JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Sept. 2016. Vol. **10**(3), p. 2199-2208

Characterization and Immobilization of Bacterial Consortium for its Application in Degradation of Dairy Effluent

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https://doi.org/10.22207/JPAM.10.3.59

(Received: 10 April 2016; accepted: 21 May 2016)

In the present investigation, the dairy industry waste water collected from three different industries was analyzed for its physiochemical characteristics. Seven bacterial strains were isolated from the effluent and their bioremediation efficiency was determined individually and in the combination as consortia. The two most efficient bacteria were identified as Bacillus sp. RD_DARAB_02 16S ribosomal RNA gene, partial sequence [Sequence ID: gb|KU597549.1] (ISD1) and Citrobacter freundii strain LCJY-002 16S ribosomal RNA gene, partial sequence [Sequence ID: gb|KC691177.1|] (ISD4) by 16S rDNA sequencing. Laboratory experiments were conducted to evaluate the biodegradation potential of ISD1 and ISD4 bacterial strains as individual isolates, free cell consortia and as immobilized consortium. Comparative analysis revealed that the combination of ISD1 and ISD4 reduced the BOD in significant amount (82.33%) in comparison to individual isolates. Furthermore, the immobilized consortia as beads showed even better reduction in BOD (91.2%) after 3days of incubation than free cell consortium. Other important physiochemical parameters viz. pH, TS, DO, BOD, COD and oil and grease (O&G) were also measured as 7.8, 383, 1.9, 147, 395 and 9.6, respectively after treatment with free cell consortia where as immobilized bacterial consortium brought the pH, TS, DO, BOD, COD and oil and grease to the levels of 7.3, 357, 2.4, 73, 247 and 7.3, respectively.

Keywords: Dairy effluent, biodegradation, immobilized bacteria, consortium.

The food industry is one of them who have highest consumptions of water and so it became the biggest producer of effluent per unit of production, additionally, these industries generate a large volume of sludge as well during the biological treatment[1]. In effluent the organic content is constituted of milk which is used as the raw material for dairy products that reflects as high chemical oxygen demand (COD) effluent with high levels of biochemical oxygen demand (BOD), oils and grease, nitrogen and phosphorus. The cleaning in place (CIP), automatic cleaning system, discard rinse waters with pH varying between 1.0 and 13.0, ultimately making the effluent treatment complicated[1].

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In the dairy effluent, dissolved sugars, proteins and fats with residues of additives are present. The parameters for untreated effluent are BOD, with an average ranging from 0.8 to 2.5 kilograms per metric ton (kg/t) of milk in the untreated effluent; COD, which is normally about 1.5 times the BOD level; total suspended solids, at 100–1,000 milligrams per litre (mg/L); total dissolved solids: phosphorus (10–100 mg/L), and nitrogen (about 6% of the BOD level). Cream, butter, cheese, and whey production are major sources of BOD in dairy wastewater. The waste load equivalents of specific milk constituents are: 1 kg of milk fat is equal to 3 kg of COD; 1 kg of lactose equals to1.13 kg of COD; and 1 kg protein makes around 1.36 kg of COD[2-3].

Dairy waste water with all the impurities decompose rapidly and deplete the dissolved

oxygen (DO) level of the receiving streams which results in anaerobic conditions and release strong foul odor due to nuisance conditions. The casein precipitation from dairy waste decomposes further into a highly odorous black sludge[4]. The dairy waste water contains soluble organics, and suspended solids which degrade to promote release of various gases, bad odor, imparts dark grey or black color, turbidity, and promotes eutrophication[5]. However, the dairy industry produces different products, such as milk, butter, yogurt, ice-cream, and various types of desserts and cheese, the characteristics of these effluents also vary widely both in quantity as well as in quality, depending on the type of system and the methods of operation used for the production. Dairy effluent consists of organics soluble in water, suspended solids and trace organics. These components contribute in high BOD and COD. The main characteristics of dairy waste water are temperature, color, pH, DO, BOD, COD, dissolved solids, suspended solids, chlorides, sulphates and oil & grease. The dairy effluent contains abundance of milk constituents like casein, inorganic salts, besides detergents and disinfectants used for washing. The dairy effluent has high sodium content due to the use of caustic soda for cleaning[6].

