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Potential of Petroleum Utilization by *Lysinibacillus sphaericus* Isolated from Oil Contaminated Site

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Abstract

Bioremediation is distinctly an effective treatment in terms of efficacy, safety, cost and simplicity of administration. This study focuses on the capability of indigenous bacterial isolates on biodegradation of petroleum hydrocarbons. Soil samples of 5 different Total petroleum Hydrocarbon (TPH) sites were collected from Changanacherry district, Kerala. A total of eight oil degrading bacterial cultures were obtained and undergone morphological and biochemical characterization. Biodegradation efficiency was further studied at 4% hydrocarbon under pH7 at room temperature. Biodegraded products were analyzed by GCMS. Of them the isolate- B6 showed 94% of degradation in Gravimetric analysis and thus selected for further studies. Molecular characterization of isolate was performed and identified as *Lysinibacillus sphaericus* (B6). The effect of environmental factors (temperature, pH and salinity) on growth of isolate was studied and the optimum pH was 7, while that of temperature was 30° C and 1% salinity. The efficiency of degradation of petroleum hydrocarbons was 80 %. Thus, *Lysinibacillus sphaericus* can be used for the effective biodegradation of petroleum hydrocarbons.

Keywords

Hydrocarbon, oil, bioremediation, pollutant.

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INTRODUCTION

Hydrocarbon based products are the predominantly used energy for industry and daily life. It was reported that nearly 600,000 metric tons per year of oil spillage throughout the world. Petroleum hydrocarbons are recalcitrant and are classified as leading major environmental pollutants. The spillage of petroleum hydrocarbon due to accidents during transportation, anthropogenic activities and various other reasons leads to contamination of air, soil and water. The frequent releases of hydrocarbon compounds into soil and ground water demands controlled release practises and strategies (Roling et al. 2002). Oil spills, especially in soil contamination have inspired cost-effective, environmentally clean up research strategies (Margesin and Schinner, 2001). Soil contamination with hydrocarbons causes extensive damage of eco system further enhance the accumulation of pollutants among biological sources leads serious life-threatening disorders. Bioremediation can be defined as application of living organisms to degrade/detoxify pollutants

(Varjani et al., 2014; Sajna et al., 2015; Varjani and Upasani, 2016). The commonly used physical remediations like adsorption and ultra-filtration along with chemical treatments like ectrolytic oxidation, ozone catalytic oxidation were widely studied and found to be high cost (Kumari *et al.* 2012). Microbial degradation studies have reported as cost effective and potent to clean up oil from different environments. Degradation by bacteria or fungi have an ultimate natural mechanism like absorption or enzymatic degradation can help the microbes to clean up the petroleum hydrocarbon pollutant as their sole source of carbon (Bisht et al., 2015).Gram negative bacteria are considered as primary degraders of spilled oil in environment and feed exclusively long chain hydrocarbons. Biodegradation by natural populations of genera Pseudomonas is frequently isolated and tolerate wide range of hydrocarbons and thus used to remove pollutants from the environment at cost effective manner (Ebadi et al.,2017). This specific study aims to identify potent soil bacteria for the effective biodegradation of petroleum hydrocarbons.

MATERIALS AND METHODS

Sample Collection

Soil samples from five different petroleum hydrocarbon (PH) sites were collected from Changanacherry district, Kerala. Soil was collected at the depth of 10-15cm from the surface of the soil using a sterile spatula and kept in sterile container. The collected samples were labelled and carefully transferred to the laboratory under ice cold condition for further analysis.

Culture Media

Bushnell Haas agar medium (0.2 $MgSO_4$, $7H_2O$, 0.02 NaCl, 1.0 K_2HPO_4, 1.0 KH_2PO_4,1.0 NH_4Cl,0.01 $FeCl_3$ and 15g/L) was prepared and enriched with filter-sterilized petrol and diesel (2ml). The pH was adjusted to 7.0 and autoclaved at 121^{0}C for 15 min.

Enrichment of Microorganisms

Microorganisms were isolated by selective enrichment technique. Enrichment of microbial culture was carried out in 500ml conical flask .30g of each soil samples were added into 250 ml of Bushnell Haas broth with 2% of petrol and diesel as carbon source and incubated for 7-10 days at 100rpm. After the first enrichment process the sample were further enriched two times to get triplicate of enrichment.

