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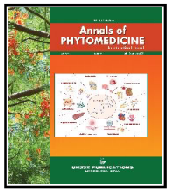

Original Article : Open Access

Crude oil degrading ability and optimization of *Bacillus velezensis* isolated from crude oil contaminated soil

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Abstract

Oil pollution is the largest serious threat to biological species. Crude oil has severe implications for creatures of all kinds. Every year, around 6 million tonnes of crude oil enter the environment, creating health risks to living things and causing environmental degradation. The greatest and most effective methods for recovering toxins in soil and water are found to be bioremediation. The present study focuses on the isolation, growth parameters optimization, molecular identification and degrading ability of bacteria that can degrade crude oil from soils collected from an automobile service center at Namakkal, Tamil Nadu. The higher significant crude oil degrading strain was isolated, and identified by screening test and 16s rRNA sequencing. technique. Cultural conditions including pH, temperature, crude oil concentration, inoculum concentration, and nitrogen source were optimized and it was statistically analyzed. Thirty-two bacterial strains were isolated using the enrichment method and the isolate named PS6 utilized crude oil in the highest amount during screening in comparison with other bacterial strains investigated in this present study. Noticeable degradation of crude oil was observed by GC-MS analysis of PS6 treated crude oil on comparing the peaks with the control. From 16s rRNA sequencing analysis, the isolated strain was identified as *Bacillus velezensis*. In statistical optimization, low $p<0.0001$ values were observed. As a result, the isolated strain can be used in the bioremediation to clean up the environment.

1. Introduction

Crude oil is a complicated collection of chemically distinct substances grouped into four major fractions: aromatic hydrocarbons, saturated hydrocarbons, asphaltenes, and resins. Crude oil plays a major role in the world's energy needs and serves as a raw material to the chemical industry, hence playing a key role in the fast-developing economy (Abosede, 2013). Accidental spills, such as pipeline ruptures and tank leaks, as well as routine oil production, can result in significant oil contamination. Every year, around 6 million tonnes of crude oil enter into the ecosystem worldwide. Upon exposure, it causes kidney and liver diseases, enhances cancer, and damages the bone marrow (Anh, 2019; Wang *et al*., 2011).

The toxicity of the oil is produced by some of the aromatic hydrocarbons which have low boiling points such as toluene, benzene, xylene, phenanthrene, naphthalene, and others. To overcome these struggles, microorganisms are implicated in the elimination of crude pollutants from the environment, called bioremediation. Microbes found in soil, water and air, from that few of them are beneficial and several microbes are harmful (Dhama *et al*., 2022). Bioremediation research and its impact on the soil environment has begun in Indonesia, but still more research in marine environments is becoming more intricate and facing many difficulties (Okoh *et al*., 2020).

Bioremediation received higher attention when compared to physical and chemical approaches because it is an environmentally acceptable process that can be applied to various situations (Harayama *et al*., 2004) is also cost-effective, operates easily, and does not have secondary pollution. Bioremediation was introduced to reduce the crude oil contaminants, whereas several microbes are confirmed that they have the capability to degrade the crude oil. Soil microbial population is the main health indicator of soil fertility but contamination of crude oil can alter the population of microbes and have an undesirable effect on other organisms. The microorganisms employed in the bioremediation approach might influence the resident microbial population (Deng *et al*., 2020). In most of the literature, biodegradation of intricate hydrocarbons such as naphthalene and pyrene was done with *Bacillus*, ranging from 20 to 60% (Borah and Yadav, 2014). Microorganisms, particularly different species of *Bacillus* have the capability to produce a varied range of enzymes that have many industrial applications (Chaudhary *et al*., 2019). The key success of the bioremediation methods relies on degrading capacity of the resident microbial community as microbes that applied exogenously failed to carry out the predictable level in the distant environment (Patowary *et al*., 2016). A range of macro and micronutrients influence the growth of microorganisms, also physical factors such as pH, and temperature play a vital role in their metabolic activity (Kanakaraju *et al*., 2022).

Three important processes occur during biodegradation, initially, they cause a small change in an organic molecule while leaving the

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key structure intact; the second step is the degradation, which can also be thought of as fragmentation of the original intact structure of the molecule but still detectable fragments are left as residuals; in the final step is the complete mineralization of the compound to an inorganic form (Janaki *et al*., 2016). To detect different compounds in a sample a type of analysis called gas chromatography mass spectroscopy (GC-MS) utilizes the capacity of mass spectroscopy with gas chromatography (Srivani and Krishna Mohan, 2023). Therefore, the present study focuses on the isolation of the potent bacterial isolates that can easily degrade crude oil and optimizing their cultural parameters to achieve maximum degradation of crude oil to save the environment.

2. Materials and Methods

2.1 Collection of soil samples

For the analysis, automobile service centers were chosen where there is a prolonged oil spillage in the soil, hence crude oil-contaminated soils were collected from those areas to isolate oil-degrading bacteria. Four samples were collected in different places in Namakkal,Tamil Nadu, India. The care should be taken to collect the samples only at a depth of 5 cm in a sterile container. The sample containers kept in an icebox were brought to the laboratory and for further process. The crude oil was obtained from Chennai Petroleum Corporation Limited, Manali, Chennai (as per the guidelines of CPCL).