The common techniques from various techniques used for dairy industry effluent treatment includes, oil and grease traps, oil water separators to separate the floatable solids, flow equalization, and some clarifiers to remove suspended solids. The biological method of effluent treatment is said to be aerobic and anaerobic treatment. Sometimes anaerobic treatment followed by aerobic treatment is employed for the reduction of soluble organic matter and biological nutrient removal is also employed for the reduction of nitrogen and phosphorus. Aerobic biological treatment involves use of microbes for degradation and oxidation of waste in the presence of oxygen. Conventional treatment of dairy wastewater by aerobic processes includes activated sludge, trickling filters, aerated lagoons, or a combination of these[7]. A comparison between aerobic and anaerobic treatment are summarized in Table 1.

Bioremediation is found to be the most efficient method for degradation of hazardous pollutants because it is natural, economic and eco-friendly. The immobilized cells of microbes may be useful to treat the waste to convert the toxicant into nutrient, biomass and CO_2 via biodegradation[7]. The microbial cells which are immobilized within a suitable matrix provide a physical support for bacterial cells, ideal size, mechanical strength, rigidity and porous characteristics to the biological material which is immobilized[8]. The biggest advantage of complete cell immobilization is that the enzymes remain active and stable for longer period of time due to their natural environment they are in[9-10].

The aim of the present study is to identify efficient bacterial isolates from waste water that could degrade dairy effluent rapidly either individually or in combination. In addition, a comparative study has been done to evaluate the bioremediation efficiency of bacterial isolates. An experimental observation is carried out using immobilized bacterial consortium, to find the potential method of bioremediation of dairy effluent that would be simple, economic and easy to use.

MATERIALS AND METHOD

Sample collection

Fresh dairy effluent sample was obtained from three different dairy industry effluent treatment plants located in Delhi-NCR (28°402 N 77°132 E). The samples were collected in a plastic container. The containers used for sample collection were pre-treated by washing with alcohol and later rinsed with distilled water, dried in an oven for 1h at 30°C and allowed to cool to room temperature. At the collection point, container was rinsed with the sample thrice and then filled, corked tightly and taken to the laboratory for further analysis. The sample was stored at a temperature below 4°C to avoid any physicochemical changes in the effluent.

Physicochemical analysis of effluent

The dairy effluent from all three industries were taken and analysis was performed by following APHA, 2005[11].The Physical parameter- Temperature, pH, TS, TDS and SS and Chemical parameter- Chloride, Dissolved Oxygen (DO), Chemical oxygen Demand (COD), Biological Oxygen Demand (BOD), Sulphate, Chloride, Oil and Grease were studied. The pH of samples was

determined at the site itself by using portable hand held pH meter and temperature in Degree Celsius on scientific thermometer.

Isolation and characterization of bacteria

For the isolation of bacteria from effluent sample, tenfold serial dilution was done with the effluents. After dilution the diluted samples were spread over nutrient agar plates and kept overnight for incubation at 37°C. Further, the single colonies of bacteria were identified on spread plate and streaked on nutrient agar plates to obtain the pure culture of isolated bacteria. For gram staining, Smear was prepared and heat fixed. The smear was treated with crystal violet for 1min and was then removed by Gram's iodine to react for 1min. The smear was then washed with water and treated with 95% ethanol for 15 sec. Smear was washed with water and again treated with safranin for 30 sec. The smear was then washed, dried and examined under microscope.

Maintenance of culture

The bacterial isolates were maintained on nutrient agar slants at 4°C and glycerol stocks of pure culture were also prepared.

Biochemical Characterization

Biochemical activities of the isolated bacteria were analyzed by different biochemical tests such as amylase production, cellulase production, degradation of pectin, hydrolysis of gelatin, casein hydrolysis, urease test, hydrogen sulfide production, carbohydrate catabolism, IMVic tests, citrate utilization, catalaste test and oxidase test. Estimation of protein and reducing sugars were done by the methods described by Lowry *et al.,*[12] and Miller *et al.,*[13]

Mutual Antagonistic effect

The isolated strains were tested for their mutual antagonistic activities in order not to influence each other. Different strains were cross-streaked but did not intersect on a Luria-Bertani (10g peptone, 5g yeast extract, 10g NaCl and 20g Agar in 1L distilled water) plate. The antagonistic phenomenon was observed after incubation at 30°C for 48 hours.

