color of the dairy wastewater and sludge samples was recorded at the site of collection. The temperature varied from 10 to 37 °C. It was due to the seasonal variations and effects, the chemical and biological reactions taking place in water [17]. pH of the samples varied between 3.4 and 6.8. Acidic nature of the effluent generated was mainly due to the production of cheese in the unorganized sector. Odor was always unpleasant due to anaerobic decomposition of organic matter. Color of the dairy waste water was pale white and the sludge samples were grey colored with large flocs of suspended matter.

3.1. Isolation of most frequently occurring micro- organisms from dairy wastewater and dairy sludge samples

Various studies were done by many workers on the microbiological

and biochemical characterization dairy wastewater of organized sector [1,8,17,18,19,20]. Whereas, only limited information is available about the bacterial diversity of unorganized sector dairy effluent and dairy sludge. The aims of this study was to isolate most frequently occurring and optimally performing microbial isolates from the unorganized dairy sector wastewater and the activated sludge. Total 59 bacterial isolates were isolated (30 isolates from the dairy wastewater and 29 from dairy sludge samples of an unorganized sector) and were designated as 1,2.....59.

3.2. Characterization of the bacterial isolates

Colony and morphological characteristics of the isolates were studied.

3.2.1. Colony characteristics

Observations about colony characteristics of the isolates were presented in Table 1. The colonies of the isolates were circular to irregular. The color of colonies was generally pale white. The shape varied from regular to irregular with entire to undulate margins. The bacterial isolates were stained to observe their morphological characters and the observations are presented in Table 1. Out of the thirty isolates isolated from dairy wastewater fourteen strains were Gram negative and these were rods, cocci and coccobacilli. The arrangement of most of the cells were in pairs and in chains. Sixteen strains were found to be Gram positive with coccobacilli morphological character. These cells were mostly present in pairs.

Among 29 isolates, obtained from dairy sludge samples, Gram negative character were exhibited by six isolates. They were mainly rods. Gram positive character was exhibited by twenty three isolates. These were rods and coccobacilli. The cells were present singly, pairs, in chains and in clusters. The microscopic characteristics of the ten most efficient bacterial isolates are shown in Fig. 1.

3.2.2. pH

pH of untreated dairy wastewater was mainly acidic in nature. It varied between 3.0 and 6.5. The pH of dairy effluent depends on the nature of end product. The effluent exhibiting the acidic conditions could have a serious impacts on soil and microflora [1]. Post treatment with microbial isolates pH of dairy water was observed to be mildly acidic to alkaline.

3.3. Bioaugmenting dairy waste water with bacterial isolates

Biological methods employing indigenous microflora are generally used for the treatment of dairy wastewater, but with time biodegradative ability decreases as mortality rate increases due to huge variations in the characteristics of the effluent. The bioaugmentation strategy can be used to treat the wastewater. It enhances the treatment process by introducing specific selected strains of micro-organisms or microbial consortia to achieve desirable results [21]. 59 bacterial isolates were examined for their ability to reduce the Biological Oxygen Demand, Total Suspended Solids and Oil and grease content. Results in Table 2 represent the percentage reduction in BOD_3, TSS and Oil and grease content by the bacterial isolates.

3.3.1. Biological Oxygen Demand (BOD)

BOD_3 is considered to be important pollution parameter to examine the water quality. The presence of fats, nutrients, lactose, detergents, sanitizing agents, casein and inorganic salts in dairy wastewater results in its high BOD_3 values, thus making water unfit for drinking and irrigation purposes [22,23]. Therefore, BOD_3 values of dairy wastewater should be estimated before its discharge to the environment. Only 12 isolates were efficient in reducing BOD_3 content above 80%. Maximum percentage BOD_3 reduction was shown by isolate 25 i.e 89.8% (90 mg/l) where as reduction in BOD_3 of control was only 11.1% (900 mg/l). Isolate 5, 9, 32 were also efficient in reducing BOD_3 content by 87.3%, 87% and 87.8% respectively. Bioremediation of industrial wastewater using microbial isolates showed high reduction of BOD_3. The reduction in BOD_3 values could be associated with consumption of organic matter by the microbial isolates. Silambarasan et al. [24] reported that 64.67% reduction of BOD_3 was observed by bioaugmenting *Pithophora sp* in dairy wastewater. Significant reduction in BOD_3 values of dairy wastewater by microbial isolates has also been reported by Das and Santra [25], Gaikwad et al. [26]. According to Marwaha et al. [27] *Candida parapsilosis* MTCC 1965 showed the reduction in BOD content of dairy wastewater by 72%.

