Optimisation of Environmental Factors on Oil Degrading Bacteria Isolated from Coastal Water and Sediments in Sri Lanka

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Abstract

Better understanding of the mechanisms of hydrocarbon degrading microorganisms and effect of some environmental factors is critical for the optimisation of the bioremediation processes. Temperature, pH, nitrate and phosphate are the major factors that influence there mediation process of bacterium. In the present study, optimisations some selected physico-chemical parameters (temperature, pH, nitrate and phosphate) were carried out on *Bacillus cereus*, *Enterobacter* sp. and *Enterobacter ludwigii* which were previously isolated as potential oil degraders. The bacteria showed maximum degradation of crude oil at 33^{o}C where the desirable pH was 8.6 for all the isolates except *E. ludwigii* (pH 5.4). A significant degradation ($p < 0.05$) of oil was detected by *B. cereus* (80% to 98%), *Enterobacter* sp. (73% to 90%) and *E. ludwigii* (70% to 83%) respectively with increasing of nitrate concentration from 0.1 to 2.5 ppm. Significant degradation of oil was not detected in the control and when bacteria were enriched with phosphate. Results of this study revealed that the bacterial remediation of oil is governed by nutritional status with special emphasis of nitrate enrichment in the environment. Thus, the results revealed that bacteria could be a useful tool to remove oil from the contaminated environment as eco-friendly, low cost application.

Key words: bioremediation, crude oil, Bacillus cereus, Enterobacter sp., *Enterobacter ludwigii*

1. Introduction

Oil spill pollution, a severe environmental problem across the world, is growing with increased levels of oil production and global transport. Its causes are either accidental or due to operation wherever oil is produced, transported, stored and used on sea or land (Mehrasbi et al., 2003). The hydrocarbons like PAHs (Poly Aromatic Hydrocarbons) have caused extensive damage to the local ecosystems since accumulation of pollutants in animals and plant tissues may cause progeny's death or mutation (Alvarez et al., 1991). This is mainly due to an accumulation of PAHs through food chain. Ultimately the effect on human health in high because humans are frequently the last part of the food chain. Therefore, dealing with these problems and to adopt appropriate solutions, fundamental and advance researches with policies are needed. Hanson et al. (1997), Sartoros et al. (2005), Xiaojun et al. (2008) and Joel et al. (2011) documented that bioremediation is more affordable application to remove oil contamination when compare with available physical and chemical removal methods. Bioremediation is the treatment process that uses microorganisms such as yeast, fungi and bacteria to break down, or degrade hazardous substance into less toxic or nontoxic substances (Kim et al., 2005).

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During the remediation process, specific microorganisms consume organic substances in crude oil as sole carbon source for their energy requirement (Bouwer and Zehnder, 1993) and the aerobic and anaerobic respiration mechanisms convert hazarders' hydrocarbon in the environment to less harmful compounds such as carbon dioxide, methane and water (Farshid et al., 2011). *Corynebacterium ulcerans, C. amycolatum, Bacillus badius* and *Micrococus varians* were recorded as potential oil degradates (Omotayo et al., 2012) where Chin et al., (2008) documented that *Yokenella* spp., *Alcaligenes* spp., *Roseomonas* spp., *Flavobacter* spp., *Sphinogo bacterium* spp. and *Moraxella* spp. as petroleum hydrocarbon-degraders. Liyanage and Manage (2014) reported that *B. cereus*, *Enterobacter* sp. and *E. ludwigii* as crude oil degraders which were isolated from coastal environment in Sri Lanka. Bioremediation processes are governed by some environmental factors such as type and amount of crude oil present in the environment, environmental temperature, nutrients such as NO^-_3, PO_4^{3-}, pH, aeration, water acidity, type and population density of hydrocarbon degrading microorganisms and contaminant mobility (Dragun, 1998; Mace, 2012). Microbial growth rates in the environment are mainly depending on the temperature and rates of degradation decrease with decreasing temperature. (Gibb et al., 2001). Toone et al., (2013) documented that degradation mechanisms are enzyme mediated and depend on intracellular and extracellular enzyme activity of the bacteria.

Furthermore, the incomplete microbial degradation occurs when the ambient environment does not contain significant concentrations of nutrients such as nitrogen and phosphorous (Prince et al., 2002). Thus, inorganic nutrients are limiting factors for the natural biodegradation process (Trindade et al., 2002; Efsun et al., 2013). The majority of microorganisms thrive best in the pH range of 6 to 8 (Omatayo et al., 2012) and adjustment of pH range from 4.5 to 7.4 was resulted in a near doubling of biodegradation rates of hydrocarbon (Leahy and Colwell, 1990). Thus, the manipulation and optimisation of these factors are needed to enhance the remediation process of pollutants in the environment (Dragun, 1998; Boopathy, 2000). South Asian countries not only import much of oil for domestic consumption, but India, Maldives, Pakistan and Sri Lanka lie close to the main shipping route that connects the Middle East to the Far East (ITOPF, 2012).

Several oil spills were recorded around the Sri Lankan Sea during past few years (MEPA, 2012). Spillage near to Panadura Sea area was the most recent event. Considering the danger and environment impact on biodiversity, numerous solutions have been proposed by national and international agencies to remove oil from contaminated areas. However, the main limitations to use available techniques are inefficient trace level adsorption, hazardousness of chemicals and non-accessibility due to high cost (Kishore. and Ashisk, 2007). Thus, to minimise the environmental effects of oil pollution, *in situ* bioremediation has been suggested (MEPA, 2012). Therefore, the present study discusses the *in vitro* optimisation of environmental factors for three crude oil degrading bacteria strains isolated from oil contamination sites in the coastal environment in Sri Lanka. Those bacteria strains were identified as *Bacillus cereus* (KM504128), *Enterobacter* sp. (KM4055978) and *E. ludwigii* (KM504129) using 16S RNA sequences.

