Biotechnology Research and Innovation (2018) 2, 37–44

Biotechnology Research & Innovation

http://www.journals.elsevier.com/biotechnology-research-and-innovation/

RESEARCH PAPER

Isolation, characterization and identification of extracellular enzyme producer *Bacillus licheniformis* from municipal wastewater and evaluation of their biodegradability

Nilesh Sonune[a,*], **Anil Garode**[b]

[a] *Swami Ramanand Teerth Marathwada University, Nanded, India*
[b] *Shri Shivaji Science College Chikhli, Maharashtra, India*

Received 24 July 2017; accepted 29 March 2018
Available online 3 May 2018

KEYWORDS
Amylase;
Bioremediation;
BOD;
Lipase;
Protease;
Wastewater

Abstract In the present investigation, a total of 34 bacterial isolates were isolated from municipal wastewater and sludge samples. Out of 34, 5 isolates were selected based on their growth on wastewater agar medium and were identified on the basis of morphological, cultural and biochemical tests. The enzyme production study showed that all the isolates were able to produce lipase and protease while one isolate (NW1D) showed amylase production. The bioremediation potential of these enzymes producing bacteria was investigated and the results suggest that the isolate NW6 was most efficient for bioremediation and showed reduction in biochemical oxidation demand (BOD), chemical oxidation demand (COD), nitrate and phosphate by 50.65%, 20%, 56.25% and 31.13% respectively after 72 h. Further, the NW6 isolate identified as *Bacillus licheniformis* by 16S rRNA sequencing and was optimized for different parameters to achieve effective results. The results showed that overall optimum inoculum size, retention time and agitation speed for NW6 were 10% (v/v), 96 h and 200 rpm respectively. The reduction in BOD, COD, nitrate and phosphate was found to be 54.55%, 36%, 24.83% and 26.42% respectively with 10% inoculum size. At 96 h retention time with 10% inoculum, BOD, COD, nitrate and phosphate reduction was 69.16%, 42.5%, 49.89% and 39.62% respectively. The reduction in BOD, COD, nitrate and phosphate was found to be 72.08%, 51%, 51.87% and 31.13% respectively at 200 rpm with 10% inoculum. Further, the NW6 may find application in municipal wastewater treatment to prevent water pollution as well as a potential enzyme production.

* Corresponding author.
E-mail: snasonune@gmail.com (N. Sonune).

https://doi.org/10.1016/j.biori.2018.03.001

Introduction

In recent decades the risk due to water pollution has become a matter of serious concern in developing countries like India, because of limitless urbanization and lack of adequate treatment facility leads to serious water pollution problems due to direct disposal of sewage and industrial effluents to water bodies. Sewage is a waste matter resulting from the discharge into the sewers of human excreta and wastewater originating from a community and its industries (Tamil selvi, Anjugam, Archana Devi, Madhan, & Kannappan, 2012). It has a high content of both inorganic and organic matter (Sinha & Paul, 2014). In India it is reported that about 70% of the available water is polluted. The chief source of pollution is identified as sewage constituting 84 to 92 percent of the wastewater (Agarwal & Rajwar, 2010). River and aquatic ecosystems continually received a huge amount of sewage, changing the status of different water quality parameters such as dissolved oxygen, BOD, COD, nitrate, phosphate etc. (Loos et al., 2013).

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 wastewater treatment are inevitably cost intensive and cannot be employed in all industries. Hence, in recent years, the biological treatment system has become popular and helped in developing relatively efficient, low cost waste treatment systems (Garcha, Vermab, & Brar, 2016). In order to design an efficient biological wastewater treatment it is important to know the microbiota composition of the wastewater and to identify the strains which metabolize organic compounds (Ahring, Ibrahim, & Mladenovska, 2001; Janczukowicz, Zielinski, & Debowski, 2007). Hence, the main objective of the present study was to isolate bacteria from wastewater and to investigate their ability for bioremediation of municipal wastewater.

Materials and method

Sample collection and site

Municipal wastewater and sludge samples were collected from various places from Buldana district (Buldana, Chikhli, Khamgaon and Mehkar) Maharashtra, India, in pre-sterilized bottle and Zip-lock plastic bag respectively in the month of March in 2012 according to standard procedures (APHA, 2005). Physical properties like pH and temperature were recorded at the site of sample collection. The samples were transferred to the laboratory immediately and stored at 4 °C to avoid any physical-chemical changes in the wastewater.

