Nature Environment and Pollution Technology
An International Quarterly Scientific Journal

p-ISSN: 0972-6268 e-ISSN: 2395-3454	Vol. 17	No. 2	pp. 517-523	2018

Original Research Paper | Open Access

Molecular Characterization of Native Dairy Wastewater Degrading Microbes Isolated from Dairy Industry Effluent

Anupama Bhardwaj*, Jagtar Singh and Sonia Chaman**†**
*Department of Biotechnology, Panjab University, Sector 14, Chandigarh-160014, India
**Department of Biotechnology, SGGS College, Sector 26, Chandigarh-160019, India
†Corresponding author: Sonia Chaman

Nat. Env. & Poll. Tech.
Website: www.neptjournal.com

Received: 14-06-2017
Accepted: 22-08-2017

Key Words:
Biodegradation
Molecular characterization
16S rRNA
Dairy effluent

ABSTRACT

In this study, fifty two microbes were isolated from dairy effluent (DE) collected from effluent treatment plant of dairy industry. All the microbial isolates were screened for DE degradation efficiency by analysing physico-chemical parameters like COD, BOD, DO, TDS, salinity and conductivity. Four microbial isolates E3, E5, E11 and F5 showed more than 55% reduction in COD, BOD, DO and were selected for molecular characterization by 16S/5.8S rRNA sequencing. These efficient degraders E3, E5, E11 and F5 were identified as *Serratia marcescens*, *Stenotrophomonas maltophilia, Brachybacetrium muris* and *Cunninghamella echinulata* (100-99% pairwise similarity) species. The present study concluded that these strains had potential of reducing the organic load of dairy effluent and appear to be most promising organisms for bioremediation of dairy wastewater.

INTRODUCTION

We live in the world of rapid industrialization and India also embraces this approach of industrialization. Among all industries, dairy industry is one of the fastest growing industries. India's processed dairy segment is growing due to increased demand for more diversified dairy products (Dhall et al. 2012). The per capita availability of milk in India has increased from 176 grams per day in 1990-91 to 322 grams per day by 2014-15. In terms of milk production, India is the world's biggest dairy industry. In last year, India produced close to 137.70 million tonnes of milk, 50% more than the US and three times more than China (APEDA 2017).

Processing of large amount of milk generates wastewater in bulk. The management of dairy wastewater coming out from manufacturing units is a central point for the milk industries. If the dairy wastewater is discharged by industries without treatment, it will adversely affect the environment as it has high BOD (Biochemical Oxygen Demand), COD (Chemical Oxygen Demand), TDS (Total Dissolvable Solids), etc. So, before discharging the dairy wastewater, it must be treated efficiently. There are many techniques/methods to treat dairy wastewater, but we need most efficient and economical method. Work should be focused on employing biological technique with less cost (Porwal et al. 2015).

Using native microbes to treat dairy wastewater makes the process more economical, feasible and efficient. Microbes treat dairy wastewater by reducing organic load from dairy wastewater as it is mainly composed of organic contents. The progress of treatment is determined by analysing various physico-chemical parameters of dairy wastewater (Sumithrabhai et al. 2016). Thus, having knowledge of microflora present in dairy wastewater makes screening of appropriate microbes for biodegradation process easy.

So far, studies on microorganisms associated with dairy wastewater followed the classical microbe culture isolation technique and subsequent identification based on morphological and biochemical characteristics, which largely depends on the cultivation of the microbes, and thus a great variation may likely occur. These methods are also time-consuming and laborious. Plate culturing techniques reveal only a little portion of the true microbial population in a natural ecosystem (Elijah et al. 2014).

The increasing knowledge of microbial gene sequences and development of new culture-independent molecular techniques, are providing novel and effective tools to analyse the diversity of microbial communities. Using 16S/5.8S rRNA sequences, numerous bacterial/fungal genera and species have been reclassified and renamed; classification of uncultivable bacteria/fungi has been made possible, phylogenetic relationships have been determined, and the discovery and classification of novel bacterial/fungal species have been facilitated (Suardana 2014). The present study was designed to isolate efficient degrading microbes from dairy wastewater and molecular methods were em-

ployed, to characterize and identify microbial species, in order to reveal its true microbial diversity.