3.3.2. Total Suspended Solids (TSS)

TSS is also, one of the important pollution parameter used to evaluate the pollution potential for dairy wastewater and also to determine the efficiency of the treatment unit [1]. Suspended solids in the wastewater originate from gelatinous milk and the curd fines or flavorings [28]. Total suspended solids (TSS) of unorganized dairy sector wastewater ranged between 410–900 mg/l. The high level of total suspended solids are due to the organic and inorganic matter present in wastewater. The presence of total suspended solids in wastewater increases turbidity, reduces light penetration in receiving water bodies and can also effect aquatic life by clogging fish gills [29,30]. TSS in control was 450 mg/l. By bioaugmenting dairy water with bacterial isolates TSS was reduced to 100 mg/l by isolate 4, 19, 25, 32. TSS reduction above 80% was shown by eleven isolates. Highest TSS reduction (about 88.6%) was shown by isolate no. 4 and 25. Isolate 26, 32, 35, 37, 45 were also efficient in reducing the TSS, the percentage reduction was recorded to be 82.1%, 81.8%, 82.8%, 80.09% and 81.8% respectively. Priya et al. [31] reported the percentage reduction in TSS content of dairy wastewater upto 83.4% by *Strptomyces indianesis* ACT 7 isolated from dairy wastewater. Shruthi et al. [32] had also reported 75.7% reduction in TSS of rubber processing wastewater by using *Pseudomonas sp*. Gaikwad et al. [26] found similar results for the reduction in TSS content by 79.76% by using microbial consortia of various bacterial species namely *Actinomycetes, Bacillus, Pseudomonas, Staphylococcus and Streptomyces*.

3.3.3. Oil and grease Content

The presence of oil and grease content in wastewater forms films on the water surfaces and thus reduces oxygen transfer rates, creating a high oxygen demands [31]. Oil and grease content of untreated dairy wastewater were in the range of 218–700 mg/l. Bioaugmented dairy wastewater with bacterial isolates reduces oil and grease content up to 30 mg/l. 9 isolates show oil and grease content reduction above 80%. Isolate no. 27 and 45 is more efficient in reducing the oil and grease content by 88.5% and 90% respectively. Vida et al. [20] reported that the bacterial isolate having bacilli like characteristics were found to, be most effective in reducing the fat content of the dairy waste by 55%. According to Porwal et al. [1], the isolate DSI_3 was efficient in reducing oil and grease content of dairy wastewater by 96.9%.

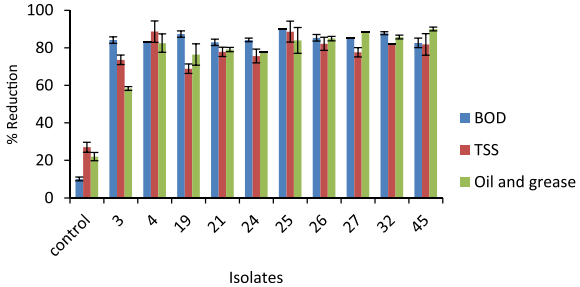

Fig. 2. Graphic representation of bioaugmentative efficacy of the ten most effective isolates.

Isolate no 3, 4, 19, 21, 24, 25, 26, 27, 32 and 45 were selected on the basis of their efficiency to reduce the three major pollution potential parameters i.e BOD, TSS and Oil and grease content. Graphic representation of bioaugmentative efficacy of the selected isolates were shown in Fig. 2.

