ISOLATION AND CHARACTERIZATION OF HALOPHILIC MICROORGANISMS FROM BEACHES OF MUMBAI FOR OIL AND PETROLEUM HYDROCARBON DEGRADATION

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Abstract: Oil spills are common problem now-a-days for Marine environment, these increasing number of marine oil spills asks for effective solutions for the environment. Bioremediation of marine oil spills has become a very practical approach to oil spill cleanup efforts in recent years. Many microorganisms possess the enzymatic capability to degrade petroleum hydrocarbons even at extreme conditions of salt in seawater.
Present study explores the hemophilic flora of beaches and seashores of Mumbai for their oil/Hydrocarbon degradation potential. Total 30 Water and 30 sand samples were collected from four different beaches of Mumbai. These samples were enriched in liquid medium containing 20% NaCl. Enriched samples were used for isolation of hemophilic organisms. Around 68 isolates were obtained and further screened for hydrocarbon degrading activity.

Keywords: Bioremediation, Halophiles, Hydrocarbon degradation, Microtitre Plate assay.

Introduction: Several microorganisms are known for their ability to decompose or transform the chemical substances present in petroleum and petroleum derivatives. A particular kind of microorganism can degrade only certain types of petroleum compounds, but a mixed population or consortia enables a higher level of degradation. Moreover, some substances can be decomposed only by co-metabolism. However, degradation of aromatic hydrocarbons seems to be restricted to microorganisms [1]. The aerobic degradation of aromatic hydrocarbons by microorganisms has been investigated extensively [2].Marine environments such as sea shores, salt lakes, salt flats, solar salterns, industrial effluents, oil fields, coastal ecosystems, areoften contaminated with high levels of petroleum hydrocarbons [3].Halophiles with biodegradative potential can be used in the bioremediation of saline environments contaminated with organic pollutants [4]. Several researchers studied biodegradation of aromatic compounds by halophilic bacteria has been well studied [5-7]. Halophiles are classified into three groups according to their optimal salt concentration for growth: slightly halophilic (1-3% w/v); moderately halophilic (3-15% w/v); and extremely halophilic(>15 % w/v) [8,9]. The present study has been focused on isolation of halophilic and halotolerant organisms from beaches and seashores of Mumbai. Further project aims at characaterization of these isolates for their oil and petroleum hydrocarbon degradation potential.

Materials and Methods:

Sample collection: Total Thirty sand samples and Thirty water samples were collected from 4 different Beaches of Mumbai, India.

Enrichment and isolation: Samples were enriched for 7 days in Modified Halophilic broth containing 200g NaCl, 7.5g Casamino acid, 10g Yeast extract, 3g Trisodium Citrate, 2g KCl, 20g $MgSO_4.7H_2O$ per litre (pH= 7) [10-11]. After enrichment loopfulof enriched broth was streaked on solid plate medium, for solid medium, 1.5% (w/v) agar was used. Plates were incubated for 48 hrs, at room temperature. Different colonies were picked and restreaked to obtain pure cultures. The pure cultures were stored at 10°C in the isolation medium slant.

Salt tolerance of the isolates: The isolates were screened for their salt tolerance level by growing them on to Luria Bertini broth (HiMedia) tubes with concentrations of salt (NaCl) ranging from 5% to 35% and subsequently observing their turbidity [12]. pH was maintained at 7.0 and temperature at room temperature.

Preliminary screening of isolates for oil and petroleum hydrocarbon degradation: [Substrates used: Edible oil, Kerosene, Petrol, and Diesel]Preliminary screening of hydrocarbon-degrading activity was performed using the Bushnell-Haas agar plate assay [14]. The Bushnell-Haas agar plates were incorporated with one of the above carbon sources prior to solidification; aloopful of the bacterium was then inoculated on the agar and the inoculated plates were incubated at room temperature for 3 days.