Identification of bacteria

Isolated bacteria were identified in the present study by routine morphological, microbiological and biochemical methods followed by 16S rRNA Gene Sequencing.

Development of Bacterial Consortium

Bacterial consortium was developed by inoculating more than one strain (added in equal ratios; 100 µl in 100 ml) in Luria-Bertani Broth (10g peptone, 5g yeast extract and 10g NaCl in 1L distilled water). Effect of pH and temperature influencing bacterial growth were also observed.

Immobilization of bacterial consortium in beads

The bacterial consortium (individual strains and consortium raiment ISD1 & ISD4 strain) was immobilized as beads according to the procedure of Leung *et al*[14] 100 ml of 2% sodium alginate solution is prepared in sterile distilled water by heating it to 60°C and mixing it thoroughly on a magnetic stirrer. Later sodium alginate solution is cooled to room temperature and 10% (10ml culture in 100ml sodium alginate solution) of the cell culture is added. The contents were mixed well by vigorous shaking to get a homogenized mixture. The sodium alginate containing cell culture suspension was extruded drop wise through a syringe and allowed to fall in a separate beaker containing 100ml of 0.1M calcium chloride solution. The beads of sodium alginate gel formed are left in the beaker overnight for hardening. Then beads were washed and stored in distilled water at 28 ± 2°C.

Biodegradation study of dairy industry effluent

10% of the inoculum comprised of immobilized beads of consortium made up of ISD1 and ISD4 were added to the dairy effluent in 500ml conical flask and kept on a rotator shaker. The degradation studies of the treated effluent were evaluated at intervals of 12 hrs.

RESULTS AND DISCUSSION

This study was undertaken to detection of the important pollution parameters in dairy industry waste water. The mean values of physicochemical parameters of collected samples from different industries are shown in Table 2. The study revealed that dairy effluent is alkaline in nature. The high BOD and COD values of the analyzed dairy effluent indicate the presence of heavy load of organic matter. The discharge of waste water to the environment without any treatment plays significant risk to public health and environmental pollution. The industrial wastes leads of the water, soil and air when they are

discharged without being subjected to treatment or when they are treated using inappropriate methods.

Mean values of physical characteristics such as pH, TDS and TSS are 9.2, 1580mg/L and 252mg/L, respectively, and mean values of chemical characteristics such as DO, BOD, COD and O&G are 1.2mg/L, 787mg/L, 1528mg/L, and 13mg/L, respectively. Mean value for total solids were found 1832mg/L. However, alkalinity (as $CaCO_3$) and temperature were calculated 600mg/L and 36°C, respectively.

In the present study, total seven (ISD 1, ISD 2, ISD 3, ISD 4, ISD 5, ISD 6, ISD 7) bacterial isolates were obtained as pure culture. After Gram method and microscopic observation characteristic specification of bacteria isolated from dairy effluent was carried out (Table 3). The isolates ISD 1, ISD 2, ISD 3, ISD 4, ISD 5 and ISD 7 were found Gram Negative whereas ISD 6 was found Gram Positive. The colony appearance on agar plate and shape of the isolates were microscopically observed. ISD 1 was thick, white, round and opaque on agar plate while its shape was found cocci, ISD 2 was thin,

Table 1. Comparison of aerobic and anaerobic treatment of dairy industry wastewaters