MATERIALS AND METHODS

Sampling: Composite sampling of dairy wastewater was done from a dairy wastewater treatment plant of Verka located in Mohali (Punjab), India. Dairy wastewater samples were collected according to standard procedures from American Public Health Association (APHA 1989).

Analysis of physico-chemical parameters of dairy wastewater: Collected dairy wastewater samples were analysed to determine their physico-chemical parameters (PCP) like salinity, conductivity, total dissolved solids, BOD, DO and COD. Analysis was done according to APHA (Sonune et al. 2015). Physico-chemical parameters salinity, conductivity and total dissolved solids were determined using "Water Quality Analyser" kit (Electronics India make) with resolution value for salinity 0.01 ppm, conductivity 0.01 mS and TDS 0.01 ppm. The organic load was determined by analysing BOD, DO and COD using titrimetric methods (Sawyer et al. 2003, Prasad & Manjunath 2010).

Isolation of the microbes: Dairy wastewater microorganisms were isolated by Koch method based on the serial dilution and spreading on nutrient agar and potato dextrose agar media. From the individual colonies resulted on the media, bacterial and fungal pure cultures were obtained by inoculation in test tubes with sloping medium surface at 37 ± 1.0°C, 48 hrs and 25 ± 1.0°C, 72 hrs respectively.

Bacterial/fungal isolates were characterized morphologically. Bacterial cells were differentiated on the basis of Gram stain property while fungal cells were characterized on the basis of lactophenol cotton blue stain property (Gaikwad et al. 2014).

Screening of dairy wastewater degrading microbial isolates: Reduction potential of microbial isolates was analysed in terms of PCP for treatment of dairy wastewater. Erlenmeyer flask (250 mL) containing 100 mL of dairy wastewater was inoculated with individual microbial isolate with 2.5% inoculum concentration at pH 6-7 at 32°C/25°C for 24 h/72 h on a rotary shaker at 150 rpm. After 24 h (bacteria) and 72 h (fungi), samples were taken from each flask and physico-chemical analysis was done (Jain et al. 2001).

Percent reduction in COD, BOD, DO, TDS, salinity and electrical conductivity were calculated and the microbial isolates exhibiting more than 55% reduction ability were selected for further characterization at molecular level using 16S rRNA and 5.8S rRNA sequencing.

Identification of efficient degrading strains by 16S and 5.8S rRNA sequence analysis: With the invention of polymerase chain reaction (PCR) and automated DNA sequencing, the genome of some bacteria has been sequenced completely. A comparison of the genomic sequences of bacterial and fungal species showed that the 16S/5.8S ribosomal RNA (rRNA) gene is highly conserved within a species and among species of the same genus and, hence, can be used as the new gold standard for the specification of bacteria and fungi (Woo et al. 2000). To study bacterial/fungal phylogeny and taxonomy, the 16S/5.8S rRNA gene sequences are very useful (Patel 2001).

The sequencing of 16S/5.8S rDNA gene of efficient microbes was done from Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh, India (http://www.mtcc.imtech.res.in). For the molecular characterization of bacterial/fungal isolates, chromosomal DNA of efficient degrader strains were isolated (Rainey et al. 1996). The 16S rDNA gene was amplified with primers 8-27f (5'-AGAGTTTGA TCCTGGCTCAG-3') and 1500r (5'-AGAAAGGAGG TGATCCAGCCA-3'). The 5.8S rRNA gene was amplified with universal primers ITS1 (52-TCCGTAGGTGAACC TGCGG-32)-ITS4 (52-TCCTC CGCTTATTGATATGC-32) (White et al. 1990). The amplified DNA fragment was separated on 1% agarosegel, eluted from the gel and purified using a QIA quick gel extraction kit (Qiagen). The purified PCR product of bacteria was sequenced with four forward and three reverse primers, namely 8-27f, 357f (5'-CTCCTACGGGAGGCAGCAG-3'), 704f (5'-TAGCGGT GAAATGCGTAGA-3'), 1114f (5'-GCAACGAGCGCAACC-3'), 685r (5'-TCTACGCATTT CACCGCTAC-3'), 1110r (5'-GGGTTGCGCTCGTTG-3') and 1500r (*Escherichia coli* numbering system). Sequencing of the amplified product was done by dideoxy chain terminator method using the Big Dye terminator kit followed by capillary electrophoresis on an ABI 310 genetic analyser (Applied Biosystems, USA). DNA sequencing of fungal PCR product was performed using the above mentioned primers in an Applied Biosystem 3130xl analyser. The identification of phylogenetic neighbours and the calculation of pairwise 16S/5.8S rDNA gene sequence similarities were achieved using the EzTaxon server (Kim et al. 2012). The 16S/5.8S rDNA gene sequence of bacterial, fungal isolates and the members of the closely related genera were retrieved from EzTaxon server and aligned using the MEGA software version 5.0 (Tamura et al. 2011).