2. Methodology

2.1. Materials and reagents

Crude oil (100%) required for the present study was received from Sri Lanka Petroleum Corporation (Pvt) Ltd. Bacteriological grade chemicals were purchased from Sigma, Aldrich© while molecular grade chemicals were purchased from Promega©, USA.

2.2 Bacteria for degradation studies

Previously isolated oil degrading bacteria (Liyanage and Manage, 2014), identified as *Bacillus cereus* (KM504128), *Enterobacter* sp. (KM4055978) and *E. ludwigii* (KM504129) were used in the present study to optimise some selected physio-chemical environmental factors for bacterial degradation kinetics.

2.3 Preparation of bacteria cultures for optimization of environmental factors

A loop of each bacterial strain was transferred into 5 ml of liquid Lurial Bertani (LB) medium and incubated at 25^{o} C. Exponentially growing cultures were centrifuged at 1000 rpm for 15 minutes. The resulted pellets were re-suspended in 0.01M Phosphate Buffer Solution (PBS) and kept overnight to let out residual carbon content. Then the suspensions were centrifuged at 1000 rpm for 15 minutes and the pellets were washed three times using PBS. Turbidity of all bacterial suspensions were equalized (A 590=0.35) using spectrophotometer (SPECTRO UV-VIS double beam PC) (Manage et al., 2009). Equalised bacterial suspensions were used to optimisation some of the physic-chemical factor such as temperature, pH, phosphate and nitrate.

2.4 Effect of temperature on crude oil degradation

Filtered sterilised sea water was inoculated by 0.5 ml of overnight starved bacteria culture. Then 1% (v/v) crude oil was added to make final volume of 10 ml in universal bottles. Samples were incubated in three different temperatures at 23^{o} C, 28^{o} C and 33^{o} C respectively in the shaking incubator at 100 rpm. One milliliter of sub-samples was collected at two days intervals for a period of 14 days to determine the concentration of crude oil.

2.5 Effect of phosphate and nitrate concentration on crude oil degradation

Phosphate concentration in sea water varies between 0.002-0.05 ppm while the nitrate levels were between 0.1-2.5 ppm (James, 2009; Liyanage and Manage, 2014). In order to determine the effect of phosphate on the crude oil degradation, filtered sterile sea water was prepared at final concentrations of 0.002 ppm and 0.05 ppm supplemented with KH_2PO_4. Filter sterilised sea water was inoculated by 0.5 ml of overnight starved bacteria culture and 1% (v/v) crude oil at final volume of 10 ml in universal bottles. The bottles were incubated at 33^{o} C in 100 rpm. The temperature was determined based on the results of the present study and literature (Efsun et al., 2013). One milliliter of sub-samples was removed at two days intervals for a period of 14 days and the concentration of crude oil was measured spectrophotometrically. Same set of experiment procedure was followed to study the effect of NO_3^- on remediation of crude oil by the bacterium. NH_4NO_3 enriched sea water with varying concentrations (0.1-2.5 ppm) were used as nitrate source.

2.6 Effect of pH on crude oil degradation

Filtered sterile sea water in triplicate was supplemented with 1% (v/v) crude oil and 0.5 ml of exponentially growing culture of bacteria at final volume of 10 ml into the universal bottles. pH was adjusted to 5.4, 7.2 and 8.6 by adding 1.0M NaOH or 1.0M HCl where appropriated. The samples were incubated in the shaking incubator at 33^{o} C in 100 rpm. One millilitre of sub- samples was collected at two days intervals for a period of 14 days to determine the concentration of crude oil.

2.7 Estimation of crude oil concentration

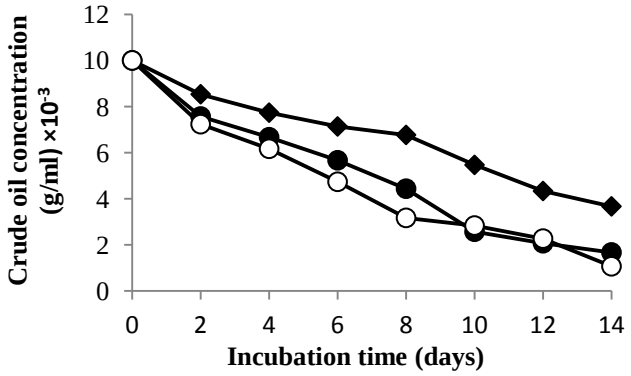
In order to estimate residual crude oil, 10 ml of analytical grade n-hexane was added to a flask containing 1 ml of sub-sample removed from the degradation experiment and then transferred in to a separating funnel to extract oil. The extracted aliquot was evaporated to dryness in the water bath at 69^{o} C and residue oil was dissolved in 10 ml of n-hexane and the concentration was measured according to Omatayo et al., (2012) using visible spectrophotometer at 400 nm. Concentration of oil in the sample was obtained using the pre-prepared standard curve and the dilution factor was considerate to calculate the actual concentration (Latha and Kalaivani, 2012). Recovery of analytical procedure was maintained to keep more than 95% before samples were subjected to final measurement (Liyanage and Manage, 2014).