3.4. Identification of Bacterial isolates

Extraction of DNA from the selected bacterial isolates were done as per the procedure described by Atashpaz et al. [13]. The quality and intactness of the extracted DNA was examined by running on 1% agarose gel which contain 1 μg/ml ethidium bromide. The A_{260}/A_{280} absorbance ratio of the extracted DNA samples were found to be nearer to 1.8 (Table 3). The extracted DNA molecules were used as templates for the amplification of 16 S rRNA genes. The universal primers 27 F and 1492 R were used for the amplification of 16 S rRNA genes at the annealing temperature of 55 °C. The intense single bands were observed on 1% agarose gel stained with ethidium bromide. (Fig. 3).

Table 3
Nucleic acid quantitation of the extracted DNA samples.

Isolate no	OD $_{260}$	OD $_{280}$	OD $_{260/280}$
3	0.048	0.031	1.54
4	0.040	0.033	1.53
19	0.060	0.033	1.71
21	0.038	0.025	1.52
24	0.062	0.038	1.63
25	0.063	0.036	1.75
26	0.040	0.026	1.53
27	0.065	0.041	1.58
32	0.044	0.026	1.69
45	0.060	0.041	1.46

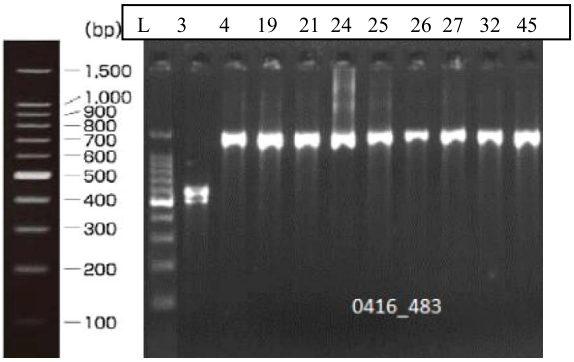

Fig. 3. PCR products of extracted DNA molecules on Agarose gel (1%).

Table 4
16S rRNA sequences analysis of the isolates.

Isolate no.	Closely related species	Phylogenetic representation
3	*Bacillus subtilis* strain SRS 35	Fig. 4
4	*Escherichia coli* strain Sam 130	Fig. 5
19	*Bacillus subtilis* GU056813.1	Fig. 6
21	*Lysinibacillus sphearicus* strain B3PO2	Fig. 7
24	*Bacillus cereus* strain N24	Fig. 8
25	*Bacillus thuringiensis* strain ODPY	Fig. 9
26	*Bacillus cereus* strain TERI – Chilika–09	Fig. 10
27	*Bacillus cereus* strain W22	Fig. 11
32	*Brevibacillus* sp. N3	Fig. 12
45	*Brevibacillus brevis* KF152965.1	Fig. 13

3.5. Sequencing results

For bacterial classification generally sequencing of 16 S rRNA gene was used as an important identification tool [33]. The reasons include its presence in almost all bacteria; its function has not changed over time and the 16 S rRNA gene (1,500 bp) is large enough to provide a genus and species identification for isolates [34]. The DNA samples of all the bacterial isolates were run on the agarose gel and the bands were visualized when observed under the Gel doc. The sequencing of the 16 S rRNA gene was done. Based on the 16 S rRNA sequences, phylogenetic dendrograms were constructed to know the genetic relationship between the bacterial isolates. The identification of the isolates were represented in the Table 4 and their phylogenetics dendrograms were shown in the (Figs. 4-13).