RESULTS

Isolation and screening of dairy wastewater degrading microbial isolates: Total 52 microbes (bacteria and fungi) were isolated from dairy wastewater sample and screened for their dairy wastewater degrading efficiency. Screening of bacteria was carried out with 2.5% inoculum concentra-

tion, pH 7.0, at 32°C for 24 h while screening of fungi was carried out with 2.5% inoculum concentration, pH 6.0, at 25°C for 72 h. Out of the total, only three bacteria i.e., E3, E5 and E11, and one fungi i.e., F5 were efficient in reducing more than 55% COD, BOD and DO.

It is evident from Table 1 that the COD, BOD and other parameters of effluent after biotreatment were significantly reduced (above 55%) by E3, E5, E11 and F5. Based on degrading capability in terms of COD, BOD, DO reduction percentage, the most efficient isolates E3, E5, E11 and F5 exhibiting the highest potential of reduction were selected for molecular characterization.

Molecular characterization of efficient microbes at species level: Selected efficient dairy wastewater degraders were subjected to molecular identification by 16S/5.8S rRNA sequencing of microbes because 16S/5.8S ribosomal(r) RNA gene analysis using sequencing for bacterial/fungal identification is becoming a confirmatory tool for microbiologists (Tewari et al. 2011).

Gene bank accession number: Full sequences (1408, 1390, 1355, 593 bp) of the 16S/5.8S rRNA gene of four isolates E3, E5, E11 and F5 have been registered in GenBank with accession numbers KX215147, KX215148, KT372349 and KX179502 respectively.

The 16S/5.8S rRNA sequencing suggested that four strains E3, E5, E11 and F5 belong to *Serratia marcescens*, S*tenotrophomonas maltophilia, Brachybacetrium muris* and *Cunninghamella echinulata* (100-99% pairwise similarity) species.

The phylogenetic tree constructed using the neighbour joining algorithm with bootstrap analysis for 1000 replicates is presented in Fig. 1. E3 bacterial isolate had shown maximum similarity of 99% with *Serratia marcescens* SM2611 followed by other *Serratia* species (up to 98%). Hence, E3 was identified as *Serratia marcescens.* Analysis of nucleotides similarity or difference, *Serratia marcescens* E3 strain was studied against some strains of *Serratia* sp., that is, *Serratia marcescens* SM2611 (KT74 1016.1), *Serratia marcescens* HX-3 (KX461911.1), *Serratia marcescens* FZSF2(KU145144.1), *Serratia marcescens* SYJ1-9 (KR262852.1), *Serratia marcescens* MUGA (KJ672369.1) as a bacterial control.