Factors	Aerobic process	Anaerobic process
Reactors	Aerated lagoons, oxidation ditches, Stabilization ponds, Trickling filters and Biological discs	UASB, Anaerobic filter, Upflow packed bed reactor, CSTR, Down flow fixed-film reactor, Buoyant Filter Bioreactor,
Reactor size	Aerated lagoons, oxidation ditches, Stabilization ponds, Trickling filters and Biological discs requires larger land area but SBR needs comparatively lower area.	Smaller reactor size is required
Effluent Quality	Excellent effluent quality in terms of COD, BOD and nutrient removal is achieved.	Effluent quality in terms of COD is fair but further treatment is required. Nutrient removal is very poor.
Energy	High energy is required.	These processes produce energy in the form of methane
Biomass yield	In comparison to anaerobic process, 6-8 times greater biomass is produced	Lower biomass is produced.
Loading rate	Maximum 9000 g COD/m3 d is reported in literature	Very high Loading rate of 31 kg COD/m3 d has been reported. This is the reason for smaller reactor volume and lesser area.
Oil and grease removal	These do not cause serious problems in aerobic processes	Fats in wastewater shows the inhibitory action during anaerobic treatment of dairy wastewaters
Shock loading	Excellent performance in this regard.	Anaerobic processes showed not good responses to this shock loading.

Source: Mrs. Bharati S. Shete, Dr. N. P. Shinkar (2013)

Table 2. Characteristics of dairy industry effluent before treatment

Sample	pH	Temp (ºC)	Alkalinity (mg/L) as CaCO3	TDS (mg/L)	TSS (mg/L)	TS (mg/L)	DO (mg/L)	BOD (mg/L)	COD (mg/L)	Oil and Grease (mg/L)
Site 1	9.6	38	614	1680	239	1919	1.2	810	1595	12
Site 2	8.6	36	556	1225	195	1420	1.5	720	1320	8
Site 3	9.4	34	630	1835	321	2156	0.9	830	1670	19
Mean	9.2	36	600	1580	252	1832	1.2	787	1528	13

white and clear with limited growth and was found rod shaped, ISD 3 was thick, white, viscous and opaque and its shape was found cocci, ISD 4 was off-white, thick, granular and translucent on plate while it was rod shaped, ISD 5 on agar plate was thin, white, granular and translucent while its shape was observed cocci, ISD 6 was thin, golden grey, shiny and transparent and it was observed as cocci and ISD 7 was off-white, thick, round and transparent while microscopic observation showed its rod shaped.

All the seven isolated bacterial strains were also characterized by biochemical tests (Table 4 & Figure 1). In the amylase production test; ISD1, ISD3, ISD4, ISD5 and ISD7 were found positive where as ISD2 and ISD6 were found negative. In carbohydrate catabolism all seven (ISD1, ISD2, ISD3, ISD4, ISD5, ISD6, ISD7) isolated strains were found positive. In casein hydrolysis test; ISD1, ISD4 and ISD7 were found positive while ISD2, ISD3, ISD5 and ISD6 were found negative. Catalase test showed all seven isolated strains positive. In citrate utilization; ISD4 and ISD6 was found positive while ISD1, ISD2, ISD3, ISD5 and ISD7 were observed negative. Gelatin hydrolysis was found positive for ISD1, ISD2, ISD3 and ISD4 while it was found negative for ISD5, ISD6 and ISD7. In hydrogen sulphide production; ISD1, ISD4 and ISD7 was positive while ISD2, ISD3, ISD5 and ISD6 were found negative. Indole test was positive for ISD1, ISD2, ISD3, ISD4 and ISD7 while negative results were found for ISD5 and ISD6. ISD2, ISD4

Table 3. Characteristics of bacterial strains isolated from dairy waste water sample

S.No	Strain	Colony appearance on plate	Shape	Gram Test
1	ISD 1	Thick, white, round, opaque	Cocci	Negative
2	ISD 2	Thin, white, limited growth, clear	Rod	Negative
3	ISD 3	Thick, white, viscous, opaque	Cocci	Negative
4	ISD 4	Thick, off-white, granular, translucent	Rod	Negative
5	ISD 5	Thin, white, granular, translucent	Cocci	Negative
6	ISD 6	Thin, golden-grey, shiny, transparent	Cocci	Positive
7	ISD 7	Thick, off-white, round, transparent	Rod	Negative

Table 4. Biochemical Characterization of bacterial isolates from dairy industry effluent