2.8 Determination of degradation rates

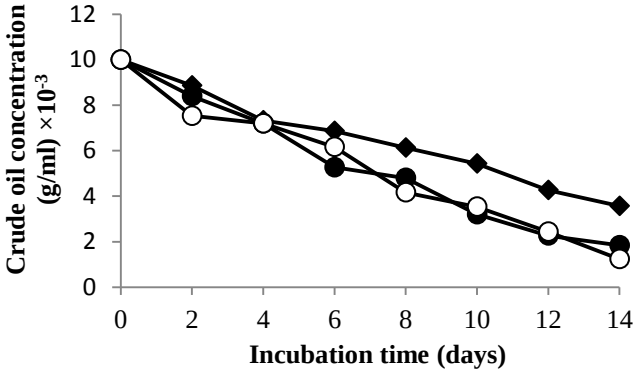
The degradation rates (*h*) of the bacteria were calculated using the equation bellow, where "C_o" and "C" are the concentration of crude oil at the beginning and at the end of the time interval "t" respectively (Manage et al., 2000).

3. Results

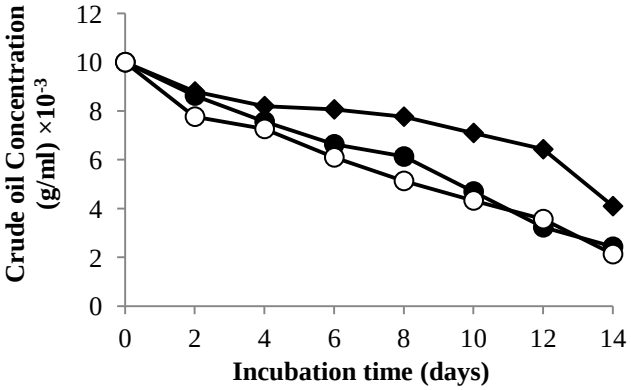
3.1 Effect of water temperature on bacterial degradation of crude oil



(a) *B. cereus*



(b) Enterobacter sp.



(c) *E. ludwigii*

Figure 1: (a) (b) (c). Degradation of crude oil (0.01 gml^{-1}) by bacteria in different temperatures. 23^{o} C (black square), 28^{o} C (close circle) and 33^{o} C (open circle).

Figure 1 (a), (b) and (c) illustrate the degradation of crude oil by *B. cereus*, *E. ludwigii* and *Enterobacter* sp. at three different temperatures. When temperature increased to 28^{o} C and 33^{o} C the degradation of crude oil by the bacteria isolates were initiated without a lag phase was detected. On the other hand, degradation at 23^{o} C was slow in all bacteria strains and remained less than 60% except *B. cereus* following 14 days of incubation.

Table 1: Degradation rates of crude oil by bacteria isolates in different temperature.

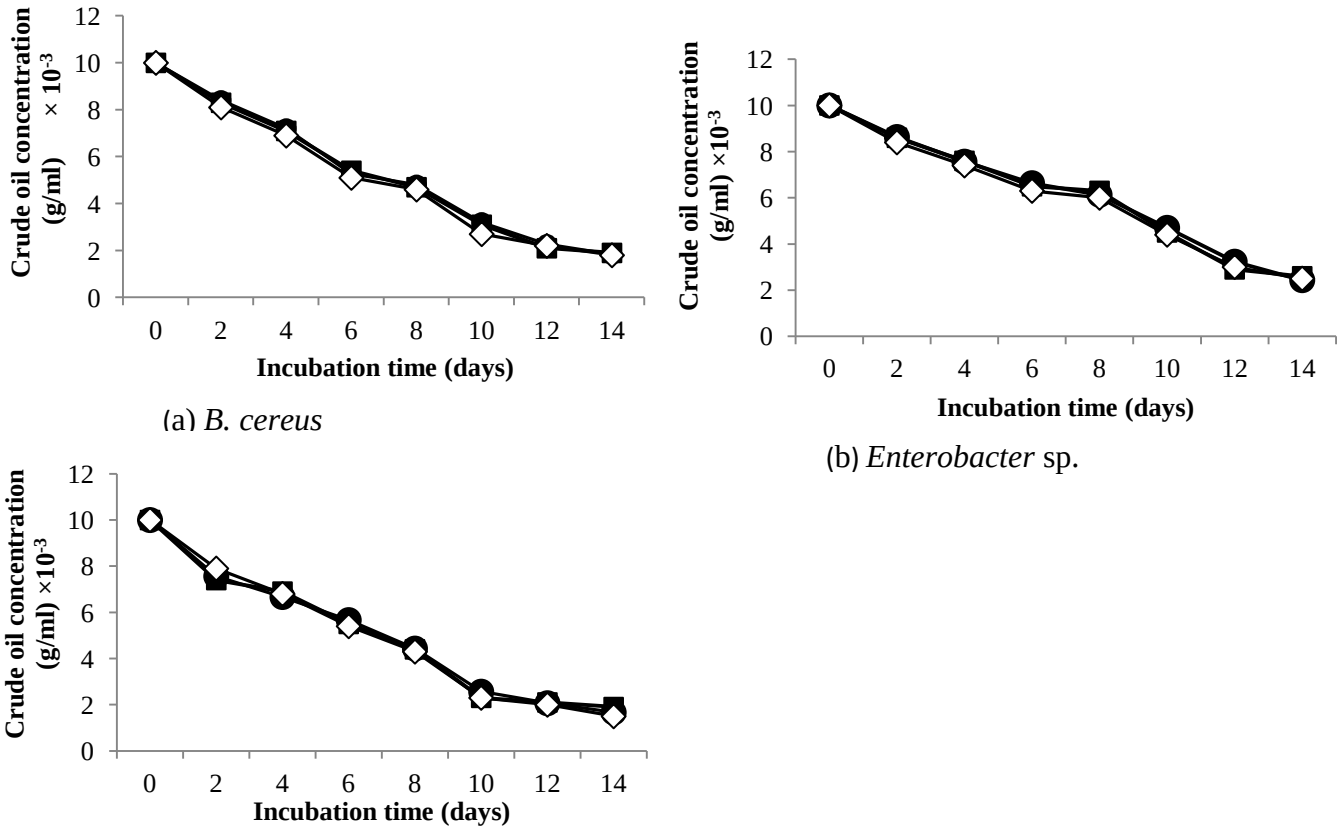
Incubation time (days)	Name of bacteria	Degradation rate (day^{-1})		
		23^{o} C	28^{o} C	33^{o} C
0-2	*B. cereus*	0.540 ±0.008	0.740 ±0.008	1.510 ±0.002
	Enterobacter sp.	0.470 ±0.007	0.652 ±0.008	0.812 ±0.011
	E. ludwigii	0.350 ±0.005	0.581 ±0.008	0.678 ±0.003
2-4	*B. cereus*	0.480 ±0.008	0.642 ±0.008	1.213 ±0.002
	Enterobacter sp.	0.450 ±0.007	0.583 ±0.008	0.585 ±0.011
	E. ludwigii	0.420 ±0.005	0.564 ±0.008	0.572 ±0.003
4-6	*B. cereus*	0.475 ±0.008	0.689 ±0.008	1.012 ±0.002
	Enterobacter sp.	0.472 ±0.007	0.595 ±0.008	0.523 ±0.011
	E. ludwigii	0.465 ±0.005	0.578 ±0.008	0.575 ±0.003
6-8	*B. cereus*	0.515 ±0.008	0.712 ±0.008	1.112 ±0.002
	Enterobacter sp.	0.532 ±0.007	0.612 ±0.008	0.586 ±0.011
	E. ludwigii	0.486 ±0.005	0.593 ±0.008	0.590 ±0.003
8-10	*B. cereus*	0.315 ±0.008	0.432 ±0.008	0.765 ±0.002
	Enterobacter sp.	0.332 ±0.007	0.392 ±0.008	0.543 ±0.011
	E. ludwigii	0.416 ±0.005	0.393 ±0.008	0.323 ±0.003
12-14	*B. cereus*	0.115 ±0.008	0.102±0.008	0.117±0.002
	Enterobacter sp.	0.087±0.007	0.088 ±0.008	0.102 ±0.011
	E. ludwigii	0.076 ±0.005	0.077 ±0.008	0.089 ±0.003