Isolation of Petrol and Diesel Degrading Bacteria

About 0.1ml of enriched broth was spread onto the Bushnell Hass agar plates with 2% of petrol and diesel. The agar plate incubated at 30^{o}C overnight. Pure cultures of bacteria isolates were maintained on sterile nutrient agar.

Identification of Bacterial Isolates

Identification of the colonies appeared on Bushnell Hass agar plates after incubation were done by morphological identification of colony characteristics and by Gram's staining.

Testing of oil degrading Bacterial Isolates

All the strains were permitted to grown in 200ml of Bushnell Hass medium supplemented with 2% of petrol and diesel. A set of control flasks were also set up containing 200ml of medium with 2% of petrol and diesel alone and incubated at 30^{0}C for 28 days. The total biodegradation rate was determined gravimetrically using the expression described by (Chaillan et al., 2004).

Gravimetric Analysis

50ml of culture broth was mixed petroleum ether: acetone (1:1) in a separating funnel and shaken vigorously to get a single emulsified layer. Top layer containing petroleum ether mixed with petrol, diesel and acetone was taken in a pre-weighed clean beaker. The petroleum ether and acetone were evaporated at 50^{o}C in a hot air oven. The gravimetric estimation of residual oil left after biodegradation was made by weighing the quantity of oil in the beaker.

Weight of residual oil:

(Weight of beaker containing extracted crude oil) - (weight of empty beaker)

Amount of oil degraded:

(Weight of crude oil added in the media) - (weight of residual oil)

Percentage of oil degradation =

Amount of crude oil degraded/Amount of crude oil added in the media*100

Optimization of Growth Conditions for Isolated Strains (Qin et al., 2012)

Bacterial culture was grown in a conical flask containing 50ml Bushnell Hass broth and 0.5% petrol and diesel at 30^{o}C for 48 hours. The bacterial growth was determined by turbidity measurement at 610 nm.

Effect Of pH on bacterial growth

The effect of hydrogen ion concentration on the growth and degradation of 2% petrol and diesel was studied. Bushnell Haas broth with petrol and diesel was prepared at pH 5.0, 6.0, 8.0 and 9.0 using 1N HCL/1N NaOH. The flask was inoculated with bacterial cultures (individual) and incubated at 30^{0}C. The population at the end of 48 h were estimated by measuring OD at 600 nm (Aislabie et al., 2006).

Effect of Temperature on Bacterial Growth

The influence of temperature (30^{0}C, 37^{0}C and 40^{0}C) on the growth and degradation of diesel and petrol

by bacterial isolates at different time intervals were studied using Bushnell and Haas broth with 2% diesel and petrol at pH 7.0. Percentage of degradation were determined at different time intervals.

Gas Chromatography-Mass Spectrometry (GC-MS)

The biodegradation products after solvent extraction using petroleum benzene and acetone in the ratio (1:1) were analysed using high resolution GC-Agilent 7890 and MS-Agilent 5975.The column used was 30m x 25mm x 0.25 µ. The temperature of the programme was 40^0C isothermal time ,heating up to 250^0C.the most relevant peaks were identified on the basis of mass spectra data bases (NIST).

Molecular Identification of Microorganisms

The selected isolates were subjected to molecular characterization using the following 16S Forward primer 5 CAGGCCTAACACATGCAAGTC 3 and Reverse primer 5 GGGCCGGGTGTACAAGGCCG 3 rRNA primer. The molecular identification of the isolate was done at Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram

RESULT AND DISCUSSION

Sample collection

The soil samples for the isolation of hydrocarbon degraders were collected from different petrol pumps of Changanacherry district which is given in image 1.