The phylogenetic tree constructed using the neighbour joining algorithm with bootstrap analysis for 1000 replicates is presented in Fig. 2. E5 bacterial isolate had shown maximum similarity of 100% with *Stenotrophomonas maltophilia* 6B2-1 followed by other *Stenotrophomonas* species (up to 99%). Hence, E5 was identified as *Stenotrophomonas maltophilia*. Analysis of nucleotides similarity or difference, *Stenotrophomonas maltophilia* E5 strain was studied against some strains of *Stenotrophomonas* species, that is, *Stenotrophomonas maltophilia* 6B2-1 (AY445079.1), *Stenotrophomonas maltophilia* 13635L (EU741084.1), *Stenotrophomonas maltophilia* LWJ3 (KT932956.1), *Stenotrophomonas maltophilia* YLZZ-2 (EU022689.1), *Stenotrophomonas maltophilia* LMG11087 (X95924.1) as a bacterial control.

Table 1: Percent (%) reduction in PCP of DWW by native microbes.

PCP	Bacteria			Fungi
	E3	E5	E11	F5
COD	66	58	62	72
BOD	64	64	65	62
DO	66	64	66	64
TDS	19	34	52	42
E.C.	60	52	22	29
Salinity	53	52	53	42

*PCP = Physico-chemical parameters; DWW = Dairy wastewater

The phylogenetic tree constructed using the neighbour joining algorithm with bootstrap analysis for 1000 replicates is presented in the Fig. 3. E11 bacterial isolate had shown maximum similarity of 100% with *Brachybacterium muris* strain C3H-21 followed by other, *Brachybacterium* species (up to 96.6%). Hence, E11 was identified as *Brachybacterium muris.* Analysis of nucleotides similarity or difference, *Brachybacterium muris* E11 strains were studied against some strains of *Brachybacterium* species, that is, *Brachybacterium muris* (NR_024571.1), *Brachy bacterium muris* C3H-21 (AJ537574.1), *Brachybacterium* sp. HC15 (EU686684.1), *Brachybacterium muris* SM-T9 (KF876891.1), *Brachybacterium muris* HBUM (KR90 6470.1) as a bacterial control.

The analysis of similarity or nucleotides difference *Cunninghamella echinulata* F5 strain was studied against some strains of *Cunninghamella* sp., that is, *Cunninghamella echinulata* A-207 (JQ683239.1), *Cunninghamella echinulata* YQ24 (GU966504.1), *Cunninghamella echinulata* CBS (JN205894.1), *Cunninghamella echinulata* (KJ183114.1), *Cunninghamella echinulata* (JN205893.1), *Cunninghamella echinulata* (JQ678764.1), *Cunninghamella echinulata* (JX661054.1), *Cunninghamella echinulata* (JQ683240.1). The 5.8S rRNA sequencing suggested that strain F5 belongs to *Cunninghamella echinulata* (100-99% pairwise similarity) species (Fig. 4).

DISCUSSION

The isolated strains on the basis of 16S and 5.8S rDNA

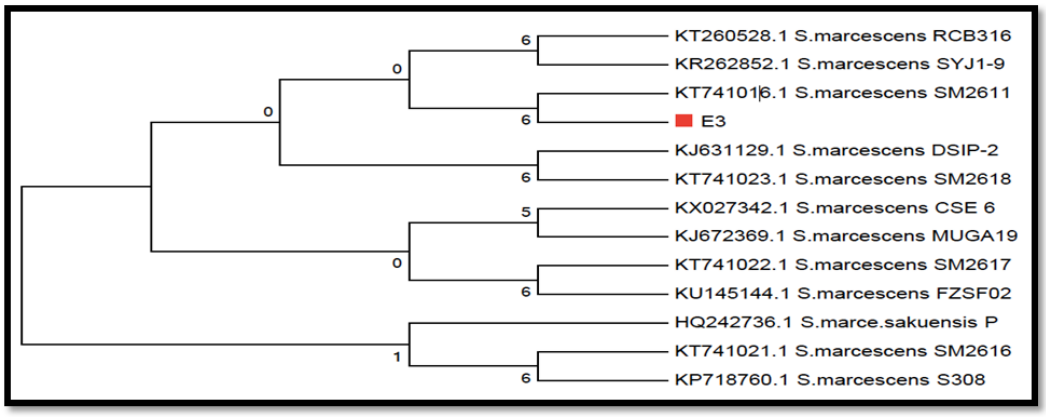

Fig. 1: Phylogenetic tree for E3 constructed using neighbour-joining algorithm of nucleotides sequence of 16S rRNA gene. The number in the branch of phylogram indicates bootstrap value (%) by 1000-replication multiple.