4. Conclusion

Environmental laws have become stringent, discharge of the effluent within the permissible limit is mandatory in the developed and developing countries. The dairy industry is practiced at large scale as well as at a small scale level all over the world. The dairy wastewater treatment methods practiced by large-scale holders comprise physico-chemical methods requiring a large surface area for the set up of effluent treatment plant and technically trained personnel with efficient management skills. It adds to the cost of the treatment process, making it cost intensive and cannot be employed in small scale industries. Therefore, biological treatment methods are considered to be ideal and economical. Dairy industry wastewater is an enriched media for the microbial growth [35]. They do not contain hazardous materials and being organically rich they are an ideal candidate for biological treatment which is carried out by indigenous microflora. Indigenous micro flora increases the efficiency of the biological treatment system as they were adapted to the wastewater physical-chemical conditions. The present investigation was carried out to isolate the most frequently occurring and optimally performing microorganisms from dairy wastewater and sludge samples. 10 bacterial isolates (Isolate no. 3, 4, 19, 21, 24, 25, 27, 32 and 45) were selected on the basis of their bioremediation efficiency to reduce BOD, TSS and Oil and grease content. 16 S rRNA sequencing results concludes that, all of the selected strains belong to *Bacillus sp* except isolate no. 4. The isolate no 4 was identified as *Escherichia coli* strain CXIB. The other strains identified as *Bacillus subtilis* strain SRS 35 (Isolate no. 3), *Escherichia coli* strain Sam130 (Isolate no. 4), *Bacillus subtilis* strain GUO6813.1 (Isolate no. 19), *Lysinibacillus sphericus* strain B3PO2 (Isolate no. 21), *Bacillus cereus* strain N24-2 (Isolate no 24), *Bacillus thuringiensis* strain ODPY (Isolate no 25), *Bacillus cereus strain* TERI– Chilika-09 (26), *Bacillus cereus* strain w22 (27), *Brevibacillus sp*. N3 (Isolate no 32) and *Brevibacillus parabrevis* (Isolate no 45). *Bacillus thuringiensis* strain ODPY (Isolate 25) was found to be more efficient in reducing the BOD_3 content by 89.8%. *Bacillus subtilis* strain GUO6813.1, *Bacillus cereus strain* TERI –Chilika-09, *Bacillus cereus* strain w22, *Brevibacillus sp*. N3 were also efficient in reducing BOD_3 content by 87.3%, 85.3%, 85.4% and 87.8% respectively. TSS content reduction above 80% were shown by isolates no. 4, 25, 26, 32 and 45. Maximum reduction in TSS content were recorded by *Escherichia coli* strain Sam130; *Bacillus thuringiensis* strain ODPY (88.6%). About 88.5% and 90% of oil and grease content reduction were shown by isolate 27 and 45 respectively. As per the standards set by Central Pollution Control Board, New Delhi for the discharge of dairy wastewater to the surface water, BOD of the treated wastewater should be not more than 100 mg/l (if applying on land), TSS content should be 150 mg/l and Oil and grease content should be 10 mg/l. Bioaugmentation of dairy wastewater by these selected isolates reduced the BOD_3, TSS and Oil and grease content upto 50 mg/l, 100 mg/l and 40 mg/l, respectively. So, the treated dairy

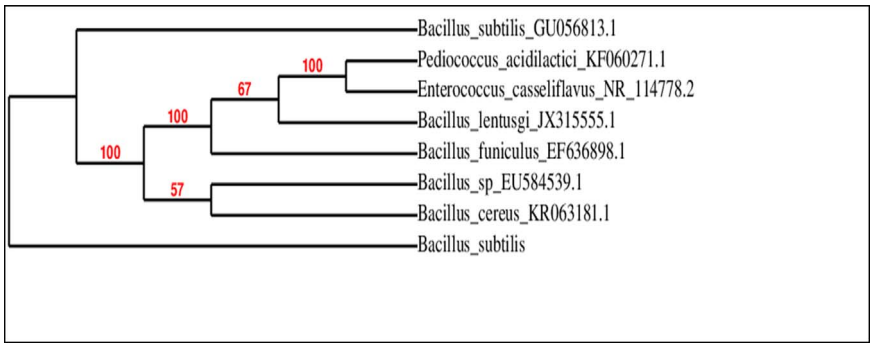

Fig. 4. Phylogenetic tree showing close homologs to *Bacillus subtilis* strain.