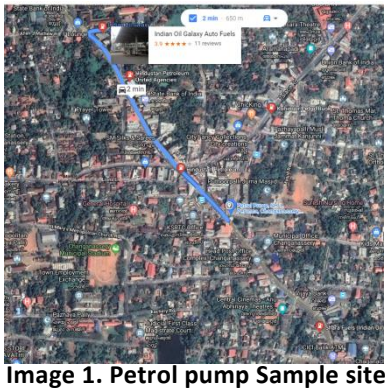

Image 1. Petrol pump Sample site

Isolation of bacterial culture

Microorganisms were isolated by selective enrichment technique. Cultures enriched in suspension of diesel and petrol, grow slowly and became turbid after 7 days of incubation. Totally 8 colonies were isolated from five different sampling sites. The CFU are 6, 8,12, 08, 06 X 10^7 respectively among SI, to SV. Most of the colonies are large, circular, translucent, entire margin, flat and few are irregular, opaque and undulate in nature. Based on colony morphology (Table 1) isolates were designated as OD1 to OD8. Out of 8 isolates four were gram negative rods and 4 isolates were gram positive. All the isolate was catalase positive and 50% were oxidase positive. Among the isolates, except OD2 all the 7 isolates were motile organisms. Enrichments using diesel and petrol found to be isolation hydrocarbon adopted microorganisms from contaminated sampling site (Ghoreishi et al., 2017). An enrichment culture leads mineralization of oil and ideal method for isolating bacteria expressing hydrocarbon degrading phenotypes (Al-Wasify and Hamed, 2014).

Screening of Oil Degrader s

After three successive enrichments the samples were transferred to BHA agar plates contain inorganic source, necessary for the growth of bacteria medium enriched with oil supports growth on the media, those bacteria that are able to decompose hydrocarbon. A total of 8 isolates were obtained and the isolates were named as OD1 to OD8 respectively. All the selected strains showed optimal growth at 30^0C and the selected isolates were preserved at 4°C for further studies. The gravimetric estimation of residual oil left after biodegradation was determined and the percentage of degradation of all the 8 isolates were calculated (Table 2). Among all the 8 isolates B6 (94%) shows maximum degradation on 28^{th} day. OD6 strain were Gram positive motile rods and shows Citrate, urease, mannitol and Catalase positive. The isolate showed negative reactions in case of Indole production, Methyl red (MR) test, Voges-Proskauer (VP) test, and Oxidase test. TSI test was observed with acid butt and acid slant without gas production and H_2S production. Grams staining and biochemical reactions confirm the isolate belongs to *Lysinibacillus*. This bacterial isolate was found to have higher potency to degrade the crude oil was selected for further study. It was reported that *Lysinibacillus sphaericus* to degrade simple to complex hydrocarbon event at 2-5% (Marquez-Rocha et al., 2001).

Microorganisms degrade the crude oil compounds by utilizing them as carbon and nitrogen sources by producing different catabolizing enzymes (Penet and Marchal, 2006). A number of bacterial isolates from soil like *Acinetobacter*, *Rhodococcus*, *Bacillus*, *Pseudomonas,* and *Sphingomonas* have been reported to utilize n-alkanes during petroleum oil degradation (Zanaroli *et al.* 2010; Hassanshahian *et al.* 2014)

Effect of pH on bacterial growth

As shown from the Figure 1 *Lysinibacillus sphaericus* showed maximum absorbance at pH 7 and satisfactory growth on pH 8 and least growth at pH 5 and 6. This indicates that these isolates are well adopted at neutral to alkaline soil environment. From the Figure 2 it is observed that 30^0C is the optimum temperature for maximum growth. Satisfactory growth was observed at 37^0C and least

growth at 40^{0}C. From the figure 3 after (48 hours) it is observed that good growth was found in 1% saline concentration. Satisfactory growth was observed at 2% and least at 3%. Thus, from the results *Lysinibacillus sphaericus* can tolerate up to 3% salt concentration. Previous reports state that most of the enzymes required for hydrocarbon degradation active at 30 to 37^{0}C under neutral pH (Mukherjee and Roy, 2013; Vaidya *et al.,* 2017). Rahman et al., (2002) pointed out that 40^{0}C as the optimum temperature for the degradation of crude oil by mixed consortium of bacterial cultures. Among the parameters that could affect biodegradation temperature is generally considered the most important factor (Delille et al., 2004)

Gas Chromatography-Mass Spectrometry (GC-MS)

To confirm the biodegrading ability of the *Lysinibacillus sphaericus,* Gas chromatography and mass spectrometry was used. The biodegradation was detected by the peak difference in the graph when compared with the control. Significant peak difference was observed between graph of control and graph after biodegradation (fig 4 and 5). Formation of extra peak among treated GCMS indicating the production of new intermediates due the biodegradation process. GCMS analysis of extracted oil showed that diesel pollutants were degraded by the *Lysinibacillus sphaericus* confirmed by disappearance of peaks related to hydrocarbon compared to pure diesel. List of compounds after degradation were given in table 3. It was noted that Octane, Heptane, Cyclotetradecane, Heptacosane derivatives were disappeared but Dodecane, Pentadecane, Hexadecane, Octadecane were not removed.