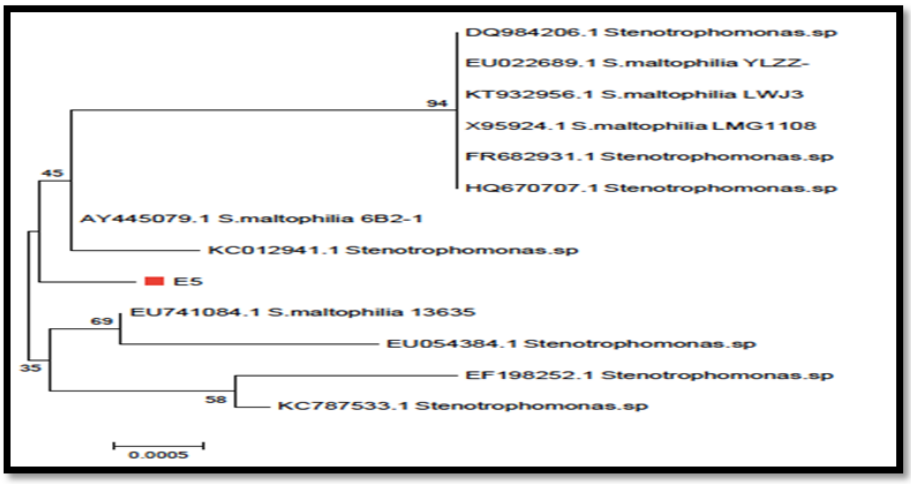

Fig. 2: Phylogenetic tree for E5 constructed using neighbour-joining algorithm of nucleotides sequence of 16S rRNA gene. The number in the branch of phylogram indicates bootstrap value (%) by 1000-replication multiple.

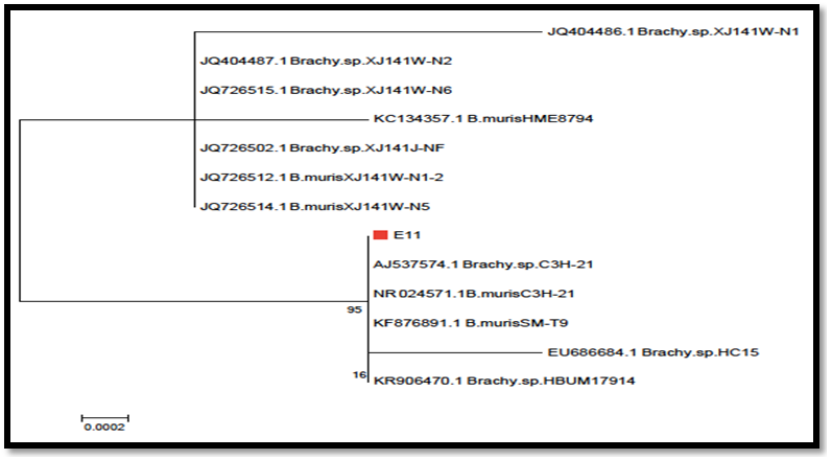

Fig. 3: Phylogenetic tree for E11 constructed using neighbor-joining algorithm of nucleotides sequence of 16S rRNA gene. The number in the branch of phylogram indicates bootstrap value (%) by 1000-replication multiple.

Table 2: List of isolated strains identified with phylogenetic analysis with accession numbers, classification, GenBank closest match, identity similarities.