S.No	Strain	Amy	Car	Cas	Cat	Cit	Gel	H_2S	Indole	MR	Oxi	Ure	VP
1	ISD 1	+++	+++	++	+	-	++	+	+	-	+	-	-
2	ISD 2	-	+	-	+	-	+	-	+	+	+	-	-
3	ISD 3	+	+	-	+	-	+	-	+	-	+	-	-
4	ISD 4	+++	+++	+++	+	+	+++	+	+	+	+	-	-
5	ISD 5	+	+	-	+	-	-	-	-	+	+	-	-
6	ISD 6	-	+	-	+	+	-	-	-	-	+	-	-
7	ISD 7	+	+	+	+	-	-	+	+	-	+	+	-

(+++ = Maximum activity,++ = Moderate activity; Amy-Amylase prduction, Car-Carbohydrate catabolism, Cas-Casein hydrolysis, Cat-Catalse test, Cit-Citrate utilization, Gel-Gelatin hydrolysis, H_2S-Hydrogen sulphide production, MR-Methyl Red test, Oxi-Oxidase test, Ure-Urease test, VP-Voges-Proskauer test)

Table 5. Dairy effluent treatment with free cell consortium and immobilized consortium after 72 hours

Sample	pH	TS (mg/L)	DO (mg/L)	BOD (mg/L)	COD (mg/L)	Oil and Grease (mg/L)
Untreated Effluent Sample	9.4	2156	0.9	830	1670	19
Treated with Free Cell Consortium	7.8	383	1.9	147	395	9.6
Treated with Immobilized consortium	7.3	357	2.4	73	247	7.3

and ISD5 were positive for MR test and ISD1, ISD3, ISD6 and ISD7 were negative. All the seven isolates were positive for oxidase test. In urease test only strain ISD7 was found positive, all other strains ISD1, ISD2, ISD3, ISD4, ISD5 and ISD6 were found negative. For VP test all the seven isolated strains showed no result and all of them were found negative.

Figure 2 shows the result of antagonistic phenomenon. The isolated strains were tested to test their tendency to influence each other by streaking them on Luria-Bertani agar plates. No

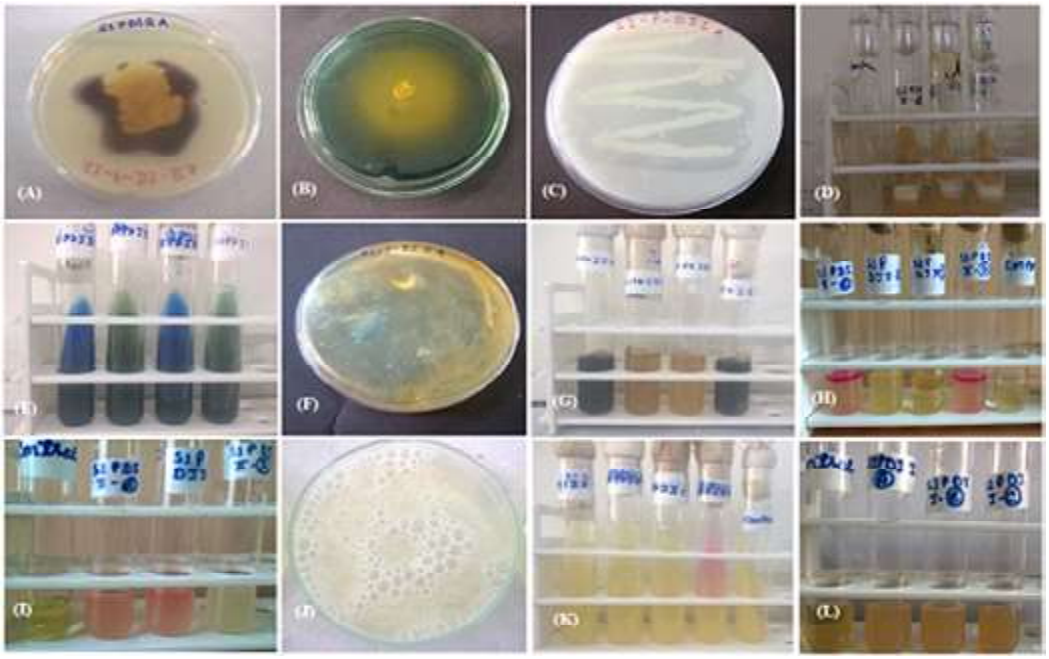

Fig. 1. Result of biochemical characterization
(A) Amylase production test (B) Carbohydrate catabolism test (C) Casein hydrolysis test (D) Catalase test (E) Citrate utilization test (F) Gelatin hydrolysis test (G) H_2S production test (H) Indole test (I) MR test (J) Oxidase test (K) Urease test (L) VP test

Fig. 2. LB Agar plate showing no antagonistic effect

antagonistic phenomenon was observed after incubation at 30°C for 48 hours.