At 33^{o} C 92% removal of oil was detected by *B. cerues*, whereas *Enterobacter* sp. and *E. ludwigii* removed 79% and 88% respectively at 14 days of incubation. The highest degradation percentage of oil was recorded at 33^{o} C and it was significantly different from the removal percentages of crude oil obtained at 23° C and 28° C respectively. The rate of crude oil degradation by any bacterial isolate relies mainly on the incubation temperature. The highest degradation rate of oil was detected for all bacteria strains at 33^{o} C (Table 1) where *B. cereus* showed the highest degradation rate (1.510 ±0.002 d^{-1}) after two days of incubation. Thus, 33^{o} C temperature was selected as optimum temperature to carry out the rest of experiments planned in the present study.

Degradation rate of *B. cereus* was 0.540±0.008 d^{-1}and 0.740±0.008 d^{-1} at 23^{o} C and 28^{o} C respectively. *Enterobacter* sp. and *E. ludwigii* showed 0.812±0.011 d^{-1} and 0.678 ±0.003 d^{-1} degradation rates at 33^{o} C after 2 days of incubation respectively. In order to evaluate the possible influence of nutrients, especially nitrates and phosphates on bacterial degradation of crude oil, bacterial cultures were incubated separately in different concentrations of nitrate and phosphate with crude oil.

3.2 Effect of nutrients on degradation of crude oil

The phosphate concentration in the sea water at the time of bacterial isolation was ranged between 0.002 to 0.05 ppm. Similar concentration range of phosphate was used to study degradation kinetics of oil *in vitro* in the present study. Significant degradation of oil with inoculation of phosphate was not recorded (Figure 2). The degradation rate of *B. cereus* varied between 0.735±0.002 d^{-1}-0.755±0.008 d^{-1} at 0-2 days of incubation and maintained more or less constant degradation rate (0.642±0.008-0.692±0.005 d^{-1}) at 2-8 days of incubation for each phosphate concentration. Then degradation rate increased from 0.642±0.008 d^{-1} to 0.756±0.003 d^{-1} at 8-10 days of incubation and then decreased to 0.102±0.002 d^{-1} at 10-14 days of incubation (Figure 2(a)). Degradation rate of oil by *Enterobacter* sp. was changed between 0.457±0.003 d^{-1}-0.682±0.021 d^{-1} at 0-8 days and was increased to 0.692±0.001 d^{-1}at 8-10 days. There after degradation rate was decreased from 0.692±0.021 d^{-1} to 0.069±0.003 d^{-1}at 10-14 days (Figure 2(b)). Significant difference of degradation rates was not detected between control and experiments setup for *Enterobacter* sp. as well. At each phosphate concentration *E. ludwigii* also showed less or more constant range of degradation (0.386 to 0.525 d^{-1}) at 0-12 days and then gradually decrease (0.072 to 0.083 d^{-1}) at 12-14 days of incubation (Figure 2(c)).



(a) *B. cereus*

(b) *Enterobacter* sp.