S.No.	Phylogenetic architecture analysis			
	Accession number/ Strain name	Microbe class	GenBank closed match	Identity similarity (%)
1.	KX215147/E3	Bacteria: *Proteobacteria* *Gamma Proteobacteria* *Enterobacteriales* *Enterobacetriaceae* *Serratia*	KT741016.1 *S. marcescens* SM2611	99
2.	KX215148/E5	Bacteria: *Proteobacteria* *Gamma Proteobacteria* *Xanthomonadales* *Xanthomonadaceae* *Stenotrophomonas*	AY445079.1 *Stenotrophomonas maltophilia* 6B2-1	100
3.	KT372349/E11	Bacteria: *Actinobacter* *Actinobacteria* *Actinomycetales* *Dermabacteraceae* *Brachybacterium*	AJ537574.1 *Brachy.* sp. C3H-21	100
4.	KX179502/F5	Fungi: *Mucorales* *Cunninghamellaceae* *Cunninghamella*	JQ683239.1 *Cunninghamella echinulata* A-207	99

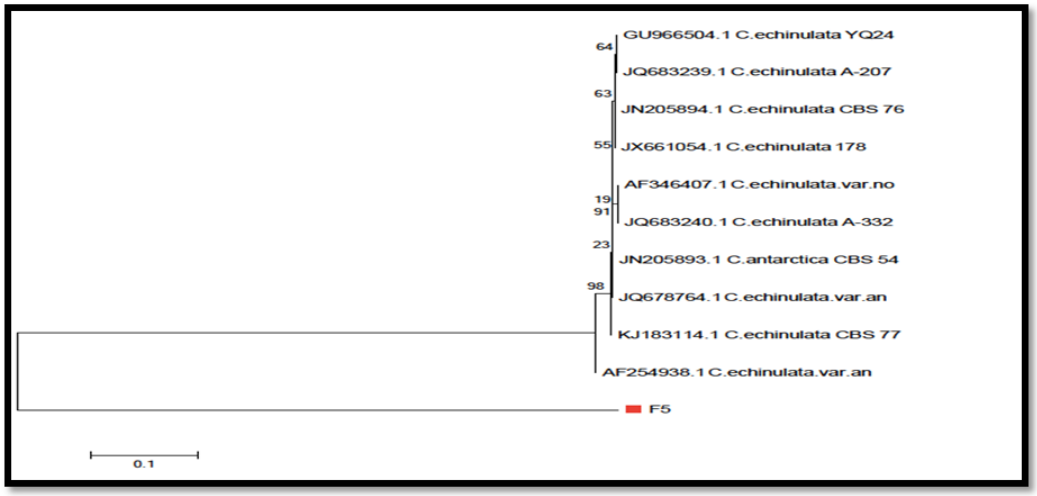

Fig. 4: Phylogenetic tree for F5 constructed using neighbour-joining algorithm of nucleotides sequence of 5.8S rRNA gene. The number in the branch of phylogram indicates bootstrap value (%) by 1000-replication multiple.

method were identified as *Serratia marcescens*, *Stenotrophomonas maltophilia, Brachybacetrium muris* and *Cunninghamella echinulata* (100-99% pairwise similarity) species and submitted to the GenBank database under NCBI Accession Numbers KX215147, KX215148, KT372349 and KX179502 respectively, as presented in Table 2.

Mazzucotelli et al. (2013) also isolated *Serratia* sp. and *Stenotrophomonas* sp. from agro-industrial by-products and waste and used these two bacteria for bioconversion of agro industrial by-products and waste into various different value-added products. Zhang et al. (2012) reported role of *Serratia marcescens* N80 in biodegradation of herbicide nicosulfuron and Kumar et al. (2013) used *Serratia marcescens* in cyanide degradation.

Mazzucotelli et al. (2014) along with three bacterial species i.e., *Lactococcus garvieae, Bacillus thuringiensis, Escherichia coli* used *Stenotrophomonas* sp. in consortium

to treat dairy wastewater. The degradation percentages with the selected consortium reached 80.67 and 83.44% at 24 and 48 h, respectively. Similarly Sonune & Garode (2015), observed that *Stenotrophomonas maltophilia* was able to reduce COD and BOD of municipal wastewater by 20 and 30% respectively.

Velmurugan & Arunachalam (2009) isolated the bacteria belonging to *Brachybacterium* genus from soil samples collected from the pharma industries and used it to degrade phenol and naphthalene. Al-Mailem et al. (2014) used *Brachybacterium* sp. along with other bacterial genera *Pseudomonas, Microvirga, Zavarzinia, Mycobacterium, Microbacterium, Stenotrophomonas, Gordonia, Bosea* and *Sphingobium* as biofilm to treat hydrocarbons contaminated sewage effluent.