Isolation, screening and identification of bacterial isolates

The collected municipal wastewater and sludge samples were serially diluted and inoculated on the nutrient agar medium separately. Morphologically different colonies were isolated, purified and maintained at 4 °C on nutrient agar slants. Screening was carried out by inoculating all bacterial isolates on wastewater agar medium (WWA medium) containing 100 ml sterilized municipal wastewater and 2% agar without addition of any external nutrients in the medium. The plates were incubated at 37 °C for 48 h (Sonune & Garode, 2015). Those isolates which showed growth on WWA medium were identified by morphological, cultural and biochemical characteristics based on Bergey's Manual of Determinative Bacteriology (Holt, 1994).

Screening for hydrolyzing extracellular enzymes

Those isolates which showed growth on WWA medium were screened for extracellular enzyme activity by plate assay method.

Plate assay for amylase activity

It was carried out by inoculating bacterial isolates on starch agar medium [(w/v) soluble starch 0.2%; peptone 0.5%; yeast extract 0.15%; beef extract 0.15%; NaCl 0.5%; agar 1.5%, pH 7.0 ± 0.2]. Screened isolates from nutrient agar slants were streaked on starch agar plate and incubated at 37 °C for 24–72 h. After incubation, iodine solution was flooded with dropper for 30 s on the starch agar plate. The clear zone of hydrolysis around the colony indicates a positive result.

Plate assay for protease activity

Screened isolates were inoculated for protease production on milk nutrient medium [(w/v) peptic digest of animal tissue 0.5%; beef extract 0.15%; yeast extract 0.15%; NaCl 0.5%; milk powder 1%; agar 1.5%, pH 7.0 ± 0.2] and incubated at 37 °C for 24–72 h. The zone of clearance was observed around the colonies indicated the proteolytic activity of that isolates.

Plate assay for lipase activity

Screened isolates were inoculated for lipase production on egg yolk nutrient agar medium [(w/v) peptic digest of animal tissue 0.5%; beef extract 0.15%; yeast extract 0.15%; NaCl 0.5%; egg yolk 1%; agar 1.5%, pH 7.0 ± 0.2] and incubated at 37 °C for 24–72 h. The zone of clearance was observed around the colonies indicated the lipolytic activity of that isolates.

Inoculum preparation

The loopfull culture of screened bacterial isolates were inoculated individually in pre-sterilized 50 ml wastewater broth (WWB) containing only sterile municipal wastewater and 0.5% peptone added for enhancement of bacterial growth. The flasks were kept in a shaker at 37 °C for 48 h with 120 rpm. The optical density (O.D.) of cell suspension was adjusted to McFarland 0.5 standard (Wayne, 2009).

Bioremediation study of bacterial isolates

Bioremediation study was carried out in a batch experiment. The inoculum of each isolates (10%) with 0.5 O.D.

was taken in 250 ml flask containing 90 ml non-sterile municipal wastewater separately. Simultaneously, one control flask was taken containing only non-sterilized municipal wastewater. These flasks were kept in a shaker at 37 °C for 72 h with 120 rpm. After treatment, all the samples were centrifuged at 10,000 rpm for 20 min at 10 °C and supernatants were used for further analysis.

Analytical methods

Municipal wastewater samples were characterized before and after the treatment. The parameters under study include pH, BOD5, COD, nitrate and phosphate. All the parameters were analyzed by using standard techniques described in Standard Methods for the Examination of Water and Wastewater (APHA, 2005).

Optimization of conditions for efficient bioremediation

The selected bacterial isolate which showed maximum bioremediation potential was optimized for different condition such as inoculum size, retention time and rotation per minutes for optimum results.

Molecular identification of bacteria

The most effective selected strain was identified by 16S-rRNA sequencing. For genotypic characterization, the DNA of the bacterial isolate was extracted from a single colony as described by Wilson and Carson (2001). Amplification of 16S rDNA by PCR was done using universal 518F forward primer (5′-CCAGCAGCCGTAATACG-3′) and 800R reverse primer (5′-TACCAGGGTATCTAATCC-3′). Amplification was carried out in a 50 μl reaction volume. The thermal cycle (PCR) steps were applied as follows: 5 min initial denaturation at 94 °C, followed by 30 cycles of 1 min denaturation at 94 °C, 1 min primer annealing at 55 °C, 1 min extension at 72 °C and a final 10 min extension at 72 °C. The amplified DNA fragments were separated on 1% (w/v) agarose gel electrophoresis eluted and purified using the Qiaquick gel extraction kit (Qiagen, Germany) following the manufacturer's protocol (Nimnoi, Pongsilp, & Lumyong, 2010). The purified PCR product was sequenced using the Big-Dye terminator kit ABI 310 Genetic Analyzer (Applied Biosystems, USA). Sequence data of partial 16S rDNA was aligned and analyzed for finding the closest homologous microbes. The unknown query 16S rRNA nucleotide sequence was compared to nucleotide databases using BLAST program. Then multiple sequence alignment was developed for these homologous sequences using the algorithm described in Clustal Omega. A phylogenetic tree was constructed by using MEGA software version 4.0 by neighbour joining method. The bacterial sequence was submitted to the National Centre for Biotechnology Information (NCBI) Gene bank and the accession numbers were taken for further correspondence.