2.2 Isolation of hydrocarbon-degrading bacteria

2.2.1 Enrichment method

The collected soil samples were subjected to enrichment for the isolation of crude oil-degrading bacteria. In this method, from the collected soil samples, 1g was added to the sterilized bushnell haas broth mixed with crude oil, and the mixture was incubated at 30°C in a shaking incubator at the speed range of 180 rpm for 3 days. The enriched broth was serially diluted and transferred to the bushnell haas medium mixed with crude oil as a source of carbon by spread plate technique and it was incubated. The bacterial colonies on the agar plates were taken and purified for further studies.

2.3 Screening

During the screening phase, the capacity of the isolated bacterial strains to breakdown the crude oil was assessed. The isolated bacterial colonies (100 µl/ml) were inoculated in 100 ml of bushnell haas broth with 2% crude oil as a source of carbon and for incubation the broth was kept in a shaking incubator at the speed of 180 rpm for 7 days which were maintained at 37°C. By evaluating the broth turbidity, the bacterial culture growth was quantified. Gravimetric analysis was used to extract the crude oil remaining in the cultured broth.

2.3.1 Gravimetric analysis

The remaining crude oil in the broth was extracted by vigorous mixing of petroleum ether with the liquid broth and kept undisturbed in a separating funnel. The petroleum ether separates the oil from, the broth and settles at the top layer whereas the bottom layer consists of the spent medium. Then, the petroleum ether oil mixture was passed through sodium sulfate to remove the moisture and allowed to evaporate the solvent (Gupte and Sonawdekar, 2015). After this, the separated oil was subjected for GC-MS (gas chromatography mass spectrometry) analysis.

2.3.2 GC-MS analysis

GC-MS analysis of petroleum ether extract was performed with GC Clarus 500 Perkin Elmer system and gas chromatograph interfaced with a mass spectrometer utilizing an Elite-1 combined silica capillary column in the size of 30 mm x 0.25 mm 1D x 1 µm df, composed of 100% dimethyl polysiloxane and helium as carrier gas at a constant flow of 1 ml/min. During the run, the temperature of the injector was maintained at the range of 260°C and it was injected with 1 µl of sample, the initial temperature was 60°C for 2 min; the temperature was increased at the speed of 10°C/min to 300°C and the same was maintained for 6 min. Mass spectra were collected at 70 eV, fragments ranging from 45 to 450 Da, and a scanning interval of 0.5 sec. The relative amount (%) of every component was considered by comparing its regular peak area to the total peak areas, the mass spectra and chromatograms were processed using the software turbomass. The series of the components was compared with the components spectrum of the database stored in the GC-MS NIST (2008) library.

2.4 Molecular identification of the isolate

2.4.1 Isolation of genomic DNA

DNA was extracted from bacterial cultures using the cetyltrimethyl ammonium bromide process (Lawniczak *et al*., 2020). The bacterial broth was centrifuged for 7 min at 7000 rpm and the pellet was concentrated. The pellet was resuspended with CTAB buffer containing 5% CTAB, 25 mM Tris-HCl, 5 M NaCl, 50 mM EDTA and 2% beta mercaptoethanol, and then incubated at 48°C for 2 h. After completing 10 min of incubation, centrifugation at the speed of 10,000 rpm was done for the separation of supernatant, then it was mixed with chloroform and isoamyl alcohol (24:1) and centrifugation was repeated at the speed of 12,000 rpm for 10 min to divide the supernatant from the broth, followed by addition of 1ml isopropanol in the collected supernatant then the whole mixture was kept at –20°C for 10 min, final centrifugation was done at the range of 12,000 rpm to collect the pellet and washed using 70% ethanol. The obtained pellet that contains the DNA was suspended in 1X TE buffer.

2.4.2 PCR amplification and sequencing

The isolated DNA has been amplified with the bacterial Universal-16S rRNA primers with the UF 5'-GAGTTTGCTGCTCAG-3' and UR 5'-ACGGCTACCTTGACTTT-3' (Greisen *et al*., 1994). The PCR reaction was carried out in 20 µl of the mixture containing 2 µl of DNA, 2 µl of 10X PCR buffer, 0.2 µl of taqpolymerase [2 U/µl], 2 µl of each [4 pmol/µl] primer. Amplification was done at 94°C as the initial temperature for 10 min, then 30 cycles were completed at 94°C for 1 min, followed by 48°C for 1 min, 72°C was maintained for 2 min and to end the process a final extension was done at 72°C for 10 min. The final PCR products were separated using 1% (wt/vol) agarose gel for 60 min at a constant voltage of 50 V in 1X TAE buffer. The amplified substance was identified by visualizing it in a UV transilluminator. The amplicon was fed into a sequencer; forward and reverse sequences were identified. The identified sequences were combined and subjected to BLAST analysis for species identification.

2.5 Optimization of growth conditions

The cultural conditions such as pH, temperature, inoculum concentration, incubation time, and nitrogen source were optimized for the maximum growth of the isolated bacteria. In addition to these parameters, different concentrations of crude oil were also optimized.

2.5.1 Effect of different percentages of crude oil concentrations

To find the crude oil optimum concentration, the standard inoculum was inoculated in the mineral salt medium with the addition of various concentrations of crude oil such as 0.5%, 1%, 1.5%, 2%, and 2.5% separately in each conical flask containing medium. Then, the flasks were incubated at 37°C at 200 rpm for 7 days. After incubation optical density (OD), values were taken at 600 nm using a spectrophotometer.