16S rRNA gene cloning and phylogenetic analysis

Two bacterial isolates ISD1 and ISD4 from the seven isolates were selected for the identification on the basis of the result of the biochemical assay. These strains were chosen for further studies because of their maximum degradation and moderate degradation activity towards different substrate observed during the biochemical assay.

For ISD 1, 1227bp 16S rRNA gene was sequenced. ISD1 was found to be most similar to *Bacillus sp.* RD_DARAB_02 16S ribosomal RNA gene, partial sequence [Sequence ID: gb|KU597549.1]. The next closest homologue was found to be *Bacillus thuringiensis* strain VKK-

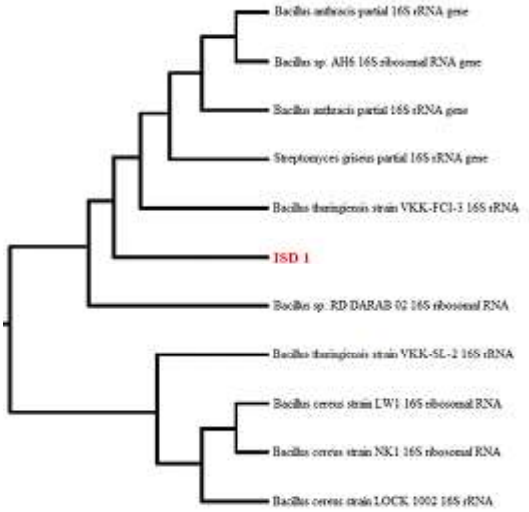

Fig. 3. Phylogram obtained after 16S rRNA sequencing for isolated strain ISD1

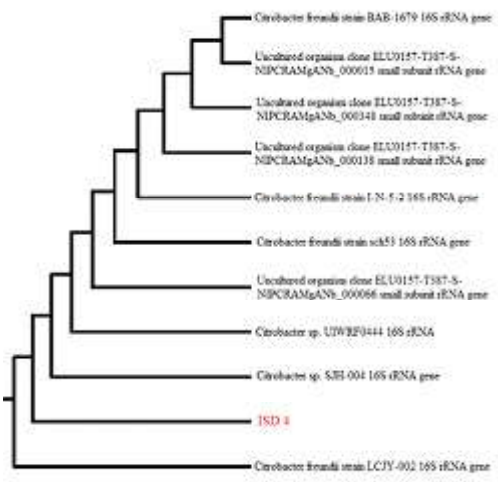

Fig. 4. Phylogram obtained after 16S rRNA sequencing for isolated strain ISD4

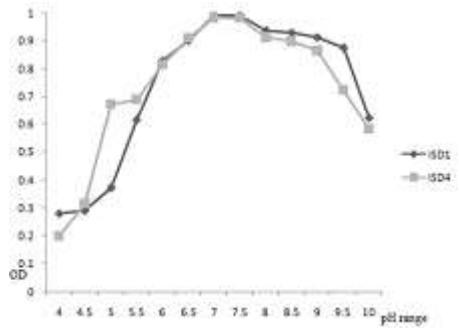

Fig. 5. Bacterial growth at different pH range (OD at 600nm, incubation time 24hours)

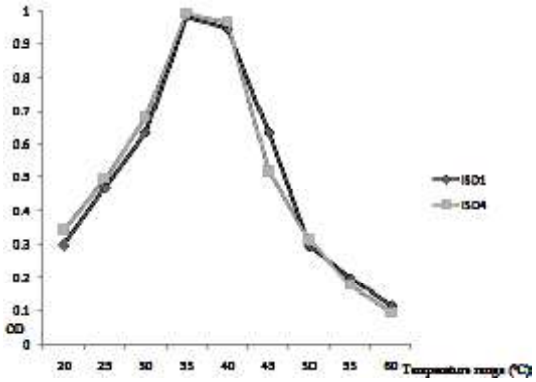

Fig. 6. Bacterial growth at different temperature range (OD at 600nm, incubation time 24hours)