Statistical analysis

Each experiment was performed in triplicates. The data obtained from the experiments were analyzed and expressed as mean and standard deviation.

Results and discussion

In the present investigation, a total of 34 bacterial isolates were isolated, among them 16 were from wastewater samples which were designated as NW and 18 were from sludge samples designated as NS. Out of 34 isolates, 5 isolates were screened on WWA medium which devoid of any external nutrients. These isolates showed good growth this may due to their ability to utilize organic material from wastewater as nutrients. Hence, these isolates were further identified, tested for enzyme production and bioremediation study. The morphological, cultural and biochemical characteristics suggested that these isolates were *Bacillus cereus*, *Bacillus licheniformis*, *Enterobacter aerogenes* and *Enterobacter intermedius* (Tables 1 and 2).

Among the screened bacterial isolates, only *B. cereus* NW1D showed amylase production. The results showed that amylase production was increased from 24 to 72 h of incubation and was found to be 16, 28.4 and 38 mm at 24, 48 and 72 h of incubation respectively. On the other hand the remaining 4 isolates were unable to produce amylase (Table 3). The similar results were reported from sewage enriched soil (Pokhrel et al., 2013) and domestic wastewater (Garode & Sonune, 2013), while all these five bacterial isolates showed protease production. The results showed that protease production increased from 24 to 72 h of incubation in all the isolates. Among 5 isolates, *B. cereus* NW1D showed highest protease production at 72 h of incubation (76 mm), while protease production by *B. cereus* NS1 (30, 43 and 51 mm), *B. licheniformis* NW6 (18, 33 and 41 mm), *E. aerogenes* NS14 (10, 25 and 30 mm), *E. intermedius* NW1A (18, 32 and 46 mm) and *B. cereus* NW1D (16, 33 and 76 mm) was observed at 24, 48 and 72 h of incubation respectively (Table 3). Similar results were reported from waste dump sites of municipality using bacteria (Saha & Santra, 2014). All the isolates showed lipase production (Table 3), among them, *B. licheniformis* NW6 and *E. intermedius* NW1A showed highest lipase production (41 mm each) at 72 h incubation, while lipase production by *B. cereus* NS1 (5.2, 16 and 25 mm), *B. licheniformis* NW6 (8, 33 and 41 mm), *E. aerogenes* NS14 (0, 13 and 31 mm), *E. intermedius* NW1A (6.5, 26 and 41 mm) and *B. cereus* NW1D (9, 23 and 32 mm) was observed at 24, 48 and 72 h incubation respectively. These results correlate with the previous reports (Garode & Sonune, 2014; Manh, 2008; Odeyemi, Aderiye, & Bamidele, 2013; Verma & Baiswar, 2013).

The initial pH of the sample was slightly acidic whereas it was slightly alkaline after treatment (Table 4). The results suggest that the increase in pH in experimental samples was slightly higher as compared to control sample. This increase may be associated with degradation of proteins and amino acids present in wastewater into ammonia that increases the pH of sample to slightly alkaline. However, the pH value of samples before and after treatment was within the permissible limit.

Table 1 Morphological and colony characteristics of selected bacterial isolates.

Isolate code	Source	Gram staining	Shape	Pigment	Opacity	Motility	Morphology	Spore
NS1	S	+	Rod	White	Opaque	+	Circular	+
NW6	W	+	Rod	White	Opaque	+	Circular	+
NS14	S	–	Rod	White	Opaque	+	Circular	–
NW1A	W	–	Rod	White	Opaque	+	Irregular	–
MW1D	W	+	Rod	White	Opaque	–	Irregular	+

S, sludge; W, wastewater; +, positive; –, negative.

Table 2 Biochemical characteristics of the selected bacterial isolates.