2.5.2 Effect of pH

To optimize the pH value, 100 ml of the mineral salt medium was prepared in five different flasks, and 2% crude oil (optimized with the above-said procedure) was incorporated into the medium. The pH value for each medium was set at different values that includes 5,6, 7, 8 and 9. After setting the pH of the medium 1% culture was inoculated into each flask and incubated at 37°C at 200 rpm for 7 days. After incubation, the OD values were measured at 600 nm using a spectrophotometer.

2.5.3 Effect of temperature

To optimize the temperature, 100 ml of the mineral salt medium was prepared in five different flasks, and 2% crude oil (optimized already) was incorporated into the medium and kept at different temperatures such as 32°C, 35°C, 37°C, 40°C and 42°C at 200 rpm for 7 days. After incubation, the OD values were recorded at 600 nm using a spectrophotometer.

2.5.4 Effect of nitrogen source

For nitrogen source optimization, the standard inoculum was inoculated in crude oil (2%) supplemented mineral salt medium (100 ml) and it was added with various concentrations of ammonium sulfate including 0.1%, 0.2%, 0.3%, 0.4% and 0.5% in different flasks. Then, the flasks were incubated at 37°C at 200 rpm for 7 days. After incubation, the OD values were assessed using a spectrophotometer at 600 nm.

2.5.5 Effect of different percentages of inoculum concentrations

The concentration of inoculums was optimized by varying the quantity of inoculums added. For 100 ml of mineral salt medium with 2% crude oil incorporated, the percentage of inoculums added was varied such as 0.2%, 0.4%, 0.6%, 0.8% and 1% each to 5 different flasks and each flask was labeled according to the concentration of the inoculums. Then, the flasks were incubated at 37°C at 200 rpm for 7 days. After incubation, the OD values were recorded at 600 nm using a spectrophotometer.

2.5.6 Statistical analysis

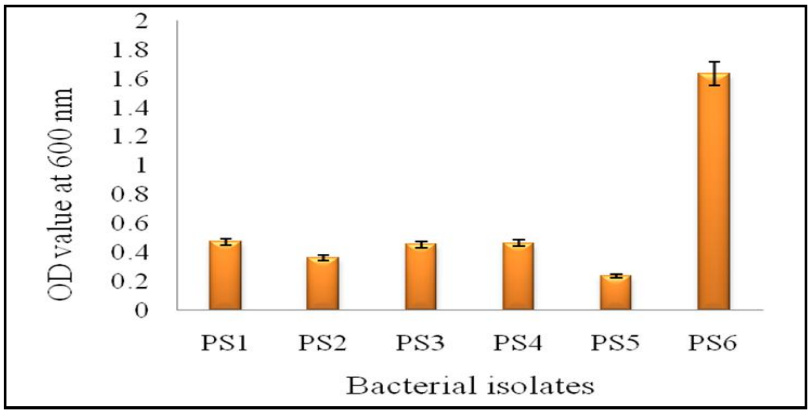
Statistical optimization of cultural conditions were performed using prism for the analysis of variance and the significance was determined by F test and its probability. Finally, an experiment was done under the optimized growth conditions. The residual oil was analyzed through GC-MS analysis.

3. Results

The current study focused mainly on isolating bacteria, especially from oil-contaminated soil (Figure 1) and these isolated organisms were inoculated into bushnell haas broth enriched with crude oil supplement.

Figure 1: Sample collection site.

Four soil samples were collected and processed. From the first sample, 0.3×10^5 CFU/ml, 0.25×10^5 CFU/ml in second sample, 0.2×10^5 CFU/ml in third sample and 0.13×10^5 CFU/ml in last sample bacterial colonies were obtained, in that thirty-two different strains includes the genus of *Bacillus*, *Pseudomonas*, *Mycobacterium*, *Serratia*, *Staphylococcus*, *Micrococcus* were isolated, *Bacillus* are more dominant in the samples and named PS1-PS6, NS1-NS7, RS1-RS11, and R1-R8. Among these isolated strains of PS1-PS6, four belonged to Gram-positive and the other two were Gram-negative group of bacteria. In NS1-NS7 three belonged to Gram-positive and the remaining were Gram-negative, from RS1-RS11 six Gram-positive and five Gram-negative were obtained, in R1-R8 five belonged to Gram-positive and the others were Gram-negative. The growth of all the isolates was measured and evaluated by its OD value at 600 nm (OD values of isolated strains on triplicates) and the results are recorded in a graphical representation (Figure 2). Among these isolates, PS6 showed significant growth and was used for further analysis.



