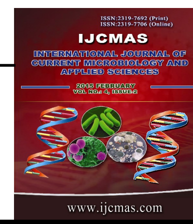
Int.J.Curr.Microbiol.App.Sci (2015) 4(2): 322-328

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 2 (2015) pp. 322-328**
http://www.ijcmas.com



Original Research Article

Parametric Optimization of Media for the Crude Oil Degrading Bacteria Isolated from Crude Oil Contaminated Site

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A B S T R A C T

With the increasing population and rapid urbanization of mankind, it is very much important to keep our agricultural land safe and free of any type of pollutant. For a developing country like India this becomes mandatory to keep a check on their agricultural land. Crude oil is a major pollutant of land as the cases of crude oil spillage are increasing day by day. Bioremediation is a better option to keep our land free of any type of pollutant. Bacteria capable of degrading crude oil were isolated from the soil samples contaminated with crude oil. Various parameters affecting the bacteria were studied and parametric optimization of the bacteria was done. The bacteria isolated from the soil samples were *Bacillus cereus*, *Pseudoxanthomonas mexicana, Halomonas daqingensis* and *Parapusillimonas granuli.* Effect of each parameter on degradation efficiency for each microbe is discussed based on the results obtained from various experiments.

Keywords

Crude oil, Bioremediation, Parametric Optimization, Gas Chromatography

Introduction

Due to rapid growth of human population, demand for crude oil and its other derivatives have increased. Thus in order to fulfill this demand transportation of crude oil is carried out on a great scale and therefore the oil spillages become a consequence for this. Because of the oil spillage the land becomes polluted and it becomes almost impossible for us to utilize the land again, especially for the agricultural use. Biodegradation of the crude oil done by the microorganisms is a safer way to get rid of the pollutants as this is cheap method as well as safe for the environment as the end products of the microbial degradation are not lethal for the environment (Das *et al.,* 2014; Guru *et al.,* 2013; Toledo *et al.,* 2006; Vincent *et al.,* 2011). Bioremediation of waste materials, which contain hydrocarbons and their derivatives, is based on the ability of microorganisms to increase their biomass growing on these substrates and to degrade them to non-toxic products, such as H_2O and CO_2 (Toledo *et al.,* 2006).

Petroleum hydrocarbon can be degraded by various microorganisms such as bacteria, fungi and yeast. Studies have shown that most potential bacteria for petroleum hydrocarbon degradation have been isolated

from areas contaminated with oil. (Guru *et al.,* 2013; Kuyukina *et al.,* 2005) The application of bacterial isolates in degrading crude oil involves the manipulation of environmental parameters to allow microbial growth and degradation to proceed at a faster rate (Vidali, 2001) The present study is focused on optimization of parameters to enhance the degradation efficiency of crude oil by the bacterial isolates from the crude oil contaminated site.

Materials and Methods

Isolation of bacteria

Soil samples of different depth were collected from the oil field (ONGC). The samples were collected in pre-sterilized sample bottles and also in whirl pack bags following aseptic conditions. The soil samples were stored at 4^0C for further analysis. The initial isolation was carried out as per the method described by Prescott. In the process, 10 grams of soil sample were mixed with 100ml of 0.09% N-saline and vortexed for 10 minutes; the supernatant was collected in a fresh tube and was used as an inoculum after serial dilution. The inoculums were streaked on N-agar plates and were incubated at 37^0C for 48 hours. (Prescott, 2002).

Different colonies were selected depending upon the morphological characters and further isolation of the crude oil degrading bacteria was done by enrichment method. The isolated bacteria were grown on Bushnell Hass medium. Only crude oil degrading bacteria were capable of growing on this medium (Guru *et al.,* 2013). The isolated bacteria were used as inoculum and added in 250ml flask containing 100ml Bushnell Hass broth and 1% crude oil, only four organisms showed potency to degrade the crude oil within 24–48 hours whereas others took a period of about 120–146 hours.

Identification of bacteria

Identification of the isolated bacteria was done by morphological characterization. Gram staining of the isolated bacteria was done in order to identify whether the isolated bacteria were Gram positive or negative. Motility test of the isolated bacteria was performed as well as spore forming capacity of the bacteria was also checked.

All these procedures were carried out as per standard procedure (Prescott, 2002). For confirmation of microbes 16s rRNA sequencing analysis was done. Obtained sequences were BLAST on NCBI to identify the microbes. Sequences were also submitted to NCBI databank.

Determination of potential carbon source

Carbon sources such as glucose, sucrose, yeast extract and crude oil were used for this study. Each of these carbon sources was added to 20 mL of Bushnell Hass broth at a concentration of 0.5% (w/v) at the optimum pH determined previously. One percent of crude oil was added separately into the medium.

The cultures were incubated at 37° C in an orbital shaker operated at 150 rpm. The cultures were centrifuged for few minutes and supernatant was taken and the O. D was taken at 600 nm using UV visible spectrophotometer. The O.D was noted at 24, 48 and 72 hours period of time.