Quantitative screening of isolates for oil and petroleum hydrocarbon degradation: Screening of isolated microorganisms was done by microtiter plate technique [14]. The addition of indicator enables to measure microbial hydrocarbon degrading ability by monitoring a color change. Pure bacterial isolates were inoculated in 10 ml Luria Bertini medium (pH 7.2) and incubated for 48 hours at Room temperature. After incubation, cells were aseptically placed in sterile centrifuge tubes and centrifuge for 10 minutes at 15,000 rpm to pellet the cells. After washing the cells in 5 ml of Bushnell-Haas medium

and recentrifugation for another 10 minutes, the cells were resuspended in 4 ml of Bushnell-Haas medium. Microtiter plates in our experiment were set up with each well contained 200 µl of sterilized Bushnell-Haas medium (pH 7.2), 50 µl of cells and 5 µl of hydrocarbon. Respective controls were maintained. Plates were incubated for 3 days at room temperature, non-shaking. Growth was measured before and after incubation at 600 nm. At the end of theexperiment, 50 µl of p-iodonitrotetrazolium (INT) indicator was added to each well. Plates were incubated for 4 hours after addition of indicator. Each microtiter plate was scored for positive results. A positive result was indicated by red precipitate or brown precipitate.

Sr. no.	Sampling site	Number of sand samples	Number of water samples
1	Gorai Beach, Mumbai	07	06
2	Juhu Beach, Mumbai	10	11
3	Dadar Chowpatty, Mumbai	06	06
4	GirgaonChowpatty, Mumbai	07	07

Table-1.Sampling Details

Results and Discussion: Sampling and Isolation: Sixty environmental samples were obtained from four famous beaches of Mumbai, India. Sampling details are shown in Table 1 and figure 1. Total 68 different isolates were obtained from these samples. These isolates were labeled as H-1 to H-68 and were maintained in Luria Bertini agar slants with 10% salt concentration at 10ºC

Fgure 1. Sampling sites

Table 2.Halotolerance of the isolates

Isolate	Growth till % NaCl	Isolate	Growth till % NaCl	Isolate	Growth till % NaCl
H-1	**25**	**H-25**	**15**	**H-49**	**20**
H-2	**25**	**H-26**	**30**	**H-50**	**15**
H-3	**30**	**H-27**	**25**	**H-51**	**30**
H-4	**20**	**H-28**	**25**	**H-52**	**30**
H-5	**30**	**H-29**	**15**	**H-53**	**15**
H-6	**25**	**H-30**	**25**	**H-54**	**20**
H-7	**25**	**H-31**	**30**	**H-55**	**35**
H-8	**25**	**H-32**	**15**	**H-56**	**35**
H-9	**20**	**H-33**	**25**	**H-57**	**30**
H-10	**25**	**H-34**	**15**	**H-58**	**30**
H-11	**20**	**H-35**	**15**	**H-59**	**30**
H-12	**20**	**H-36**	**30**	**H-60**	**30**
H-13	**25**	**H-37**	**25**	**H-61**	**25**
H-14	**20**	**H-38**	**25**	**H-62**	**35**
H-15	**20**	**H-39**	**15**	**H-63**	**15**
H-16	**30**	**H-40**	**15**	**H-64**	**30**
H-17	**25**	**H-41**	**15**	**H-65**	**25**
H-18	**25**	**H-42**	**20**	**H-66**	**15**
H-19	**25**	**H-43**	**20**	**H-67**	**20**
H-20	**20**	**H-44**	**15**	**H-68**	**30**
H-21	**35**	**H-45**	**35**		
H-22	**20**	**H-46**	**20**		
H-23	**30**	**H-47**	**30**		
H-24	**25**	**H-48**	**15**		

Salt Tolerance of the isolates:

Out of 68 isolates around 5 isolates were found to be capable of growing at the salt concentration of 35% and 60 isolates showed good growth till 30% NaCl,

while 25 isolates required minimum of 10% of NaCl for their growth (no growth were observed for these isolates in 5% NaCl). The salt tolerance of the isolates is summarized in the Table 2.

Preliminary screening of isolates for oil and petroleum hydrocarbon degradation:

The activity of isolates was observed in BH-plate assay supplied with sole carbon source. The concentration of all carbon source was maintained at 5%. Out 68 isolates, 13 showed growth on plates supplied with edible oil as carbon source, 22 isolates showed growth on Kerosene plates while only 5 isolates were able to grow on plates containing Petrol as carbon source. 27 isolates showed growth on Diesel containing plates. These isolates were selected and further used for Micro-Titer Plate assay.