Cunninghamella echinulata is a lignolytic fungi and the microbial degradation by lignolytic fungi has been intensively studied during the past few years and due to the irregular structure of lignin, lignolytic fungi produce extracellular enzymes with very low substrate specificity, making them suitable for degradation of different compounds (Haritash & Kaushik 2009).

Dairy wastewater degrading activity has rarely been described for the genus *Serratia, Stenotrophomonas, Brachybacterium* and *Cunninghamella*. Reduction of PCP of dairy wastewater by these microbial isolates has not been described until now, thus making this study novel in the use of these microbial isolates in dairy wastewater treatment. Hence, along with other biological strategies our research work could contribute to find a biotechnological approach for dairy wastewater treatment.

CONCLUSION

This investigation resulted in isolation and molecular characterization of native dairy effluent degrading microbes. *Serratia marcescens* (E3), *Stenotrophomonas maltophilia* (E5) *Brachybacetrium muris* (E11) and *Cunninghamella echinulata* (F5) exhibited COD, BOD and DO percentage reduction potential in the range 64-72%, 64-65% and 64-66% respectively. To the best of our knowledge these strains have not been reported for biodegradation of dairy wastewater except *Stenotrophomonas* sp. used in consortium to treat dairy effluent. However, earlier studies reported these strains in degradation of various xenobiotic compounds like phenol, naphthalene, hydrocarbons, dyes, cyanide and herbicides. Present study concluded that along with the bioremediation of effluent of pharmaceutical, textile and agro-based industries, these efficient strains are suitable candidate for future use in biotreatment of effluent generated by food industry. Hence, our research work opens scope for sustainable treatment of the huge amount of wastewater generated by dairy industry.

ACKNOWLEDGEMENT

This research was financially supported by the University Grant Commission (UGC) New Delhi under major research project grant (F.No. 39-268/2010 (SR)). The authors express gratitude to Principal, Head Department of Biotechnology, SGGS College and Panjab University, for their valuable support during the entire study.

REFERENCES

APHA 1989. Standard Methods for the Examination of Water and Wastewater. American Public Health Association, Washington DC.

Al-Mailem, D.M., Kansour, M.K. and Radwan, S.S. 2014. Bioremediation of hydrocarbons contaminating sewage effluent using man-made biofilms: effects of some variables. Applied Biochemistry and Biotechnology, 174(5): 1736-1751.

APEDA 2017. Dairy Products. Available at http://apeda.gov.in/apedawebsite/SubHead_Products/Dairy_Products.html [Accessed June 13, 2017].

Dhall, P., Siddiqi, T.O., Ahmad, A., Kumar, R. and Kumar, A. 2012. Restructuring BOD:COD ratio of dairy milk industrial wastewaters in BOD analysis by formulating a specific microbial seed. The Scientific World Journal, pp. 1-7.

Elijah, A.I., Atanda, O.O., Popoola, A.R. and Uzochukwu, S.V.A. 2014. Molecular characterization and potential of bacterial species associated with Cassava waste. Nigerian Food Journal, 32(2): 56-65.

Gaikwad, G.L., Wate, S.R., Ramteke, D.S. and Roychoudhury, K. 2014. Development of microbial consortia for the effective treatment of complex wastewater. Journal of Bioremediation & Biodegradation, 5(4): 1-6.

Haritash, A.K. and Kaushik, C.P. 2009. Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review. Journal of Hazardous Materials, 169(1): 1-15.

Jain, N., Nanjundaswamy, C., Minocha, A.K. and Verma, C.L. 2001. Isolation, screening and identification of bacterial strains for degradation of predigested distillery wastewater. Indian Journal of Experimental Biology, 39: 490-492.

Kim, O.S., Cho, Y.J., Lee, K., Yoon, S.H., Kim, M., Na, H., Park, S.C., Jeon, Y.S., Lee, J.H., Yi, H. and Won, S. 2012. Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. International Journal of Systematic and Evolutionary Microbiology, 62(3): 716-721.