Determination of potential nitrogen sources

Nitrogen sources such as peptone, ammonium nitrate and glycine were used for this study. Each of these nitrogen sources was added to 20 mL of Bushnell haas broth at a concentration of 0.5% (w/v) at the

optimum pH determined previously. One percent of crude oil was added separately into the medium. The cultures were incubated at 37° C in an orbital shaker operated at 150 rpm. The cultures were centrifuged for few minutes and supernatant was taken and the O. D was taken at 600 nm using UV visible spectrophotometer. The readings were taken at the interval of 24, 48 and 72 hours.

Determination of optimum pH

Standardized inoculum was prepared in 20 ml of Bushnell Hass broth, 1% crude oil was added to each test tube. The adjusted pH of the medium was 5.0, 6.0, 7.0 and 8.0 with 1N NaOH or 1N HCl.

The cultures were incubated at 37°C on orbital shaker at a speed of 150 rpm. The cultures were centrifuged for few minutes and supernatant was taken and the O.D. was taken at 600 nm using UV visible spectrophotometer. The readings were taken for 24, 48 and 72 hours.

Determination of optimum salt concentration

In many studies it was found that salts also have effect on crude oil degradation therefore salt concentration was also determined for optimum activity. NaCl was used as salt. Different concentration of NaCl was added to 20 mL of Bushnell Haas broth at a concentration of 5.0%, 10.0%, 15.0% and 20.0% (w/v)

The cultures were incubated at 37° C in an orbital shaker operated at 150 rpm. The cultures were centrifuged for few minutes and supernatant was taken and the O. D was taken at 600 nm using UV visible spectrophotometer. The readings were taken at the interval of 24, 48 and 72 hours.

Result and Discussion

Isolation and identification of bacteria

Four potential microbes capable of crude oil were isolated from the collected samples. Table 1 shows the characteristics of isolated microbes. From 16s rRNA sequencing, it was found that the isolated microbes are *Bacillus cereus*, *Pseudoxanthomonas mexicana, Halomonas daqingensis* and *Parapusillimonas granuli*. Sequence of these microbes were submitted to NCBI and given unique identification numbers which are KM192258, KM192259, KM192260 and KM192261 respectively.

From the obtained results it was found that yeast extract is the most efficient carbon source for the growth of all the microbes during the first 24 hrs. (Table 2) Upon extension of incubation period rapid decrease in the growth was observed. In case of glucose steady growth of microbes was observed for longer period of times.

As compare to other sources, crude oil has shown least tendency for microbial growth. However the growth was slow but it was steady for longer period of time. The reason behind lesser growth might be slow degradation of crude oil. This slow degradation results into slower liberation of monomers required for the growth and development of microbes.

Maximum degradation of crude oil was achieved by *Pseudoxanthomonas mexicana* with optical density of 0.823 at 600nm followed by *Halomonas daqingensis, Parapusillimonas granuli* and *Bacillus cereus*. Surprisingly *Bacillus* species was found least efficient, otherwise in the most of the study it has shown a very potential effect (Darvishi *et al.,* 2011; Gopinathan *et al.,* 2012; Pandya and Chandra, 2013).

Among the various sources used for nitrogen source, peptone has shown significant effect on the microbial growth (Table 3). This is because of amino acids, peptides and other growth promoters present in the peptone which are readily available for growth of microbes. Glycine and ammonium nitrate belong a pure salt cannot be utilized by the microbes directly.

They have to be converted into other some element required for the growth and normal functioning of microbes. When microbes were incubated with peptone for longer period of time they have shown very good growth of microbes as compare to other to sources (Darvishi *et al.,* 2011; Pandya and Chandra, 2013; Prabhakaran *et al.,* 2014)

Apart from carbon and nitrogen, pH of the medium is also another important parameter which will affect the growth of microbes. Results of the study have shown that all the microbes can grow optimally at neutral pH (7.0) (Table 4).

When various salt concentrations were tried to determine the optimum concentration for growth, there was no direct relation found between salt concentration and microbial growth (Table 5). All the microbes have shown unique growth pattern independent of salt concentration. (Darvishi *et al.,* 2011; Prabhakaran *et al.,* 2014)

From the overall study, it was found the yeast extract is one the most potential carbon source. However, each of these microbes is also able to degrade crude oil efficiently. Among the various nitrogen sources peptone was found most efficient. Salt concentration does not show any significant effect on growth.