Molecular analysis of the selected bacterial isolate.

The molecular characterization of B6 isolate were done by 16s rRNA sequencing at Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvanthapuram. Blastn analysis of 16S rRNA sequencing showed that B6 strain is identical to *Lysinibacillus sphaericus*(Fig 6). The sequences aligned with the BLAST search of NCBI data bases were found to show 88.59% similarity for *Lysinibacillus sphaericus* strain TMB1(GU129141) and 88.43% with Bacillus sp(EF188283.1). Occurrence of *L. sphaericus* and *Geobacillus* sp consortium as an optimum treatment for contaminated soils reportd by Lina and Jenny (2014).

CONCLUSION

Bioremediation is a rapidly growing area of environmental Microbiology. The present study unveiled that the isolate *Lysinibacillus sphaericus* (B6) was the most efficient one with a percent of degradation of 80.10% with optimum pH 7 at 30°C. It is apparent from the results that the *Lysinibacilluss phaericus* degrading hydrocarbon pollutants effectively even at 3% salt concentration.

Table 1. Colony morphology of isolated bacterial colonies on nutrient agar

Isolates	Colony Characteristics						
	Size	**Shape**	**Texture**	**Opacity**	**Pigment**	**Margin**	**Elevation**
OD1	Large	Irregular	Smooth	Translucent	White	Undulate	Flat
OD2	Large	Irregular	Smooth	Translucent	White	Entire	Flat
OD3	Pinpoint	Round	Smooth	Translucent	Cream	Entire	Slightly Raised
OD4	Medium	Round	Rough	Translucent	White	Entire	Flat
OD5	Large	Round	Rough	Translucent	White	Entire	Flat
OD6	Small	Round	Smooth	Opaque	White	Entire	Slightly raised
OD7	Large	Irregular	Rough	Translucent	White	Undulate	Flat
OD8	Pinpoint	Round	Smooth	Translucent	White	Entire	Slightly Raised

Table 2. Percent of Degradation by Gravimetric Analysis

Isolate code	Percent of Degradation (28th Day)
OD1	86%
OD2	84%
OD3	85.7%
OD4	83%
OD5	87%
OD6	94%
OD7	86.5%
OD8	86%

Table 3. NIST library match of GCMS

PEAK	R. TIME	AREA%	NAME	BASE
1	7.957	11.33%	Ethylbenzene	91.1
2	10.221	15.62%	2- pentanethiol, 2-methyl	43.1
3	10.873	38.51%	Pentanal, 2,2-dimethyl	43.1
4	11.657	4.29%	Ethanone, 1-3ethyloxiranyl	43.1
5	12.015	4.51%	Hexane, 2-nitro	43.1
6	12.420	7.80%	Cyclopropane, 2-bromo 1,1,3-trimethyl	83.1
7	20.161	11.97%	Undecane,2,6-dimethyl	57.1
8	21.869	25.42%	Tridecane,7-methyl	57.1
9	23.125	15.88%	Tridecane,6- methyl	57.1
10	23.289	25.08%	Naphthalene, 1,2,3,4 tetrahydro-2,6-dimethyl	118.1
11	24.715	50.25%	Dodecane, 2,6,10-trimethyl	57.1
12	31.387	62.45%	Pentadecane,2,6,10-trimethyl	57.1
13	32.665	54.54%	Pentacane,2,6.10,14-tetramethyl	57.1
14	34.897	100.0%	Hexadecane, 2,6.10,14-tetramethyl	57.1
15	36.970	32.62%	7,9-di-tert-butyl 1-oxaspiro (4,5) deca-6,9-diene,2-8 dione	205.1
16	37.923	37.86%	Dibutyl phthalate	149.0
17	38.659	34.44%	Hexdecanoic acid, ethyl ester	88.0
18	42.358	21.25%	Octadecanoic acid, ethyl ester	88.1