Biochemical tests		Isolates				
Isolate code		NS1	NW6	NS14	NW1A	NW1D
Catalase		+	+	+	+	+
Oxidase		+	+	–	–	+
Indole production		–	–	–	–	–
Methyl red		+	+	–	+	–
Voges proskauer		+	+	+	+	+
Citrate utilization		–	–	+	–	-
Nitrate		–	–	+	+	–
H_2S		–	+	–	–	–
Sugar fermentation						
Glucose	A	+	+	+	+	+
	G	–	–	–	+	–
Lactose	A	+	–	+	+	–
	G	–	–	–	–	–
Mannitol	A	+	+	+	+	+
	G	–	–	–	+	–
Sucrose	A	+	+	+	+	+
	G	+	–	+	+	+
Trehalose	A	+	+	+	+	+
	G	+	–	–	–	+
Name of bacteria		*B. cereus*	*B. licheniformis*	*E. aerogenes*	*E. intermedius*	*B. cereus*

+, positive; –, negative; A, acid; G, gas.

Table 3 Enzyme production efficiency of isolates.

Enzyme	Isolates					
	Time (h)	NW1A	NW1D	NW6	NS1	NS14
Amylase	24	–	16	–	–	–
	48	–	28.4	–	–	–
	72	–	38	–	–	–
Lipase	24	6.5	9	8	5.2	–
	48	26	23	33	16	13
	72	41	32	41	25	31
Protease	24	18	16	18	30	10
	48	32	33	33	43	25
	72	46	76	41	51	30

In parenthesis, data represents zone of clearance in millimetre (mm), –, negative.

BOD is an important parameter to examine water quality. The initial BOD of the sample was higher than the permissible limit. High BOD indicates a high amount of organic matter, it leads to oxygen depletion and creates anaerobic conditions which would result in reduction of diversity and distribution of aquatic fauna. Organic matter will support anaerobic action leading to the accumulation of toxic compounds in water bodies. In present study, the highest BOD reduction was observed with *B. licheniformis* NW6 in 72 h (50.65%) that reduced BOD from 61.6 ± 5.11 to 30.4 ± 3.17 mg/l, followed by *E. aerogenes* NS14, *E. intermedius* NW1A and *B. cereus* NW1D (24.68% each) and reduced the BOD up to 46.4 ± 7.73, 46.4 ± 2.62 and 46.4 ± 9.69 mg/l respectively from the initial value. The least reduction was observed with NS1 (5.19%) which was similar to control sample (Table 4). Sonune and Garode (2015) and Ravi Kumar, Lakshmi Prasad, Srinivasa Rao, and Sambasiva Rao (2013) reported that 42.86% and 36.41% reduction in BOD of municipal wastewater by *B. licheniformis* respectively. Vasconcellos et al. (2009) reported that

Table 4 Bioremediation efficacy of municipal wastewater.

Isolates	pH	BOD mg/l (% Reduction)	COD mg/l (% Reduction)	Nitrate mg/l (% Reduction)	Phosphate mg/l (% Reduction)
Initial	06.82 ± 0.02	61.6 ± 5.11	200 ± 20.78	47.89 ± 5.47	1.06 ± 0.16
Control	7.04 ± 0.06	58.4 ± 7.62 (5.19)	198 ± 35.44 (1.0)	80.81 ± 2.46 (−40.74)	0.75 ± 0.11 (29.25)
NW1A	07.15 ± 0.01	46.4 ± 2.62 (24.68)	166 ± 15.72 (17)	209.52 ± 32.72 (−77.14)	01.71 ± 0.09 (−38.01)
NW1D	7.44 ± 0.06	46.4 ± 9.69 (24.68)	150 ± 5.29 (25)	77.82 ± 5.73 (−38.46)	01.3 ± 0.04 (−18.46)
NW6	7.45 ± 0.02	30.4 ± 3.17 (50.65)	160 ± 7.0 (20)	20.95 ± 3.24 (56.25)	0.73 ± 0.06 (31.13)
NS1	07.88 ± 0.07	58.4 ± 7.41 (5.19)	180 ± 22.61 (10)	62.85 ± 7.27 (−23.8)	01.45 ± 0.23 (−26.9)
NS14	7.05 ± 0.02	46.4 ± 07.73 (24.68)	170 ± 14.42 (15)	53.87 ± 4.20 (−11.1)	1.68 ± 0.12 (−36.9)

In parenthesis, data represents mean (±) standard deviation.