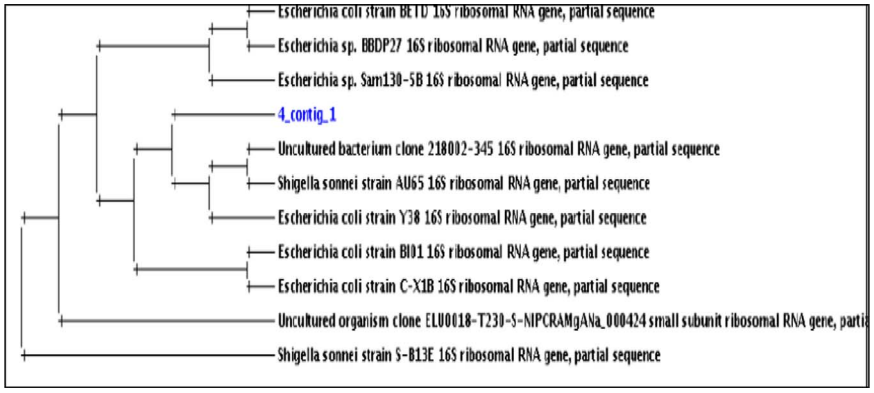

Fig. 5. Phylogenetic tree representing close homologs to strain *Escherichia coli* strain Sam 130.

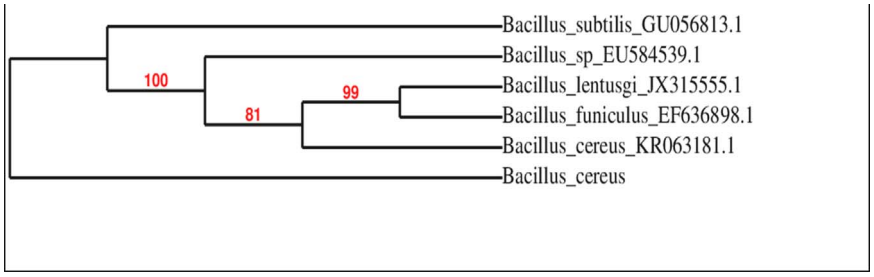

Fig. 6. Phylogenetic tree representing close homologs to strain *Bacillus subtilis* strain GUO6813.1.

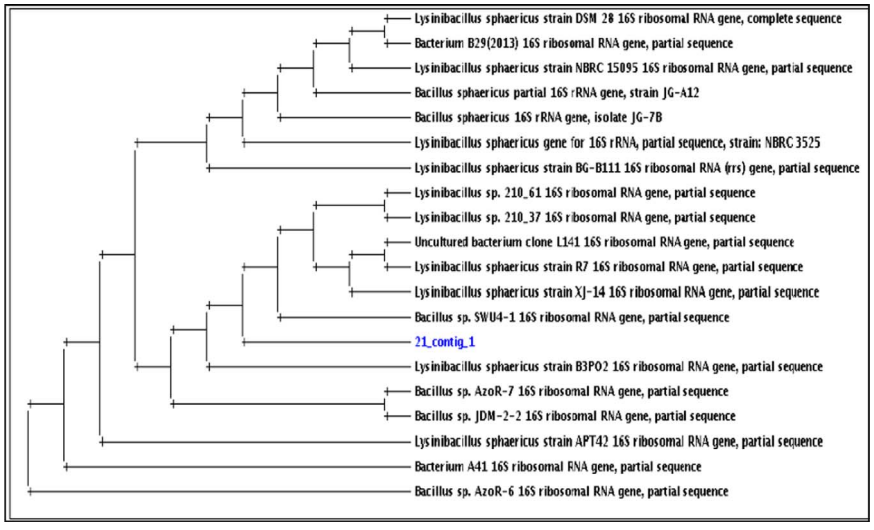

Fig. 7. Phylogenetic relationship between *Lysinibacillus sphearicus* strain B3P02 and other strains.