Kumar, V., Kumar, V. and Bhalla, T.C. 2013. In vitro cyanide degradation by Serretiamarcescens RL2b. International Journal of Environmental Sciences, 3(6): 1969-79.

Mazzucotelli, C.A., Ponce, A.G., Kotlar, C.E. and Moreira, M.D.R. 2013. Isolation and characterization of bacterial strains with a hydrolytic profile with potential use in bioconversion of agroindustial by-products and waste. Food Science and Technology (Campinas), 33(2): 295-303.

Mazzucotelli, C.A., Durruty, I., Kotlar, C.E., Moreira, M.R., Ponce, A.G. and Roura, S.I. 2014. Development of a microbial consortium for dairy wastewater treatment. Biotechnology and Bioprocess Engineering, 19(2): 221-230.

Patel, J.B. 2001. 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. Molecular Diagnosis, 6(4): 313-321.

Porwal, H.J., Mane, A.V. and Velhal, S.G. 2015. Biodegradation of

dairy effluent by using microbial isolates obtained from activated sludge. Water Resources and Industry, 9: 1-15.

Prasad, M.P. and Manjunath, K. 2011. Comparative study on biodegradation of lipid-rich wastewater using lipase producing bacterial species. IJB-Niscair, 10: 121-124.

Rainey, F.A., Ward-Rainey, N., Kroppenstedt, R.M. and Stackebrandt, E. 1996. The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete lineage: proposal of Nocardiopsaceae fam. nov. International Journal of Systematic and Evolutionary Microbiology, 46(4): 1088-1092.

Sawyer, C.N., McCarty, P.L. and Parkin, G.F. 2003. Chemistry for Environmental Engineering and Science. 5th ed., New York: McGraw Hill.

Sonune, N.A., Mungal, N.A. and Kamble, S.P. 2015. Study of physico-chemical characteristics of domestic wastewater in Vishnupuri, Nanded, India. Int. J. Curr. Microbiol. App. Sci., 4(1): 533-536.

Sonune, N.A. and Garode, A.M. 2015. Bioremediation potential of bacterial isolates for municipal wastewater treatment. Current World Environment, 10(2): 619-625.

Suardana, I.W. 2014. Analysis of nucleotide sequences of the 16S rRNA gene of novel *Escherichia coli* strains isolated from feces of human and Bali cattle. Journal of Nucleic Acids, pp. 1-7.

Sumithrabhai, K., Thirumarimurugan, M., Sivakumar, V.M. and Sujatha, S. 2016. Expedient study on treatment of dairy effulent in fluidized bed reactor using immobilized microalgae. Int. J. Adv. Engg. Tech., 7(2): 231-235.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution, 28(10): 2731-2739.

Tewari, D., Cieply, S. and Livengood, J. 2011. Identification of bacteria recovered from animals using the 16S ribosomal RNA gene with pyrosequencing and Sanger sequencing. Journal of Veterinary Diagnostic Investigation, 23(6): 1104-1108.

Velmurugan, A.M. and Arunachalam, C. 2009. Bioremediation of phenol and naphthalene by *Bacillus* species and *Brachybacterium* species isolated from pharma soil sample. Current World Environment, 4(2): 299-306.

White, T.J., Bruns, T., Lee, S.J.W.T. and Taylor, J.W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protocols: A Guide to Methods and Applications, 18(1): 315-322.

Woo, P.C.Y., Leung, P.K.L., Leung, K.W. and Yuen, K.Y. 2000. Identification by 16S ribosomal RNA gene sequencing of an Enterobacteriaceae species from a bone marrow transplant recipient. Journal of Clinical Pathology-Molecular Pathology, 53(4): 211-215.

Zhang, H., Mu, W., Hou, Z., Wu, X., Zhao, W., Zhang, X., Pan, H. and Zhang, S. 2012. Biodegradation of nicosulfuron by the bacterium *Serratia marcescens* N80. Journal of Environmental Science and Health, Part B, 47(3):153-160.