B. licheniformis for biodegradation of cassava processing wastewater. The significant decrease in BOD and COD values could be associated with the consumption of organic material as nutrients by microbes.

The COD test is a rapid method for estimation of organic matter present in the wastewater sample. Out of 5 isolates, highest reduction in COD was showed by NW1D (25%) that reduced COD from initial value 200 ± 20.78 to 150 ± 5.29 mg/l, followed by NW6 (20%), NW1A (17%), NS14 (15%) and NS1 (10%). They showed a reduction from initial value to 160 ± 7.0, 166 ± 15.72, 170 ± 14.42 and 180 ± 22.61 mg/l respectively, whereas the reduction of control was negligible (1%). These results were agreed with the results of Ordaz-Diaz et al. (2014) and Hamza, Mohammed, and Sale (2012). These bacteria are capable of producing a wide variety of enzymes that can degrade complex organic compounds into CO_2 and water present in the wastewater (Claxton & Houx, 1995; Kumari, Mehta, Shukla, John, & Mehta, 2008). The bacterial species present in the wastewater has no significant effect on the removal of BOD and COD as observed in the case of control. Gaikwad, Wate, Ramteke, and Roychoudhury (2014) and Zhao, Hu, Chen, Zhao, and Liang (2009) found that *Pseudomonas* sp. and *Bacillus* sp. were able to reduced COD and BOD.

The nitrate concentration was found to be above the permissible limit. The results showed that the *B. licheniformis* NW6 showed reduction in nitrate from initial value 47.89 ± 5.47–20.95 ± 3.24 mg/l (56.25%), this may be due to denitrification process. Sonune and Garode (2015) reported nitrate reducing *B. licheniformis* from municipal wastewater whereas Rajakumar et al. reported *Bacillus* sp. was most efficient for nitrate reduction.

The phosphate is one of the most serious environmental problems because of its contribution to the eutrophication process of lakes and other natural waters. It occurs in natural water, wastewater, sediments and sludge. The possible entry of this ion into aquatic environment is through household sewage. In the present investigation the *B. licheniformis* NW6 reduced phosphate from initial value 1.06 ± 0.16–0.73 ± 0.06 mg/l (31.13%). Krishnaswamy, Muthuchamy, and Perumalsamy (2011) found that the *Bacillus* sp RS-1 was found to be efficient in phosphate reduction. Usharani et al. (2009) observed the phosphate removal efficiency of 38–55% by *Bacillus* sp. from wastewater. However in the present study, phosphate concentration was found to be lower than the permissible limit. Similar findings were reported by many studies (Garode & Sonune, 2015; Sonune, Mungal, & Kamble, 2015).

Optimization of NW6 for bioremediation of municipal wastewater

Effect of inoculum size: The efficient bioremediation ability of NW6 was studied by using different inoculum size of NW6 (5, 10, 15, 20 and 25%). The results showed that the highest reduction in BOD (54.55%) and COD (36%) was achieved at 10% (v/v) inoculum whereas the reduction decreases as further increase in inoculum size. The maximum reduction in nitrate (35.27%) and phosphate (33.09%) was achieved at 15% inoculum size (Fig. 1). Hence, from these results, overall optimum inoculum size was considered as 10%. At

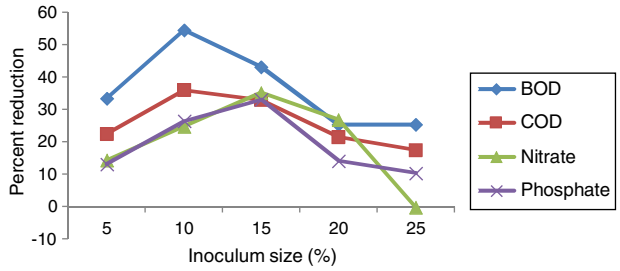

Figure 1 Graphical representation of bioremediation efficacy of *B. licheniformis* NW6 at different inoculum size.

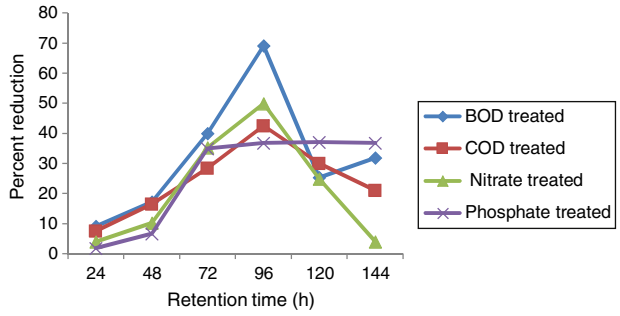

Figure 2 Graphical representation of bioremediation efficacy of *B. licheniformis* NW6 at different retention time.

higher inoculum size reduction percentage were lower it may be due to low nutrient availability and high mortality rate. Our results were comparable to Garcha, Brar, and Sharma (2014) who reported optimum inoculum size for consortium consisting of bacteria and yeast was 11% for BOD reduction of dairy wastewater.