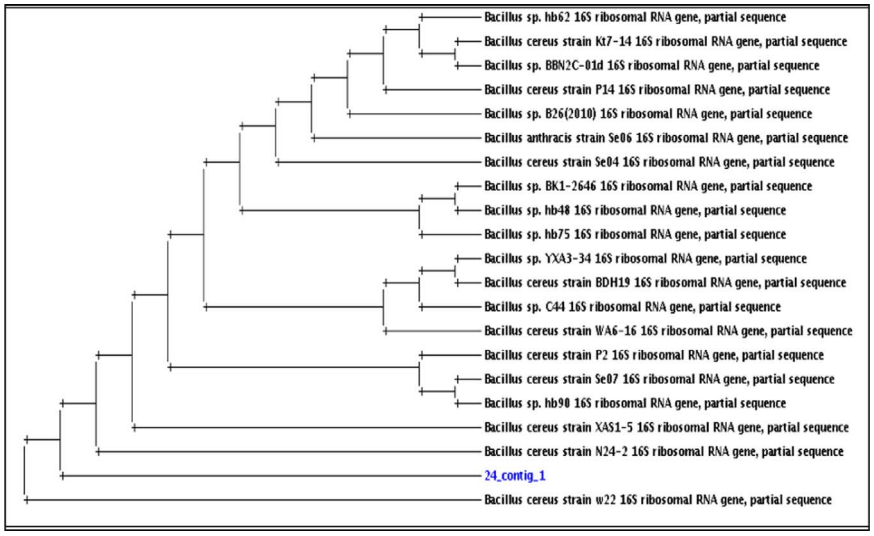

Fig. 8. Phylogenetic tree showing close homologs to *Bacillus cereus* strain N24.

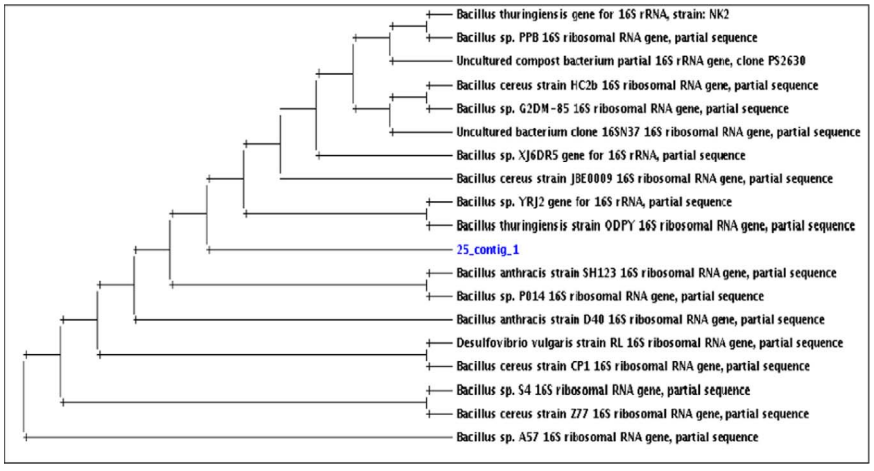

Fig 9. Phylogenetic tree showing close homologs to *Bacillus thuringiensis* strain QDPY.

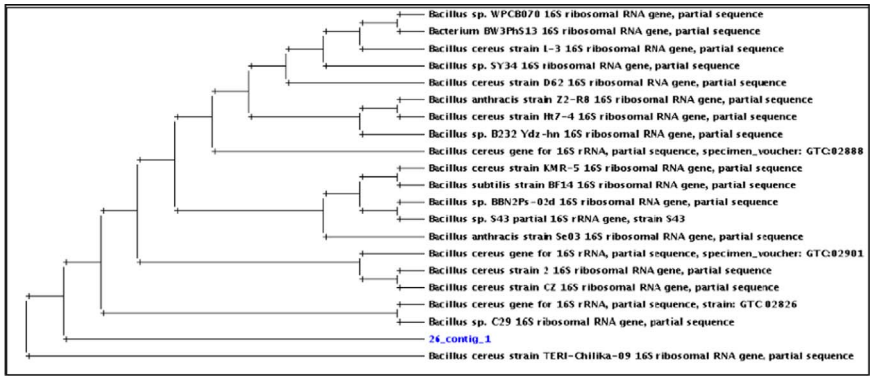

Fig. 10. Phylogenetic tree showing close homologs to *Bacillus cereus* strain TERI –Chilika-09.

effluent is suitable for applying on the land for the irrigation purposes. The identification of these active strains will leads to the development of suitable, eco-friendly method for the treatment of dairy wastewater. These findings are of great concern as overall efficiency of the treatment process will be increased by bio-augmenting dairy wastewater with optimally performing strains isolated from the same source As dairy wastewater exhibits dynamic characteristics, it is always better to use consortium over single culture [36,37]. Currently work is