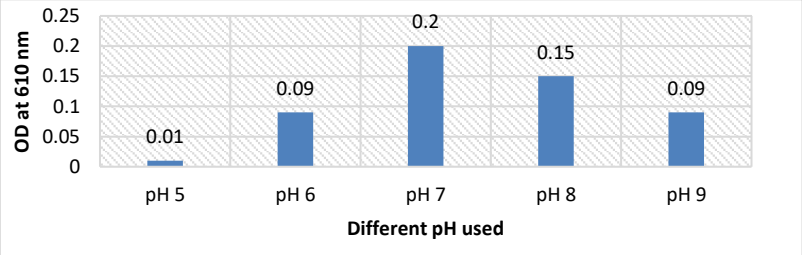

Figure 1. Effect of pH on bacterial growth

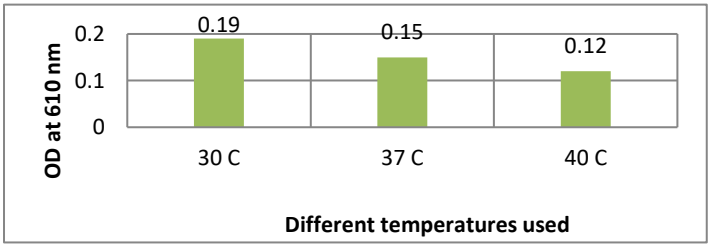

Figure 2. Effect of pH on bacterial growth

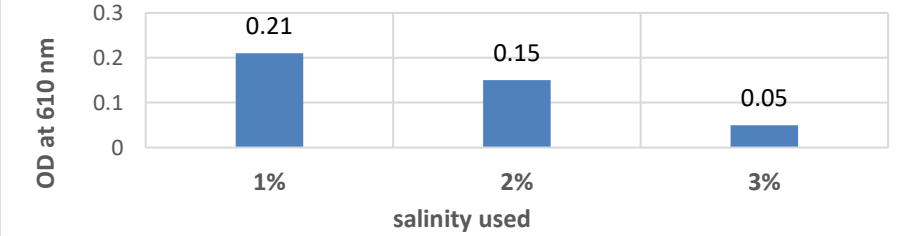

Figure 3. Effect of salinity on bacterial growth

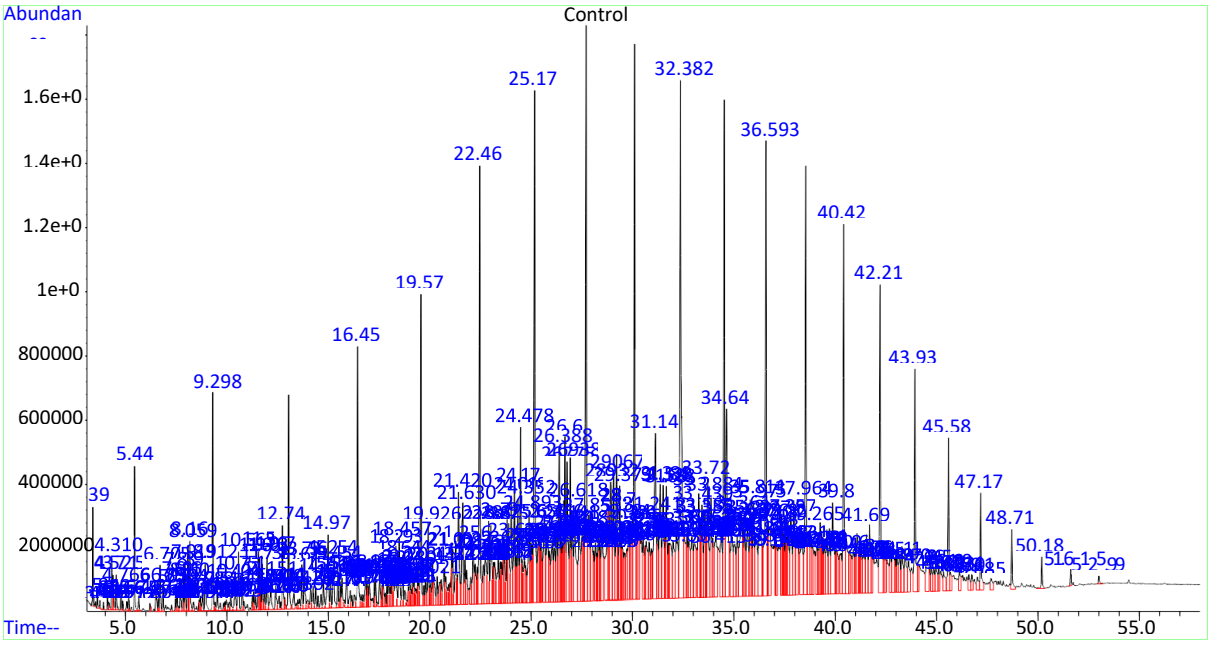