Effect of retention time: Different time periods (24, 48, 72, 96, 120 and 144 h) were used to check maximum bioremediation ability of NW6 with 10% (v/v) inoculum size. The results indicate that the highest reduction in BOD (69.16%), COD (42.5%), nitrate (49.89%) and phosphate (39.62%) was achieved at 96 h. At 96, 120 and 144 h of incubation comparable phosphate reduction (37%) was noticed (Fig. 2). The results indicate that the optimum retention time for NW6 was 96 h. Our results were promising than Bestawy, AL-Hejin, Amer, and Kashmeri (2014) who reported the *Bacillus amyloliquefaciens* reduced BOD up to 31.9% after 7 days. The reduction decreased with time reflects toxicity of the wastewater that led to the death of some bacterial cells adding their organic matter in the wastewater (Bestawy et al., 2014).

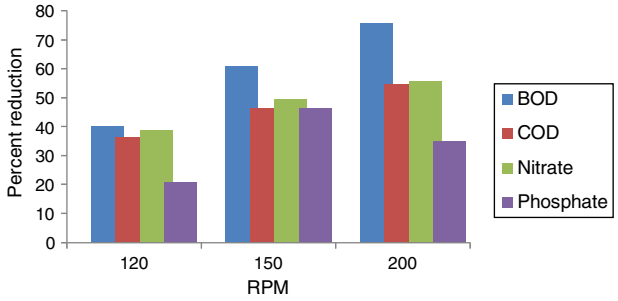

Figure 3 Graphical representation of bioremediation efficacy of *B. licheniformis* NW6 at different rotation per minutes.

Effect of rotation per minutes: *B. licheniformis* NW6 was tested for different agitation speed such as 120, 150 and 200 rpm. The results indicate that as agitation speed increased the reduction in BOD, COD and nitrate was also increased. The maximum reduction was found at 200 rpm in BOD (72.08%), COD (51%) and nitrate (51.87%) while maximum reduction in phosphate (42.45%) was achieved at 150 rpm (Fig. 3). Hence, optimum agitation speed for NW6 was considered as 200 rpm. Aiswarya and Anu (2017) showed 80.3% and 72.28% reduction in BOD and COD respectively of ayurvedic hospital wastewater at agitation speed 180 rpm by *Bacillus cereus*.

Out of the five isolates, NW6 showed effective bioremediation results as compared to other isolates. Hence, it was further identified by 16S rRNA sequencing method. Based on 16S rRNA gene analysis, isolate NW6 was grouped into genus *Bacillus* and the sequence was most closely related to *B. licheniformis* with similarity of 90%. The isolate *Bacillus licheniformis* NW6 has accession number KR261405 (Fig. 4).

Conclusion

In the present investigation, all the screened bacterial isolates showed ability to utilize organic material as nutrients as they showed growth on WWA medium which was devoid of any external nutrients. These isolates include *Bacillus cereus*, *Bacillus licheniformis*, *Enterobacter aerogenes* and *Enterobacter intermedius*. Among them, *B. licheniformis* strain KR261405 was found to be most efficient bacteria as compared to other isolates. In optimization, the addition of 10% (v/v) inoculum gave appreciable biodegradation of municipal wastewater in reasonable residence time of 96 h and 200 rpm in term of reduction in BOD, COD, nitrate and

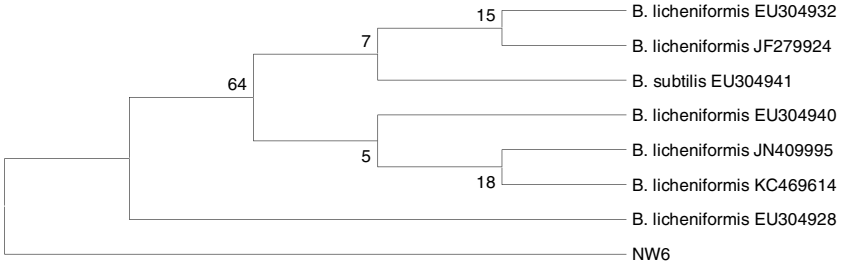

Figure 4 Neighbour joining tree based on 16S rRNA sequence of genus *Bacillus* obtained from BLAST search showing the position of NW6 isolate and related strains.

phosphate. Hence it can be recommended for treatment of municipal wastewater.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

Authors are grateful to Head, P.G. Department of Microbiology, Shri Shivaji Science College Chikhli for providing the facilities for the study.