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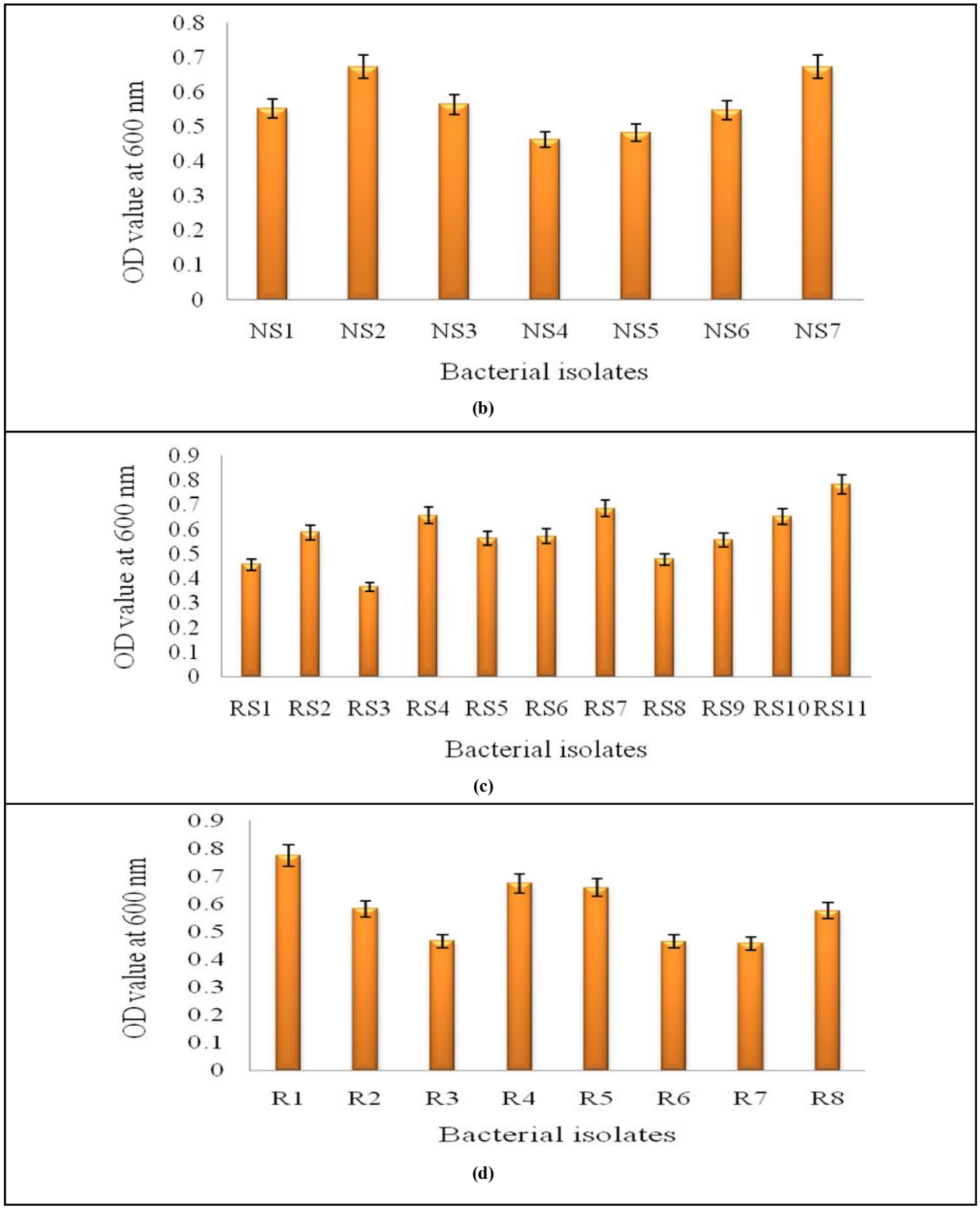


Figure 2: Growth of bacterial isolates from oil-contaminated soil samples: (a) PS1-PS6, (b) NS1-NS7, (c) RS1-RS11, (d) R1-R8.

3.1 Analysis of oil degradation

PS6 strain identified after screening was further analyzed for its ability to degrade crude oil using GC-MS (Figure 3). In this analysis, evident variations of the components (control crude oil) (Figure 4) and PS6-treated crude oil were analysed.