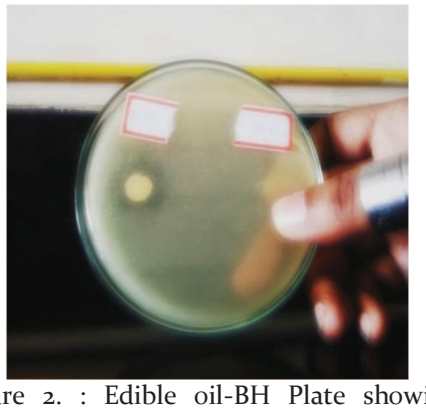

Figure 2. : Edible oil-BH Plate showing growth of isolate alongwith zone of clearance

Quantitative screening of isolates for oil and petroleum hydrocarbon degradation: The major parameter of this assay is the use turbidity or absorbance to monitor biomass growth. The method offers fast, cheap and easy detection. The addition of indicator enables to measure microbial hydrocarbon degrading ability by monitoring a color change. The results are mentioned in below tables along with statistical evaluation the data[15]. Isolate H-54 for Edible oil, H-68 for Kerosene, H-47 for Diesel, and H-56 for Petrol were found to most potent isolates.

Figure 3 Depicts results of INT assay.

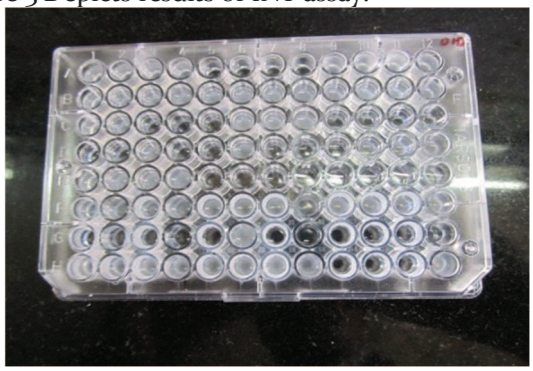

Plate before adding INT

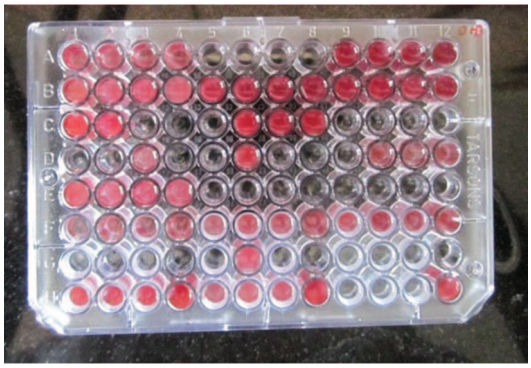

Plate after adding INT

Figure 3: INT assay

Table-3.Quantitative screening of isolates for oil degradation

Isolate	Initial Optical density	Optical density after 3 Days
H-2	0.121 ± 0.005	0.384 ± 0.001
H-22	0.065 ± 0.013	0.054 ± 0.010
H-29	0.119 ± 0.023	0.626 ± 0.020
H-33	0.065 ± 0.009	0.564 ± 0.084
H-40	0.138 ± 0.018	0.596 ± 0.057
H-47	0.125 ± 0.017	0.653 ± 0.095
H-52	0.059 ± 0.003	0.379 ± 0.275
H-54	0.105 ± 0.015	0.744 ± 0.076
H-55	0.059 ± 0.005	0.047 ± 0.001
H-56	0.063 ± 0.003	0.710 ± 0.379
H-60	0.082 ± 0.007	0.349 ± 0.191
H-61	0.062 ± 0.003	0.393 ±0.110
H-67	0.139 ± 0.020	0.515 ± 0.069
Control	0.067 ± 0.026	0.070 ± 0.023

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Table-4.Quantitative screening of isolates for Kerosene degradation