References

Agarwal, A. K., & Rajwar, G. S. (2010). Physico-chemical and microbiological study of Tehri Dam Reservoir Garhwal Himalaya. *Journal of American Science*, *6*(6), 65–71.

Ahring, B. K., Ibrahim, A. A., & Mladenovska, Z. (2001). Effect of temperature increase from 55 to 65 °C on performance and microbial population dynamics of an anaerobic reactor treating cattle manure. *Water Research*, *35*, 2446–2452.

Aiswarya, M., & Anu, N. (2017). Bioremediation of ayurvedic hospital wastewater using *Bacillus Cereus*: Effect of agitation speed and time. *International Journal for Research in Technological Studies*, *4*(6), 64–66.

American Public Health Association (APHA), American Water Works Association (AWWA) and Water Environment federation (WEF). (2005). *Standard methods for the examination of water and wastewater* (21th ed.). Washington, DC: APHA.

Bestawy, E. E., AL-Hejin, A., Amer, R., & Kashmeri, R. A. (2014). Decontamination of domestic wastewater using suspended individual and mixed bacteria in batch system. *Journal of Bioremediation and Biodegradation*, *5*(5), 231. http://dx.doi.org/10.4172/2155-6199.1000231

Claxton, L. D., & Houx, U. S. (1995). Integration of complex mixture toxicity and microbial analysis for environmental remediation research. In *Ecotoxicity and human health*. pp. 467–468. Lewis Publishers.

Gaikwad, G. L., Wate, S. R., Ramteke, D. S., & Roychoudhury, K. (2014). Development of microbial consortia for the effective treatment of complex wastewater. *Journal of Bioremediation and Biodegradation*, *5*(4), 227. http://dx.doi.org/10.4172/2155-6199.1000227

Garcha, S., Brar, S. K., & Sharma, K. (2014). Performance of laboratory prepared microbial consortium for degradation of dairy wastewater in batch system. *Journal of Scientific and Industrial Research*, *73*, 346–350.

Garcha, S., Vermab, N., & Brar, S. K. (2016). Isolation, characterization and identification of microorganisms from unorganized dairy sector wastewater and sludge samples and evaluation of their biodegradability. *Water Resources and Industry*, *16*, 19–28.

Garode, A. M., & Sonune, N. A. (2013). Isolation and identification of amylase producing bacterium from domestic wastewater of Buldana District (M.S.). *International Journal of Scientific Research*, *2*(5), 11–12.

Garode, A. M., & Sonune, N. A. (2014). Screening and identification of lipase producing bacteria from domestic wastewater. *International Journal of Pharma and Biosciences*, *5*(2), 265–268.

Garode, A. M., & Sonune, N. A. (2015). Bacteriological and physico-chemical assessment of municipal wastewater from Buldana District, India. *International Journal of Current Microbiology and Applied Sciences*, *4*(7), 577–584.

Hamza, U. D., Mohammed, I. A., & Sale, A. (2012). Potentials of bacterial isolates in bioremediation of petroleum refinery wastewater. *Journal of Applied Phytotechnology in Environmental Sanitation*, *1*(3), 131–138.

Holt, J. G. (1994). *Bergey's manual of determinative bacteriology* (9th ed.).

Janczukowicz, W., Zielinski, M., & Debowski, M. (2007). Biodegradability evaluation of dairy effluents originated in selected sections of dairy production. *Bioresource Technology*, *99*, 4199–4205.

Krishnaswamy, U., Muthuchamy, M., & Perumalsamy, L. (2011). Biological removal of phosphate from synthetic wastewater using bacterial consortium. *Iranian Journal of Biotechnology*, *9*(1), 37–49.

Kumari, S., Mehta, A., Shukla, S., John, J., & Mehta, P. (2008). Biodegradation potential of the selected bacterial strains isolated from Sagar Lake. *Asian Journal of Experimental Sciences*, *22*(1), 43–48.