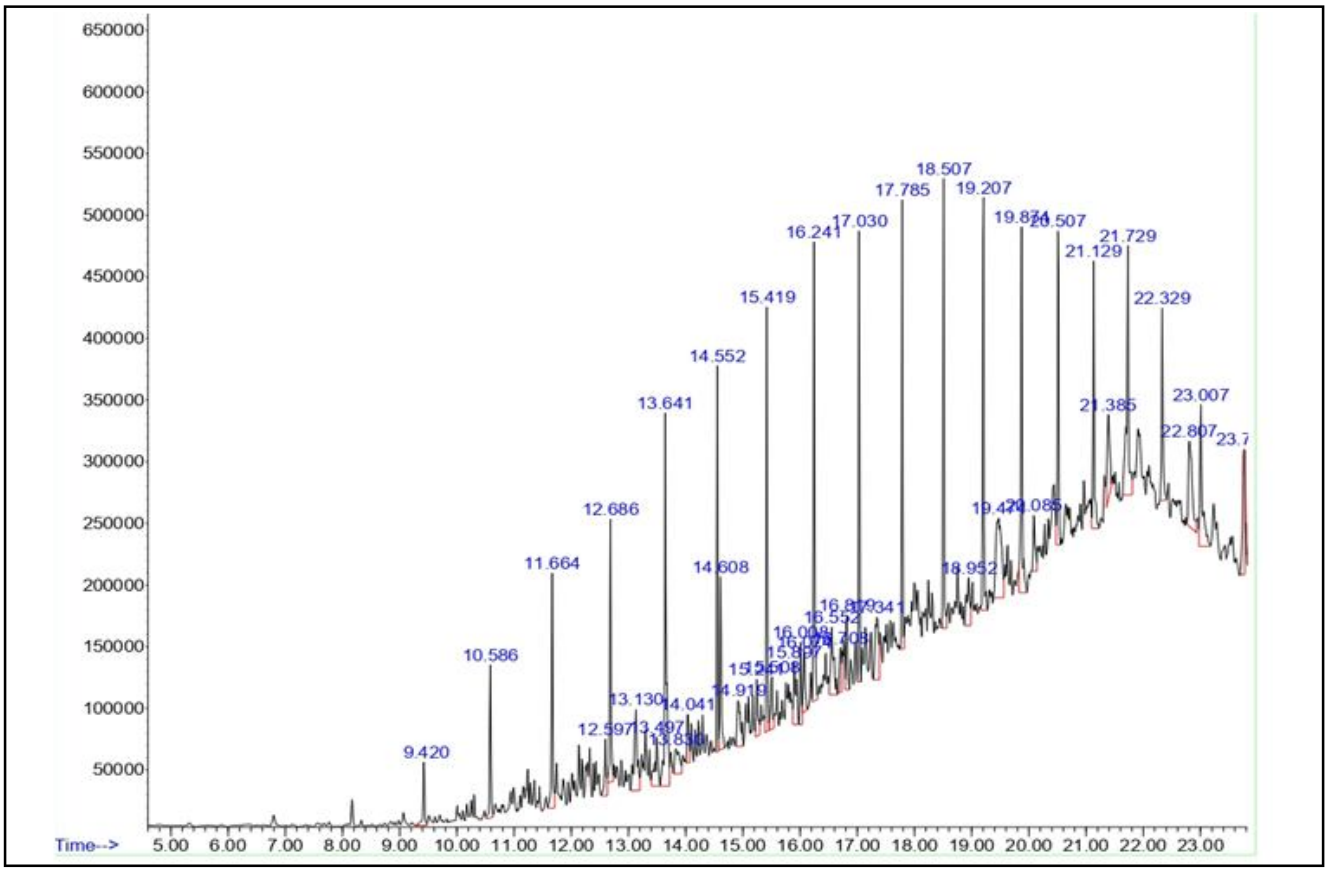


Figure 3: GC-MS spectrum of PS6-treated crude oil.

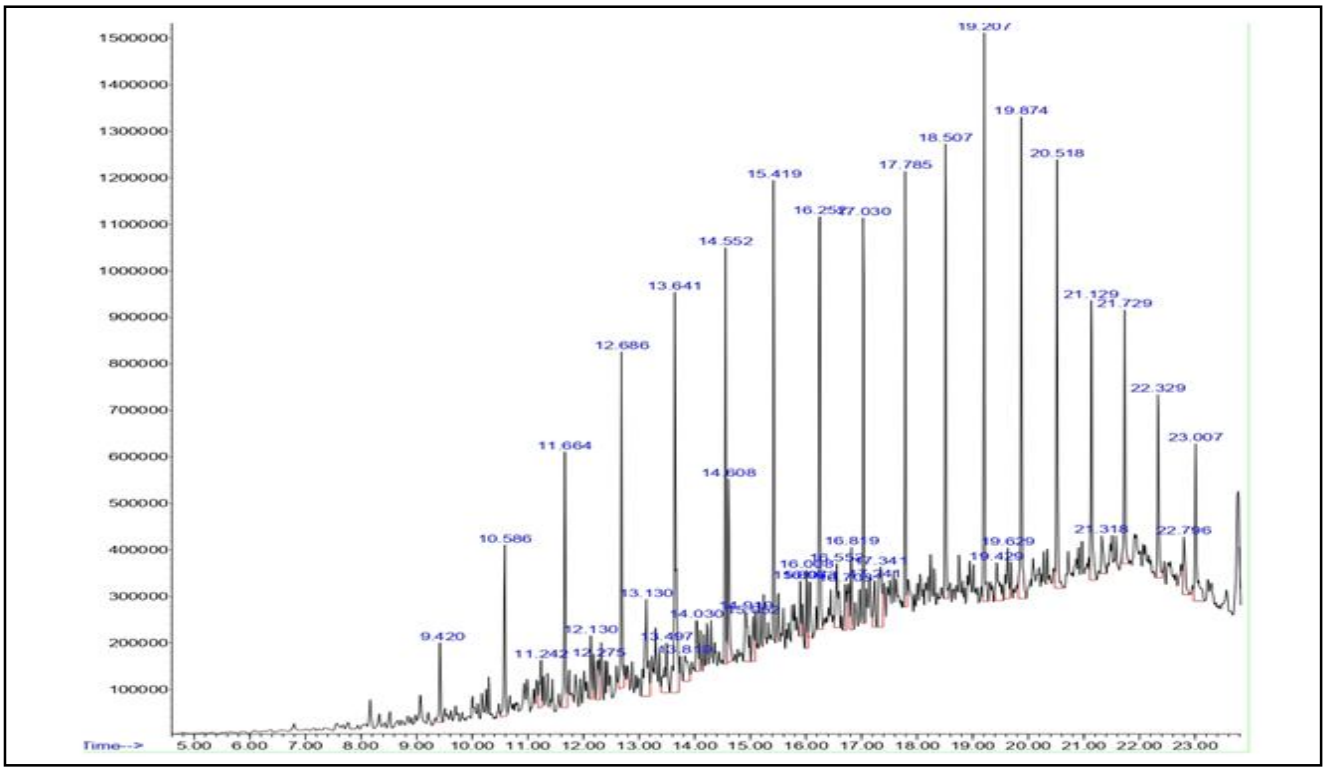


Figure 4: GC-MS spectrum of control crude oil.