Isolate	Initial Optical density	Optical density after 3 Days
H-2	0.181 ± 0.018	0.176 ± 0.005
H-3	0.121 ± 0.025	0.456 ± 0.070
H-15	0.104 ± 0.012	0.335 ± 0.021
H-19	0.058 ±0.004	0.070 ± 0.013
H-22	0.060 ± 0.005	0.060 ± 0.006
H-25	0.055 ± 0.006	0.065 ± 0.003
H-29	0.073 ± 0.005	0.057 ± 0.012
H-34	0.110 ± 0.015	0.162 ± 0.023
H-39	0.058 ± 0.029	0.154 ± 0.007
H-40	0.062 ± 0.005	0.160 ± 0.005
H-45	0.134 ± 0.030	0.141 ± 0.014
H-46	0.138 ± 0.006	0.461 ± 0.158
H-47	0.303 ± 0.010	0.552 ± 0.008
H-49	0.200 ± 0.016	0.614 ± 0.121
H-51	0.278 ± 0.107	0.792 ± 0.133
H-55	0.069 ± 0.006	0.071 ± 0.015
H-56	0.110 ± 0.020	0.506 ± 0.090
H-58	0.108 ± 0.038	0.408 ± 0.039
H-64	0.170 ± 0.007	0.467 ± 0.019
H-66	0.189 ± 0.065	0.513 ± 0.155
H-68	0.061 ± 0.002	0.656 ± 0.176
Control	0.104 ± 0.025	0.110 ± 0.016

Summary and Conclusions: Beaches of Mumbai showed diversity of various halophilic bacteria. Around 68 halophilic isolates were obtained and studied for Halotolerance. The optimum salt concentration for most of the isolates was found to be 20%. Around 5 extreme halophilic isolate with growth at 35% of salt concentration were obtained. The results have demonstrated the ability of these halophilic bacteria to degrade Edible oiland several other petroleum hydrocarbons.Halophilic microorganisms are adapted to grow and thrive under adverseconditions of oceans and salt lakes. Hydrocarbon degrading halophiles could be ideal candidates for the biological treatment of pollutedextreme habitats such as oil spills

Table-5.Quantitative screening of isolates for Diesel degradation

Isolate	Initial Optical density	Optical density after 3 Days
H-2	0.113±0.008	0.300 ±0.024
H-3	0.109 ±0.004	0.410 ±0.012
H-15	0.097±0.001	0.426 ±0.149
H-19	0.053±0.007	0.038 ±0.005
H-22	0.045±0.010	0.481 ±0.075
H-25	0.050±0.005	0.042 ±0.005
H-29	0.091 ±0.017	0.555 ±0.053
H-33	0.061±0.007	0.517 ±0.041
H-34	0.106±0.013	0.056 ±0.004
H-39	0.082±0.008	0.375 ±0.046
H-40	0.159±0.037	0.488 ±0.285
H-45	0.141±0.025	0.345 ±0.162
H-46	0.156±0.017	0.593 ±0.065
H-47	0.033±0.007	0.852 ±0.254
H-49	0.303±0.020	0.590 ±0.088
H-51	0.165±0.041	0.460 ±0.045
H-52	0.060±0.011	0.035 ±0.007
H-54	0.300±0.026	0.601 ±0.009
H-55	0.570±0.514	0.050 ±0.003
H-56	0.070±0.007	0.086 ±0.029
H-58	0.068±0.007	0.475 ±0.009
H-60	0.085±0.005	0.059 ±0.010
H-61	0.066±0.002	0.043 ±0.004
H-64	0.055±0.003	0.566 ±0.049
H-66	0.180±0.014	0.592 ±0.132
H-67	0.226±0.049	0.446 ±0.004
H-68	0.120±0.001	0.683 ±0.196
Control	0.094±0.017	0.104 ± 0.005

Table-6.Quantitative screening of isolates for Petrol degradation

Isolate	Initial Optical density	Optical density after 3 Days
H-40	0.132 ± 0.019	0.387 ± 0.050
H-49	0.093 ± 0.012	0.420 ± 0.082
H-55	0.086 ± 0.024	0.422 ± 0.217
H-56	0.180 ± 0.003	0.874 ± 0.030
H-66	0.115 ± 0.046	0.353 ± 0.163
Control	0.077 ± 0.013	0.084± 0.021

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