Table.1 Characteristics of the bacteria yielded the following results

Organism	Gram Staining	Motility test	Spore formation
Pseudoxanthomonas mexicana	Gram negative	Motile	Non-sporulating
Halomonas daqingensis	Gram negative	Non- motile	Non-sporulating
Bacillus cereus	Gram positive	Motile	Sporulating
Parapusillimonas granuli	Gram negative	Motile	Non-sporulating

Table.2 Effect of carbon source on growth of microbes

Carbon Source	24 hrs	48 hrs	72 hrs
Bacillus cereus			
Glucose	0.440±0.018	1.320±0.078	2.143±0.103
Sucrose	0.216±0.022	1.166±0.066	1.781±0.089
Yeast extract	1.568±0.051	1.353±0.075	0.825±0.066
Crude oil	0.344±0.022	1.325±0.016	0.393±0.016
Pseudoxanthomonas Mexicana			
Glucose	0.529±0.041	1.224±0.077	1.925±0.121
Sucrose	0.370±0.023	1.333±0.092	1.542±0.079
Yeast extract	1.316±0.067	1.560±0.106	1.523±0.088
Crude oil	0.357±0.021	0.377±0.025	0.823±0.054
Halomonas daqingensis			
Glucose	0.763±0.051	1.862±0.102	1.739±0.078
Sucrose	0.359±0.024	1.236±0.087	2.179±0.121
Yeast extract	1.510±0.084	1.209±0.068	0.924±0.086
Crude oil	0.373±0.022	0.452±0.032	0.754±0.054
Parapusillimonas granuli			
Glucose	0.822±0.041	1.971±0.078	1.964±0.079
Sucrose	0.298±0.012	0.532±0.032	1.720±0.025
Yeast extract	1.281±0.069	1.915±0.089	0.762±0.034
Crude oil	0.272±0.009	0.320±0.012	0.549±0.021

Table.3 Effect of nitrogen source on growth of microbes

Nitrogen Source	24 hrs	48 hrs	72 hrs
Bacillus cereus			
Peptone	0.829±0.039	1.422±0.098	1.662±0.113
Ammonium Nitrate	0.336±0.021	0.138±0.008	0.284±0.022
Glycine	0.234±0.015	0.178±0.012	0.363±0.019
Pseudoxanthomonas Mexicana			
Peptone	0.777±0.042	0.906±0.072	1.576±0.084
Ammonium Nitrate	0.362±0.026	0.303±0.022	0.451±0.029
Glycine	0.261±0.019	0.190±0.008	0.333±0.030
Halomonas daqingensis			
Peptone	0.321±0.023	1.352±0.074	1.528±0.094
Ammonium Nitrate	0.286±0.020	0.234±0.018	0.449±0.034
Glycine	0.235±0.014	0.198±0.009	0.411±0.030
Parapusillimonas granuli			
Peptone	0.815±0.022	1.296±0.062	2.001±0.112
Ammonium Nitrate	0.357±0.031	0.231±0.010	0.385±0.022
Glycine	0.252±0.009	0.202±0.008	0.413±0.031

Table.4 Effect of pH on growth of microbes

pH	24 hrs	48 hrs	72 hrs
Bacillus cereus			
5	0.234±0.010	0.261±0.012	0.238±0.011
6	0.248±0.009	0.276±0.012	0.351±0.021
7	0.276±0.020	0.312±0.024	0.318±0.022
8	0.197±0.019	0.296±0.020	0.416±0.025
Pseudoxanthomonas Mexicana			
5	0.215±0.014	0.252±0.015	0.241±0.011
6	0.266±0.015	0.303±0.020	0.320±0.022
7	0.276±0.015	0.357±0.019	0.512±0.029
8	0.281±0.014	0.304±0.017	0.457±0.021
Halomonas daqingensis			
5	0.228±0.008	0.237±0.010	0.221±0.013
6	0.219±0.011	0.282±0.015	0.345±0.020
7	0.281±0.016	0.421±0.023	0.421±0.019
8	0.237±0.014	0.351±0.021	0.444±0.024
Parapusillimonas granuli			
5	0.227±0.018	0.288±0.014	0.260±0.013
6	0.265±0.014	0.311±0.021	0.313±0.017
7	0.315±0.011	0.378±0.016	0.428±0.026
8	0.214±0.014	0.309±0.016	0.365±0.015

Table.5 Effect of salt concentration on growth of microbes

Carbon Source	24 hrs	48 hrs	72 hrs
Bacillus cereus			
5.0%	0.202±0.011	0.270±0.007	0.303±0.018
10.0%	0.270±0.009	0.305±0.023	0.354±0.017
15.0%	0.160±0.005	0.189±0.014	0.220±0.008
20.0%	0.194±0.010	0.205±0.016	0.258±0.009
Pseudoxanthomonas Mexicana			
5.0%	0.104±0.007	0.274±0.021	0.254±0.011
10.0%	0.072±0.003	0.247±0.018	0.314±0.019
15.0%	0.150±0.011	0.137±0.009	0.203±0.015
20.0%	0.23±0.013	0.237±0.007	0.303±0.020
Halomonas daqingensis			
5.0%	0.094±0.012	0.282±0.009	0.323±0.021
10.0%	0.199±0.018	0.245±20.014	0.337±0.017
15.0%	0.222±0.012	0.338±0.021	0.304±0.012
20.0%	0.250±0.017	0.234±0.019	0.331±0.010
Parapusillimonas granuli			
5.0%	0.085±0.008	0.367±0.014	0.407±0.023
10.0%	0.129±0.010	0.193±0.010	0.319±0.017
15.0%	0.167±0.014	0.203±0.016	0.317±0.019
20.0%	0.146±0.008	0.239±0.008	0.363±0.020

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