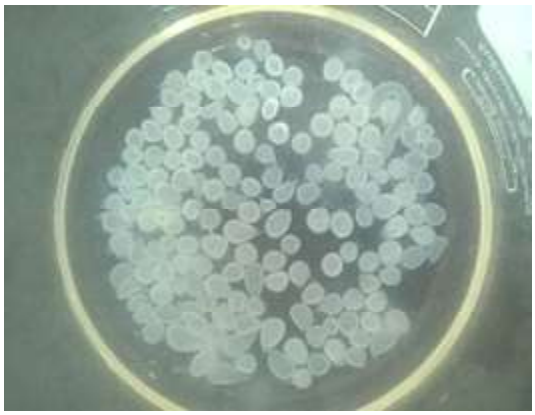

Fig. 7. Immobilized consortium as beads

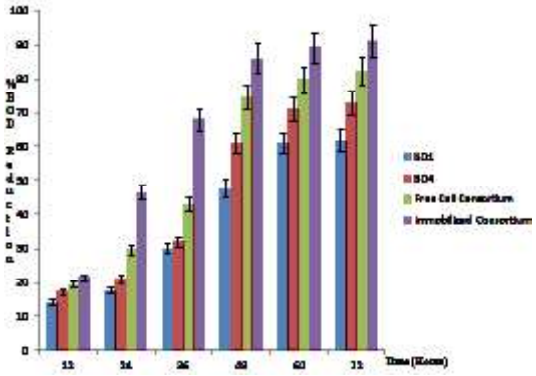

Fig. 8. % BOD Reduction of dairy effluent with time

FCI-3 16S ribosomal RNA gene, partial sequence [Sequence ID: gb|KT714037.1].

For ISD4, 1376bp 16S rRNA gene was sequenced. ISD4 was found to be most similar to *Citrobacter freundii strain LCJY-002 16S* ribosomal RNA gene, partial sequence [Sequence ID: gb|KC691177.1|]. The next closest homologue was found to be *Citrobacter sp.* SJH-004 *16S* ribosomal RNA gene, partial sequence [Sequence ID: gb|KC335138.1|].

The identified strains ISD1&ISD4 were further studied for their growth parameters at different conditions. Under the significant variation of pH and temperature, the growth of these two strains was studied after 24hours of incubation. Both the strains ISD1 and ISD4 were acting same and showing similar results at different range of pH and temperature. Optimum pH range for growth was found 6.5 to 7.5 for both the strain (Figure 5). Optimum temperature range for growth of both the strain was found between 35ºC to 40ºC (Figure 6).

Immobilized Bacterial Consortium as shown in figure 7 was prepared by inoculating equal amount (100µl in 100 ml) of each single strain culture (ISD1 & ISD4) in the same Luria Bertani Broth and further immobilized in sodium alginate beads (Figure 7).

Initially, comparative biodegradation potentials of ISD1 & ISD4 strains in different forms were studied in terms of percent decrease in highly concentrated BOD levels of dairy effluent. The different forms were ISD1 & ISD4 strains as individual, mixed ISD1 & ISD4 strains as consortium and ISD1 & ISD4 strains consortium as immobilized beads and their BOD degradation efficiency were shown in Figure 8.

BOD is the amount of dissolved oxygen needed by aerobic organisms present in a water body to breakdown the organic materials at certain temperature over a specific time period. The decrease in BOD was rapid when the consortium was used than the individual organisms. The immobilized consortium showed efficient reduction in comparison to the free cell consortium. There was a considerable decrease in BOD on the third day itself, when immobilized consortium was used for the treatment. The BOD reduced to 91.2% at the end of the treatment.