Loos, R., Carvalho, R., Antonio, D. C., Comero, S., Locoro, G., Tavazzi, S., et al. (2013). EU-wide monitoring survey on emerging polar organic contaminants in wastewater treatment plant effluents. *Water Resources*, *47*(17), 6475–6487.

Manh, L. D. (2008). Bioremediation of vegetable oil and grease from polluted wastewater in dairy factory. *VNU Journal of Science, Natural Sciences and Technology*, *24*, 56–62.

Nimnoi, P., Pongsilp, N., & Lumyong, S. (2010). Genetic diversity and community of endophytic actinomycetes within the roots of *Aquilaria crassna* Pierre ex Lec assessed by actinomycetes-specific PCR and PCR-DGGE of 16S rRNA gene. *Biochemical Systematics and Ecology*, *38*, 595–601.

Odeyemi, A. T., Aderiye, B. I., & Bamidele, O. S. (2013). Lipolytic activity of some strains of *Klebsiella, Pseudomonas* and *Staphylococcus* Spp. from restaurant wastewater and receiving stream. *Journal of Microbiology Research*, *3*(1), 43–52.

Ordaz-Diaz, L. A., Rojas-Contreras, J. A., Rutiaga-Quinones, O. M., Moreno-Jimenez, M. R., Alatriste-Mondragon, F., & Valle-Cervantes, S. (2014). Microorganism degradation efficiency in BOD analysis formulating a specific microbial consortium in a pulp and paper mill effluent. *Bioresources*, *9*(4), 7189–7197.

Pokhrel, B., Wanjare, P., Singh, S., Purushotham, B., Kumara, & Swamy, M. (2013). Isolation, screening and characterization of promising α-amylase producing bacteria from sewage enriched soil. *International Journal of Advanced Biotechnology and Research*, *4*(2), 286–290.

Ravi Kumar, B., Lakshmi Prasad, M., Srinivasa Rao, D., & Sambasiva Rao, K. R. S. (2013). Bioremediation of sewage using specific consortium of microorganisms. *International Journal of Research in Applied, Natural and Social Sciences*, *1*(6), 15–26.

Saha, A., & Santra, S. C. (2014). Isolation and characterization of bacteria isolated from municipal solid waste for production of industrial enzymes and waste degradation. *Journal of Microbiology and Experimentation*, *1*(1), 00003. http://dx.doi.org/10.15406/jmen.2014.01.00003

Sinha, S. N., & Paul, D. (2014). Biodegradation potential of some bacterial strains isolated from sewage water. *International Journal of Environmental Biology*, *4*(2), 107–111.

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

Sonune, N. A., Mungal, N. A., & Kamble, S. P. (2015). Study of physico-chemical characteristics of domestic wastewater in

Vishnupuri, Nanded, India. *International Journal of Current Microbiology and Applied Sciences*, *4*(1), 533–536.

Tamil selvi, A., Anjugam, E., Archana Devi, R., Madhan, B., & Kannappan, S. (2012). Isolation and characterization of bacteria from tannery effluent treatment plant and their tolerance to heavy metals and antibodies. *Asian Journal of Experimental Biological Sciences*, *3*, 34–41.

Krishnaswamy, U., Muthusamy, M., & Perumalsamy, L. (2009). Studies on the Efficiency of the Removal of Phosphate Using Bacterial Consortium for the Biotreatment of Phosphate Wastewater. *European Journal of Applied Sciences*, *1*(1), 6–15.

Vasconcellos, S. P., Cereda, M. P., Cagnon, J. R., Foglio, M. A., Rodrigues, R. A., Manfio, G. P., et al. (2009). In vitro degradation of linamarin by microorganism isolated from cassava wastewater treatment lagoons. *Brazilian Journal of Microbiology*, *40*, 879–883.

Verma, T., & Baiswar, V. (2013). Isolation and characterization of extracellular thermoalkaline protease producing *Bacillus cereus* isolated from tannery effluent. *International Journal of Engineering and Science*, *2*(7), 23–29.

Wayne, P. A. (2009). *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard* (8th ed.). M07-A8 Clinical and Laboratory Standards Institute.

Wilson, T., & Carson, J. (2001). Rapid, high-throughput extraction of bacterial genomic DNA from selective-enrichment culture media. *Letters in Applied Microbiology*, *32*, 326–330.

Zhao, S., Hu, N., Chen, Z., Zhao, B., & Liang, Y. (2009). Bioremediation of reclaimed wastewater used as landscape water by using the denitrifying bacterium *Bacillus cereus*. *Bulletin of Environmental Contamination and Toxicology*, *83*, 337–340.