Figure 4. GC-MS Analysis of control

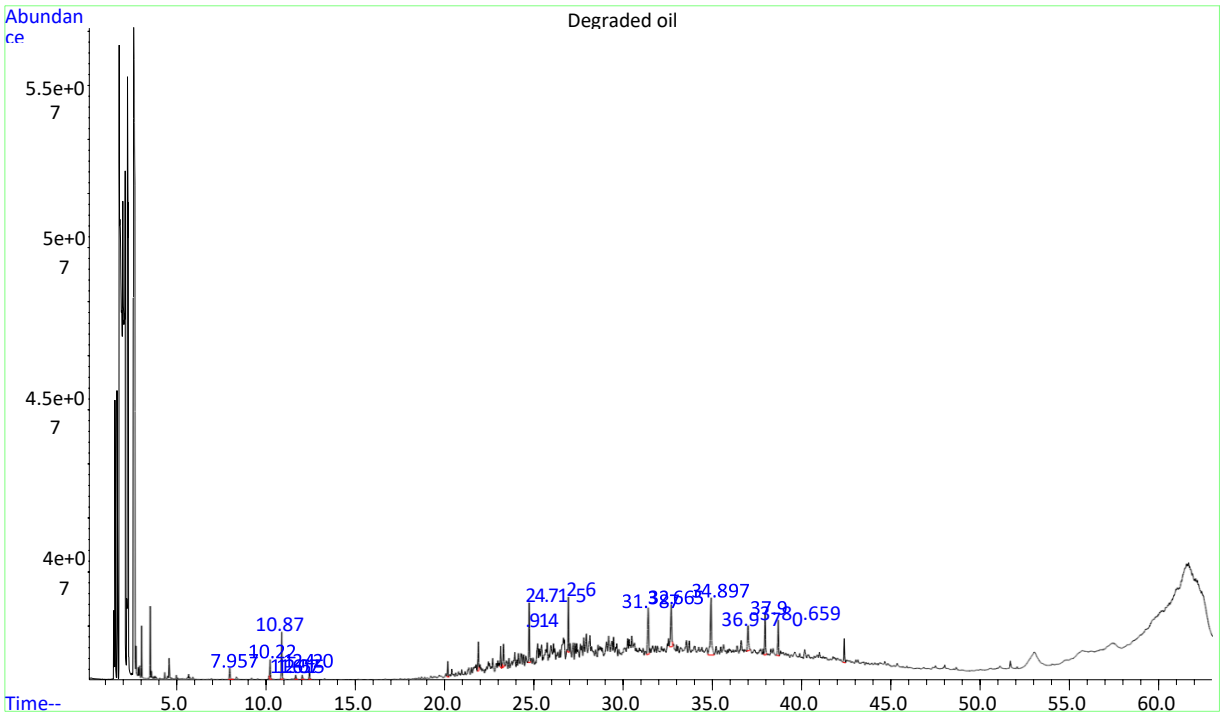

Figure 5. GC-MS Analysis of biodegraded crude oil by *Lysinibacillus sphaericus.*

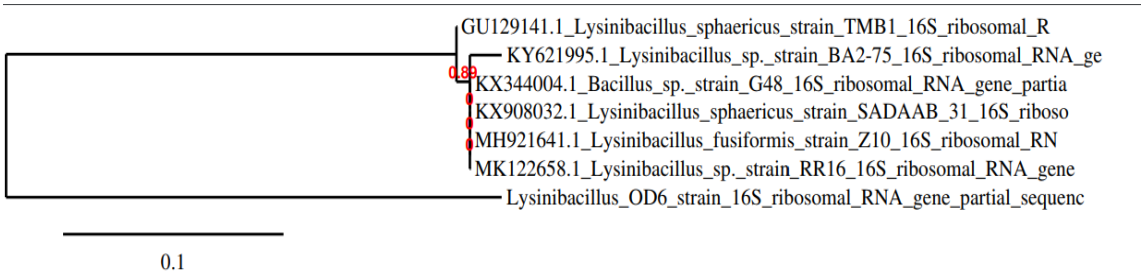

Figure 6. Phylogenetic relatedness of active isolate

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