(c) *E. Ludwigii*

Figure 2: Degradation of crude oil (0.01g/ml) by bacteria isolates with phosphate enrichment. (a) *B. cereus* (b) *Enterobacter* sp. (c) *E. ludwigii*; 0.002 ppm (close square), control (close circle) and 0.05 ppm (open square).

Table 2: Degradation rates of crude oil by bacteria with phosphate enrichment.

Incubation time (days)	Name of bacteria	Degradation rate (day^{-1})		
		Control	0.002 ppm	0.005 ppm
0-2	*B. cereus*	0.735 ±0.008	0.740 ±0.008	0.755 ±0.002
	Enterobacter sp.	0.682 ±0.007	0.652 ±0.008	0.675±0.011
	E. ludwigii	0.479 ±0.005	0.481 ±0.008	0.478 ±0.003
2-4	*B. cereus*	0.657 ±0.008	0.642 ±0.008	0.659 ±0.002
	Enterobacter sp.	0.595 ±0.007	0.603 ±0.008	0.585 ±0.011
	E. ludwigii	0.429 ±0.005	0.432 ±0.008	0.439 ±0.003
4-6	*B. cereus*	0.675 ±0.008	0.689 ±0.008	0.654 ±0.002
	Enterobacter sp.	0.587 ±0.007	0.593 ±0.008	0.579 ±0.011
	E. ludwigii	0.458 ±0.005	0.461 ±0.008	0.455 ±0.003
6-8	*B. cereus*	0.683 ±0.008	0.692 ±0.008	0.678 ±0.002
	Enterobacter sp.	0.457 ±0.007	0.476 ±0.008	0.467 ±0.011
	E. ludwigii	0.397 ±0.005	0.393 ±0.008	0.386 ±0.003
8-10	*B. cereus*	0.789 ±0.008	0.756 ±0.008	0.765 ±0.002
	Enterobacter sp.	0.687±0.007	0.692 ±0.008	0.674 ±0.011
	E. ludwigii	0.495 ±0.005	0.512 ±0.008	0.525 ±0.003
10-12	*B. cereus*	0.105 ±0.008	0.108±0.008	0.111±0.002
	Enterobacter sp.	0.079±0.007	0.083 ±0.008	0.087 ±0.011
	E. ludwigii	0.503 ±0.005	0.512 ±0.008	0.499 ±0.003
12-14	*B. cereus*	0.105 ±0.008	0.102±0.008	0.107±0.002
	Enterobacter sp.	0.078±0.007	0.069 ±0.008	0.083 ±0.011
	E. ludwigii	0.072 ±0.005	0.083 ±0.008	0.082 ±0.003

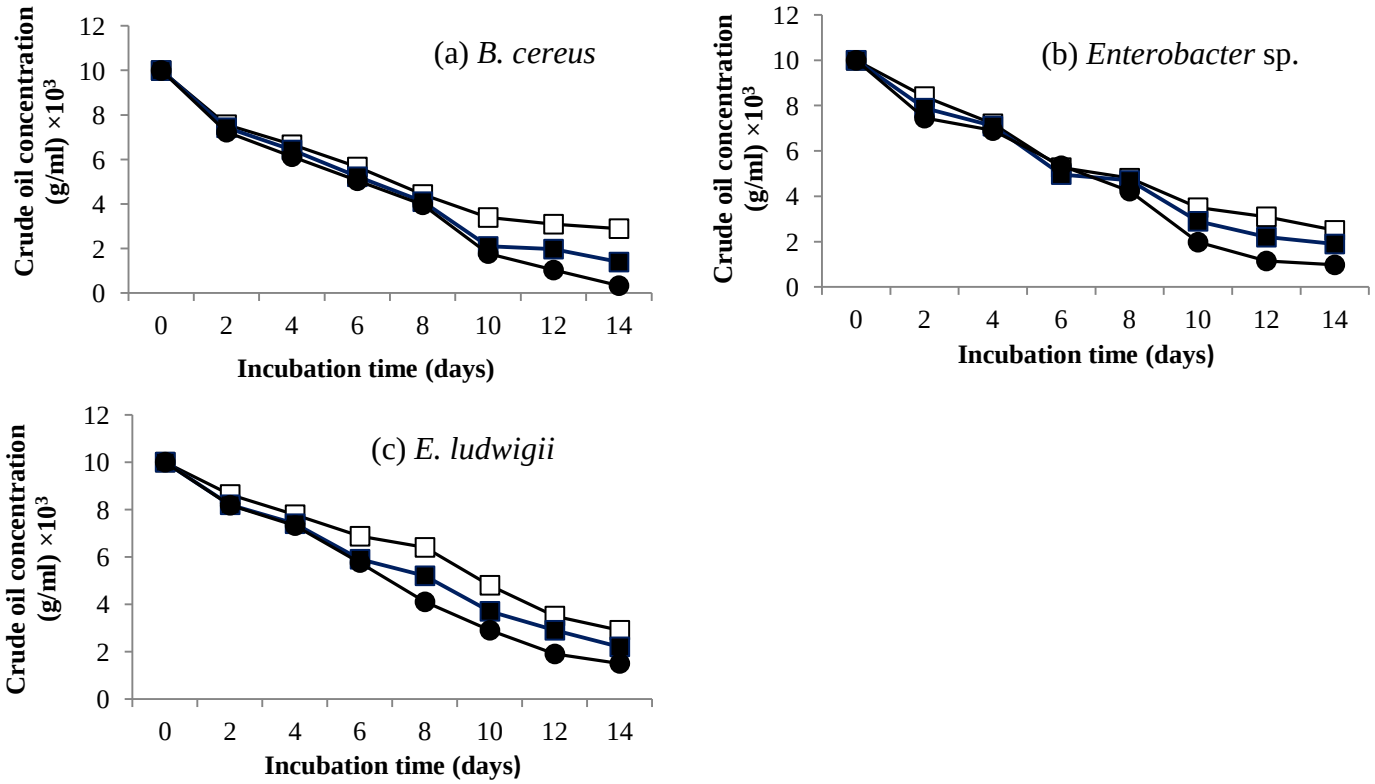


Figure 3: Degradation of crude oil (0.01 g/ml) by bacteria in the presence of nitrate. (a) *B. cereus* (b) *Enterobacter* sp. (c) *E. ludwigii*; 1.0 ppm (black square), 0.1 ppm (open square) and 2.5 ppm (close circle).