3.2 Molecular identification of bacterial strain

For molecular identification, the amplicon obtained through 16S rRNA amplification was sequenced, and aligned.The bacterial species was identified using BLAST analysis of 16s rRNA sequence based on the obtained sequences and the organism was confirmed as *B. velezensis* VCMB01 and a phylogenetic tree was constructed (Figure 5). The obtained bacterial sequence was submitted to the gene bank and the accession number is OR467344.

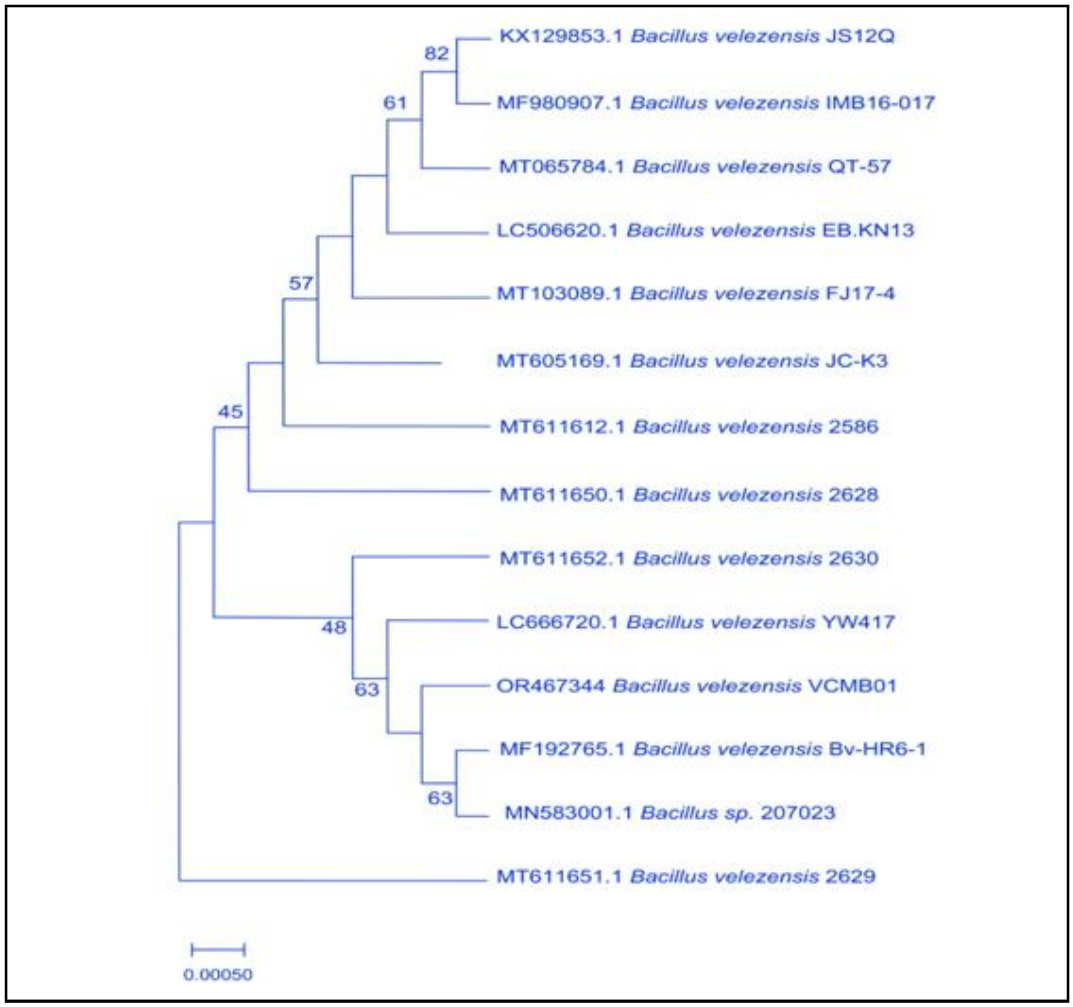
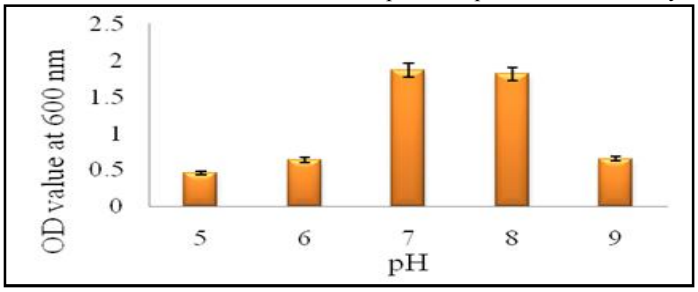


Figure 5: Phylogenetic tree.

3.3 Optimization of growth conditions

The growth conditions like pH, temperature, nitrogen source, inoculum concentrations and crude oil concentrations were optimized for the isolated bacteria. The optimization results showed that at pH 7, 37°C temperature with crude oil 2% and 1% inoculum concentration were the optimum conditions to obtain good growth of the isolated strain. Higher growth was observed on a medium incorporated with 0.1% of ammonium sulphate as as a nitrogen source. In ANOVA, the obtained low *p* value mentioned in Figure 6 shows the model's significance. Counter plots were plotted to describe the separate and interactive effect of the tested variables (Figure 7). The residual oil obtained from the experiment done by using the optimized parameters was analyzed through GC-MS (Figure 8).