The untreated dairy effluent was analyzed for pH, total solids, dissolved oxygen, biological oxygen demand, chemical oxygen demand and oil & grease and it was 9.4, 2156, 0.9, 830, 1670 and 19, respectively. The untreated effluent was then treated with free cell consortium and immobilized consortium. After 72 hrs of incubation, the treated effluent was analyzed to check the changes in the physico-chemical parameters. The pH, TS, DO, BOD, COD and oil and grease were measured as 7.8, 383, 1.9, 147, 395 and 9.6, respectively for the effluent treated with free cell consortium where as the pH was 7.3, TS was 357, DO was 2.4, BOD was 73, COD 247 and oil and grease was measured as 7.3 for the effluent treated with immobilized consortium (Table 5). The estimation of proteins and reducing sugar present in the dairy effluent was done by methods described by Lowry *et al.,* and Miller *et al*.,. The total reduction in the amount of protein and reducing sugar were estimated after treating the effluent upto 72hours by individual

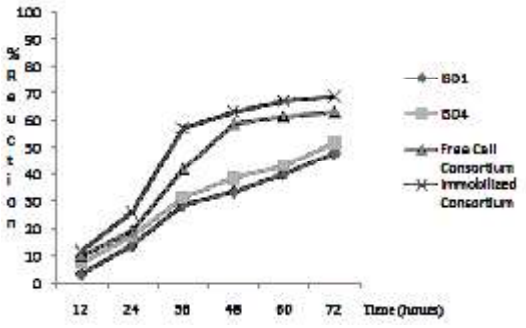

Fig. 9. %BOD Reduction of dairy effluent with time

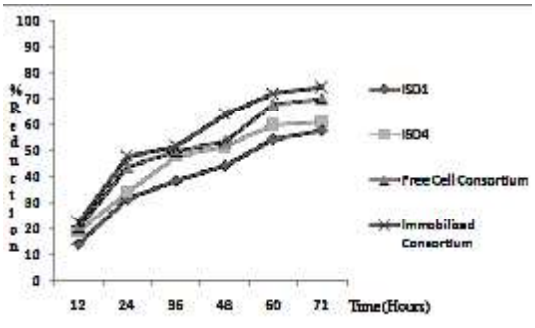

Fig. 10. Percent reduction of protein in dairy effluent after treatment

bacteria, free cell consortium and immobilized consortium. The maximum percent reduction in protein and reducing sugars after treatment was calculated as 67.3% for proteins (Figure 9) and 74.6% for reducing sugars (Figure 10), when treated with immobilized consortium.

The results obtained in present study are consistent to those reported by Dharmsthiti and Kuhasuntisook[15]. Prasad and Manjunath[16] have reported the use of *B.subtilis, B.licheniformis, B.amyloliquefaciens, S.marsescens, P.aeruginosaand S.aureusin* for waste water treatment. The average BOD value was reduced from 3200 mg/L to less than 40 mg/ L and lipid content was reduced from 25,000 mg/L to 80 mg/L, respectively within 12 days of incubation. *P.aeruginosa* showed reduction of BOD value from the day one in palm oil refinery effluent, dairy effluent and domestic water effluent.

CONCLUSION

In the present study, the dairy industry effluents were characterized and resistant bacteria were isolated from the effluent. A broad spectrum of behavior in growth of various bacterial isolates was observed. The biodegradation ability of isolated bacterial strains was studied and consortium was made by selecting the two most efficient strains (ISD1-*Bacillus sp* RD_DARAB_02 & ISD4-*Citrobacter freundii LCJY-002*) on the basis of result obtained in their biodegradation ability. The selected strains were studied for their growth on different pH and temperature to find out the optimum pH and temperature for their growth. A comparative study was done in between the immobilized consortium and free cells bacterial consortium by inoculating them into dairy effluent. Immobilized bacterial consortium has shown maximum reduction in physicochemical parameters. Present study revealed that immobilized consortium was better and efficient in removal of organic pollutants in comparison to the free cell consortium or individual bacterial strains. Results concluded that immobilized consortium of selected isolated strain (ISD1 & ISD4) is competent to treat dairy wastewater holistically.

ACKNOWLEDGEMENTS

The authors are thankful to the dairy industries located in Delhi-NCR for providing waste water samples as and when required. The authors are also thankful to Amity Institute of Biotechnology, Amity University Uttar Pradesh for providing infrastructure and scientific support to conduct this study.

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