Table 3: Degradation rates of crude oil by bacteria in the presence of nitrate.

Incubation time (days)	Bacteria	Degradation rate (day^{-1})		
		Control	0.1 ppm	2.5 ppm
0-2	*B. cereus*	0.742 ±0.008	0.738 ±0.008	0.749 ±0.002
	Enterobacter sp.	0.654 ±0.007	0.664 ±0.008	0.672±0.011
	E. ludwigii	0.572 ±0.005	0.578 ±0.008	0.583 ±0.003
2-4	*B. cereus*	0.603 ±0.008	0.598 ±0.008	0.612 ±0.002
	Enterobacter sp.	0.575 ±0.007	0.583 ±0.008	0.585 ±0.011
	E. ludwigii	0.323 ±0.005	0.357 ±0.008	0.378 ±0.003
4-6	*B. cereus*	0.597 ±0.008	0.621 ±0.008	0.632 ±0.002
	Enterobacter sp.	0.632 ±0.007	0.648 ±0.008	0.663 ±0.011
	E. ludwigii	0.356 ±0.005	0.392 ±0.008	0.401 ±0.003
6-8	*B. cereus*	0.595 ±0.008	0.626 ±0.008	0.628 ±0.002
	Enterobacter sp.	0.432 ±0.007	0.412 ±0.008	0.441 ±0.011
	E. ludwigii	0.345 ±0.005	0.357 ±0.008	0.489 ±0.003
8-10	*B. cereus*	0.621 ±0.008	0.791 ±0.008	0.845 ±0.002
	Enterobacter sp.	0.689 ±0.007	0.698 ±0.008	0.732 ±0.011
	E. ludwigii	0.534 ±0.005	0.567 ±0.008	0.592 ±0.003
10-12	*B. cereus*	0.374 ±0.008	0.432 ±0.008	0.692 ±0.002
	Enterobacter sp.	0.112 ±0.007	0.231 ±0.008	0.541 ±0.011
	E. ludwigii	0.521 ±0.005	0.572 ±0.008	0.579 ±0.003
12-14	*B. cereus*	0.015 ±0.008	0.132±0.008	0.147±0.002
	Enterobacter sp.	0.079±0.007	0.081 ±0.008	0.083 ±0.011
	E. ludwigii	0.071 ±0.005	0.076 ±0.008	0.082 ±0.003

A rapid degradation of crude oil was recorded by all three strains at 14 days of incubation with the increase of nitrate concentration in the medium from 0.1 to 2.5 ppm. Crude oil degradation percentage for *B. cereus* increased from 80% to 98% where *Enterobacter* sp. from 73% to 90% and in *E. ludwigii* from 70% to 83% respectively (Figure 3).

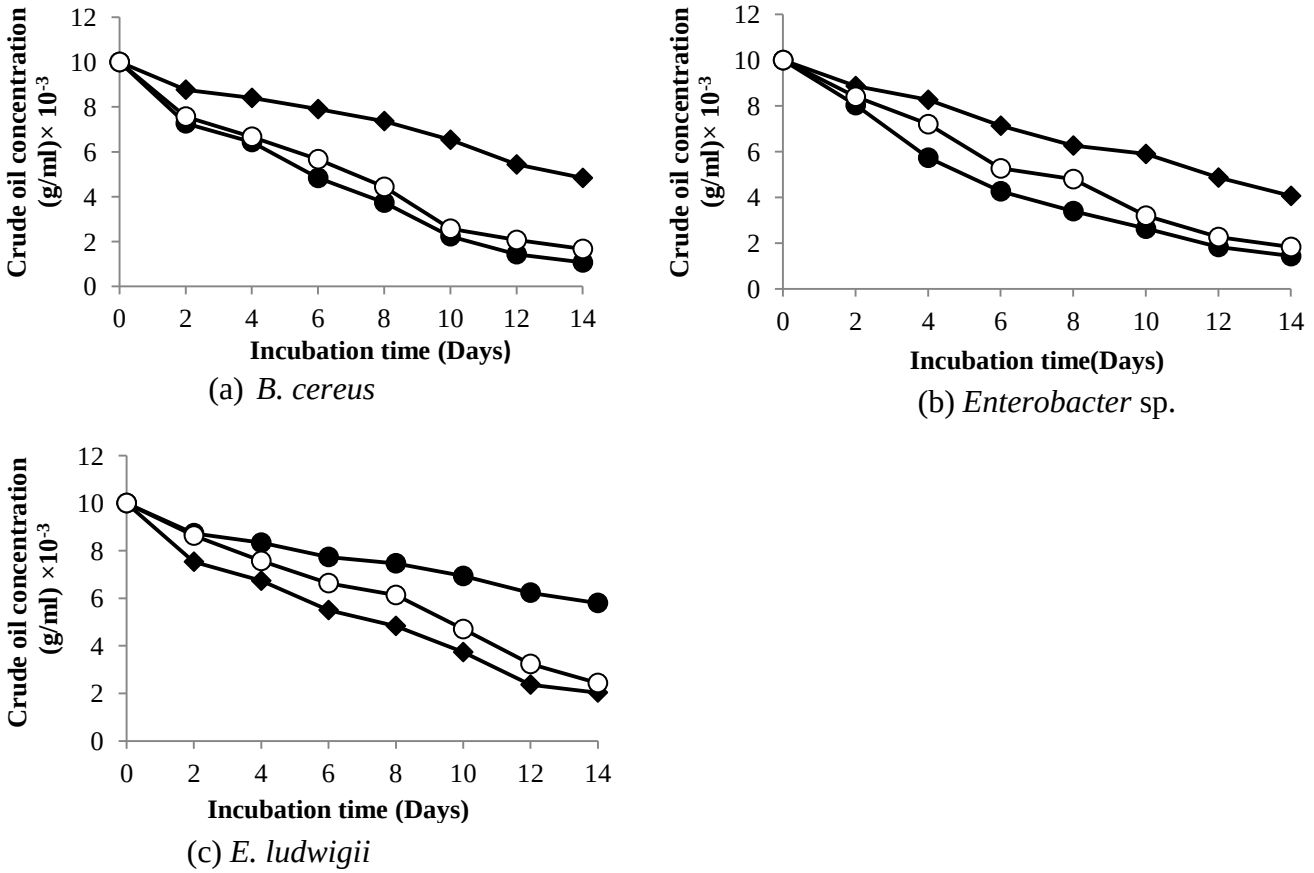
Out of two nitrate concentrations, 2.5 ppm proved to be the most potent nitrate concentration which seems accelerates bacterial growth. The degradation rate of *B. cereus* gradually decreased from 0.749±0.05 to 0.628±0.03 d^{-1} during 0-8 days of incubation and thereafter increased rapidly to 0.845 d^{-1} during 8-10 days when medium contain 2.5 ppm nitrate. The lowest degradation rate (0.147 d^{-1}) by the *B. cereus* was showed at 12-14 of incubation. The other two bacterial strains showed lower degradation rate throughout the incubation period compare to *B. cereus*.