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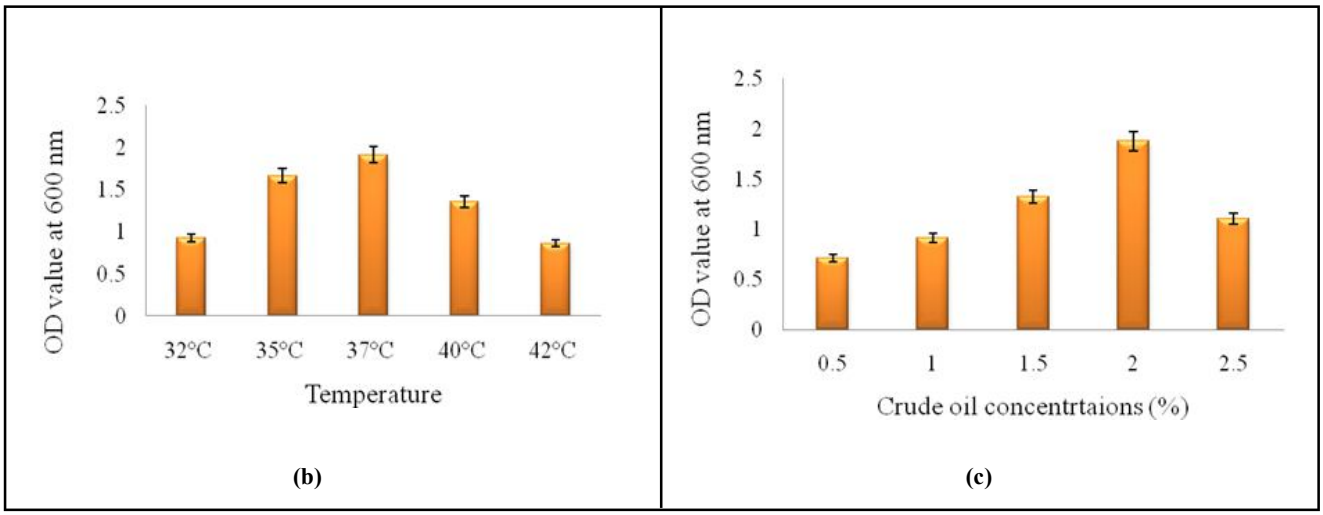
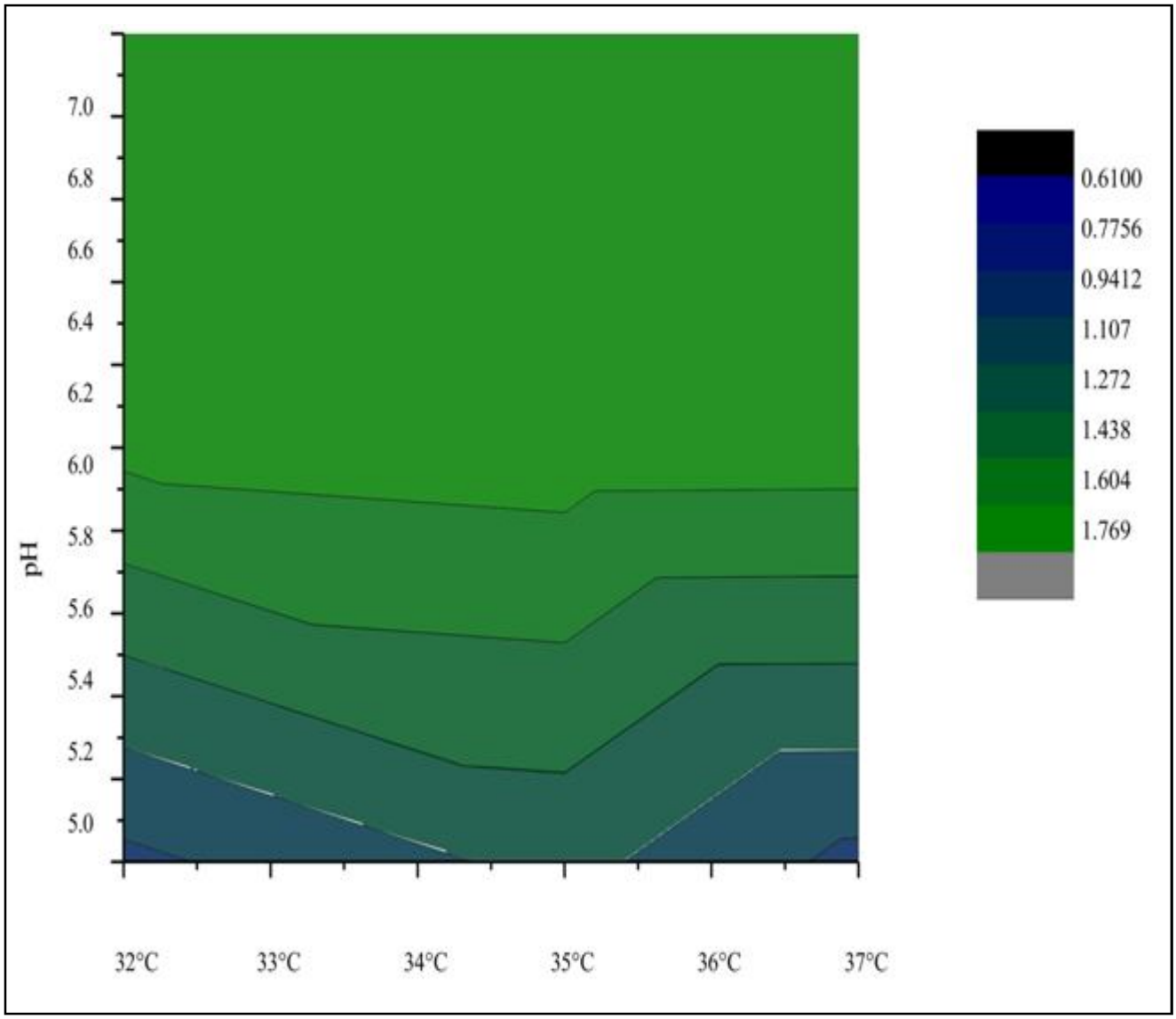