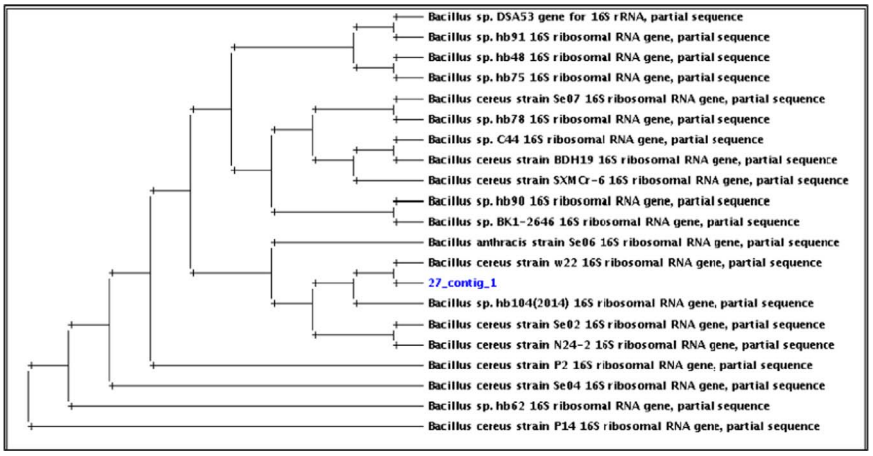

Fig. 11. Phylogenetic tree showing relationship of *Bacillus cereus* strain W22 with its homologs.

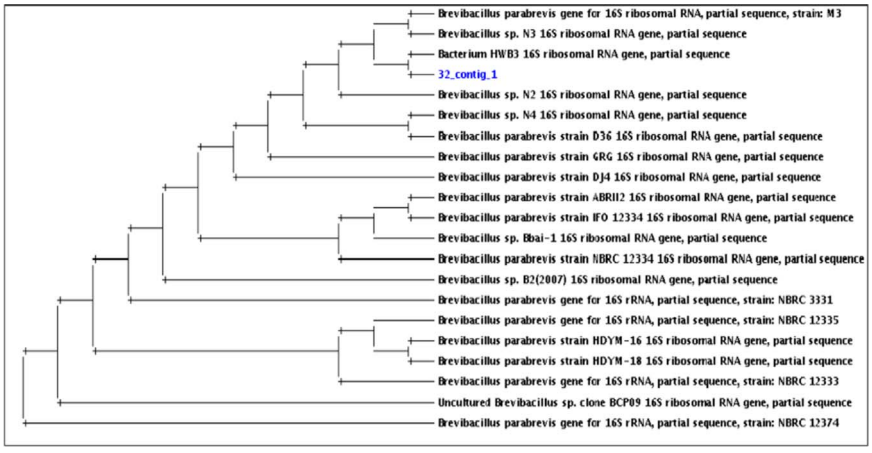

Fig. 12. Phylogenetic tree showing relationship of isolate *Brevibacillus* sp. N3 with other homologs.

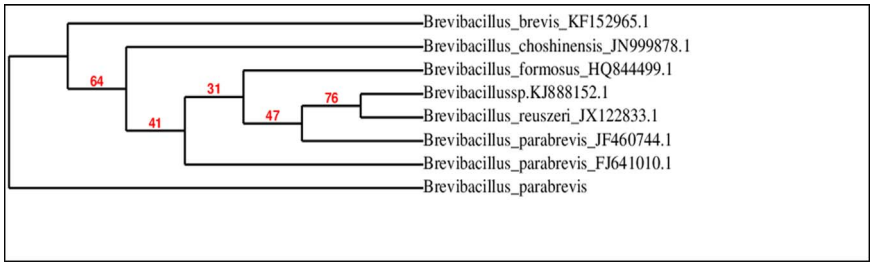

Fig. 13. Phylogenetic tree representing close homologs to *Brevibacillus brevis* KF152965.1.

underway to construct the microbial consortia based on individual efficacy of isolates.

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