At 2.5 ppm nitrate concentration, oil degradation rate of *Enterobacter* sp. decreased from 0.672 to 0.441 d^{-1}during 0-8 days of incubation where more or less similar degradation rate was recorded by *E. ludwigii* (0.583 to 0.489 d^{-1}).Thereafter, degradation rate was gradually increased by *Enterobacter* sp. (0.732d^{-1}) and *E. ludwigii* (0.592 d^{-1}) at 8-10 days of incubation and decreased again to 0.083 and 0.082 d^{-1}respectively for *Enterobacter* sp., *E. ludwigii* at 12-14 days of incubation.

3.3 Effect of pH on degradation of crude oil

Bacteria isolates employed in the present study showed different degradation rates in different pH. The highest degradation (84%) showed in acidic medium (pH 5.4) by *E. ludwigii*. In contrast *B. cereus* (94%) and *Enterobacter* sp. (88%) showed great degradation trends along with incubation in alkaline medium (pH 8.6).

The highest degradation rate (0.767 d^{-1}) was detected at 0-2 days of incubation and the degradation rate was decreased from 0.534±0.03 to 0.082±0.03 d^{-1} during 4-14 days of incubation, where degradation rate of *Enterobacter* sp. decreased gradually from 0.634±0.04 d^{-1} to 0.098±0.05 d^{-1} during 0-14 days when the medium was alkaline (pH= 8.6) at 33^{o} C (Table 4).



(a) *B. cereus* (b) *Enterobacter* sp. (c) *E. ludwigii*

Figure 4: Degradation of crude oil (0.01g/ml)by the bacteria in different pH.
(a) *B. cereus* (b) *Enterobacter* sp.(c) *E. ludwigii*; pH= 5.4 (black square), pH= 8.6 (close circle) and pH=7.2 (open circle).

Results of the present study revealed that favourable environmental factors often lead to increase the bioremediation rate of native crude oil degrading bacteria. Among the favourable factors; water temperature, nitrate and phosphate concentration and pH were considered as an essential component maintaining the balance of natural bacterial community.

Table 4: Degradation rates of bacterial strains in different pH values.

Incubation time (days)	Bacteria	Degradation rate (day^{-1})		
		5.4	Control (7.2)	8.6
0-2	*B. cereus*	0.232 ±0.008	0.740 ±0.008	0.767 ±0.002
	Enterobacter sp.	0.634 ±0.007	0.652 ±0.008	0.687 ±0.011
	E. ludwigii	0.678 ±0.005	0.581 ±0.008	0.357 ±0.003
2-4	*B. cereus*	0.213 ±0.008	0.341 ±0.008	0.349 ±0.002
	Enterobacter sp.	0.342 ±0.007	0.586 ±0.008	0.678 ±0.011
	E. ludwigii	0.572 ±0.005	0.564 ±0.008	0.327 ±0.003
4-6	*B. cereus*	0.237 ±0.008	0.225 ±0.008	0.534 ±0.002
	Enterobacter sp.	0.357 ±0.007	0.599 ±0.008	0.623 ±0.011
	E. ludwigii	0.575 ±0.005	0.578 ±0.008	0.278 ±0.003
6-8	*B. cereus*	0.132 ±0.008	0.337 ±0.008	0.421 ±0.002
	Enterobacter sp.	0.123 ±0.007	0.215 ±0.008	0.634 ±0.011
	E. ludwigii	0.583 ±0.005	0.593 ±0.008	0.221 ±0.003
8-10	*B. cereus*	0.098 ±0.008	0.339 ±0.008	0.419 ±0.002
	Enterobacter sp.	0.142 ±0.007	0.392 ±0.008	0.597 ±0.011
	E. ludwigii	0.457 ±0.005	0.393 ±0.008	0.189 ±0.003
10-12	*B. cereus*	0.087 ±0.008	0.338 ±0.008	0.415 ±0.002
	Enterobacter sp.	0.156 ±0.007	0.387 ±0.008	0.512 ±0.011
	E. ludwigii	0.323 ±0.005	0.393 ±0.008	0.106 ±0.003
12-14	*B. cereus*	0.085 ±0.008	0.079±0.008	0.082 ±0.002
	Enterobacter sp.	0.134 ±0.007	0.102 ±0.008	0.098 ±0.011
	E. ludwigii	0.066 ±0.005	0.077 ±0.008	0.057 ±0.003

4. Discussion

Sri Lanka is exposed as the worst polluter of the Indian ocean (NCEAS, 2012) and the irresponsible use of land practices, dumping oil waste to the sea, shipping activities are enhanced the contamination load of hydrocarbons, inorganic chemicals in the Indian ocean (MEPA, 2012). Therefore, the sites where the bacterial strains were isolated are more vulnerable to oil contamination with massive shipping activity and improper practices. Recently national and international agencies have received greater attention on oil pollutant status of sea and bioremediation application was suggested as an effective and eco-friendly solution (Mace, 2012).

A limited number of research has been carried out up to date on microbial degradation of crude oil. Recent time, the contamination of crude oil in coastal area is becoming a problem and poses a potential threat to human health as well. Thus, application of microbes to remediate oil is important as natural method (Thavasi et al., 2011; Hassanshahian et al., 2012). Liyanage and Manage, (2014) reported isolation and characterization of oil degrading bacteria from coastal zone in Sri Lanka as a first report, and the present study was aimed to optimize the environmental factors that effect on the bacterial isolates.

Bioremediation processes are governed by some environmental factors such as type and amount of crude oil present in the environment, environmental temperature, nutrients such as NO^{-}_{3}, PO_4^{3-}, pH, aeration, water acidity, type and population density of hydrocarbon degrading microorganisms and contaminant mobility (Dragun, 1998; Mace, 2012). Leahy and Colwell, (1990) reported the degradation

of hydrocarbon reached 29.8% after 3 days of treatment at 30^{o} C, whereas at 20^{o} C, the degradation of TPH (Total Petroleum Hydrocarbon) reached only 7%. This indicates that bacteria prefer higher temperatures, which enhance bacteria growth, following accelerates bioremediation. In the present study, at 23°C, all strains showed low degradation of crude oil and pronounced degradation was detected with increase temperature at 33 ^{0}C for all the bacterial strains. However, Alberty (2011) recorded that when temperature increases higher than 33 °C was resulted a decreasing degradation ability of bacterial strains. It is likely that at high temperatures, bacterial cells are unable to produce crude oil degrading enzymes which may result slow degradation rates (Amer et al., 2014).

The studies carried out by Zaccone et al., (2002) have clearly described the effect of seasonal variance of nutrients in water which enhance the metabolic activities of heterotrophic bacteria. Odu, (1978) documented that, after 12 weeks of incubation oil degradation by bacteria was not significantly increased in the media which was treated with phosphate. Similar results were obtained from the present study as the degradation of oil by the bacterium was not significant when the media was supplemented with phosphate (Figure 2). Zahra and Alireza, (2005) documented that NH_4NO_3 is the best nitrogen source which maximize the degradation of oil by bacteria. Results of the present study also showed that increasing of nitrate concentration in the medium from 0.1 to 2.5 ppm, enhance the oil degradation by all bacterial species (Figure 3). Ronald, (1996) documented that phosphate concentration on oil degradation is less effective compare with nitrogen. The analysis of the effect of phosphate and nitrate concentrations on crude oil degradation percentage showed that phosphate concentration does not have a potent on removal of crude oil where nitrate performs a major role on degradation process; through the degradation rate was subjected to fluctuation (Figure 3 and Figure 4). Biochemical reactions of microorganisms are catalysed by enzymes (Zahra and Alireza, 2012) and it is well known fact that enzymatic reactions occur within a suitable pH range for microorganisms which are sensitive to alterations of pH (Zahra and Alireza, 2012; Ron and Rosernberg, 2014). Results of the present study also revealed that all the bacteria isolates except *E. ludwigii* showed the highest degradation when the medium was alkaline (pH=8.6) (Figure 4) where similar condition was recorded by Diaz-Ramirez et al., (2013) for degradation of oil by the *Bacillus* sp. in alkaline media as well. Thus, the results of the present study showed the effect of pH is one of the limiting factors for bio stimulation which enhance the bacterial bio remediation process in the natural environment.

In addition to the results revealed that favourable environmental factors often lead to increase the bioremediation rate of native crude oil degrading bacteria. Further researches in this field can result in the development of most efficient and less time consuming microbial technologies which are important for developing country like Sri Lanka as accelerated development has lounged international shipping harbours and fishing harbours along the coast of the country.

Put in harnessing microbes to degradation of petroleum became world interest and scientists are being established soon remediation methods efficient, economic, versatile and environmentally sound treatment to remove hydrocarbons form the contaminated environment. The results of the present study have showed that the bacterium *B. cereus*, *Enterobacter* sp. and *E. ludwigii* can be used as microbial agents to remove crude oil from contamination sites of the environment and temperature, pH and nitrate concentrations are critically effect on natural oil degradation process in the environment.

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