Figure 6: Optimization of growth conditions significance of the differences was compared with each group by one-way ANOVA and obtained value is ***p<0.0001: (a) pH, (b) Temperature, (c) Crude oil concentrations.



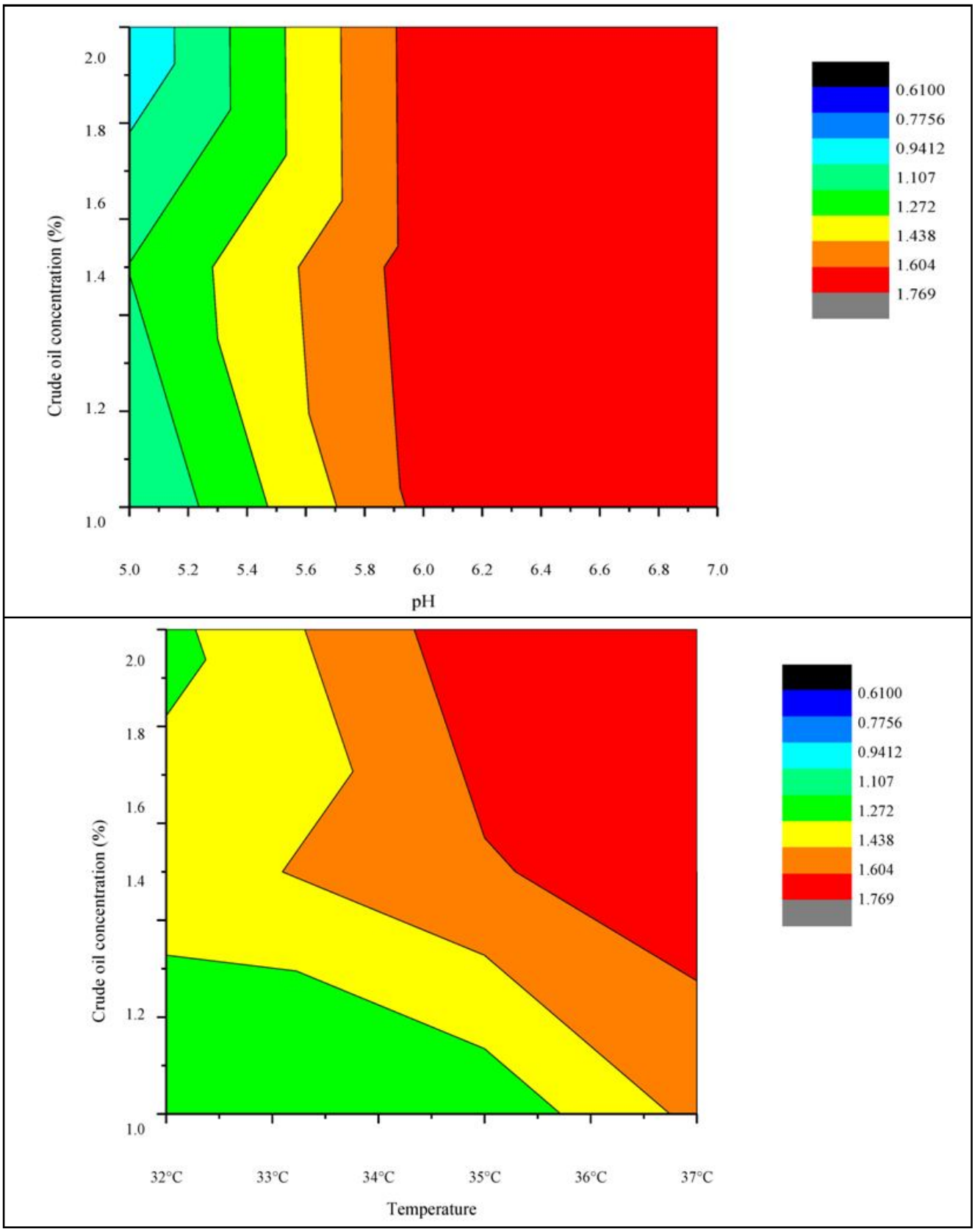


Figure 7: Counter plots and interactive effects of pH, temperature, and crude oil concentrations.

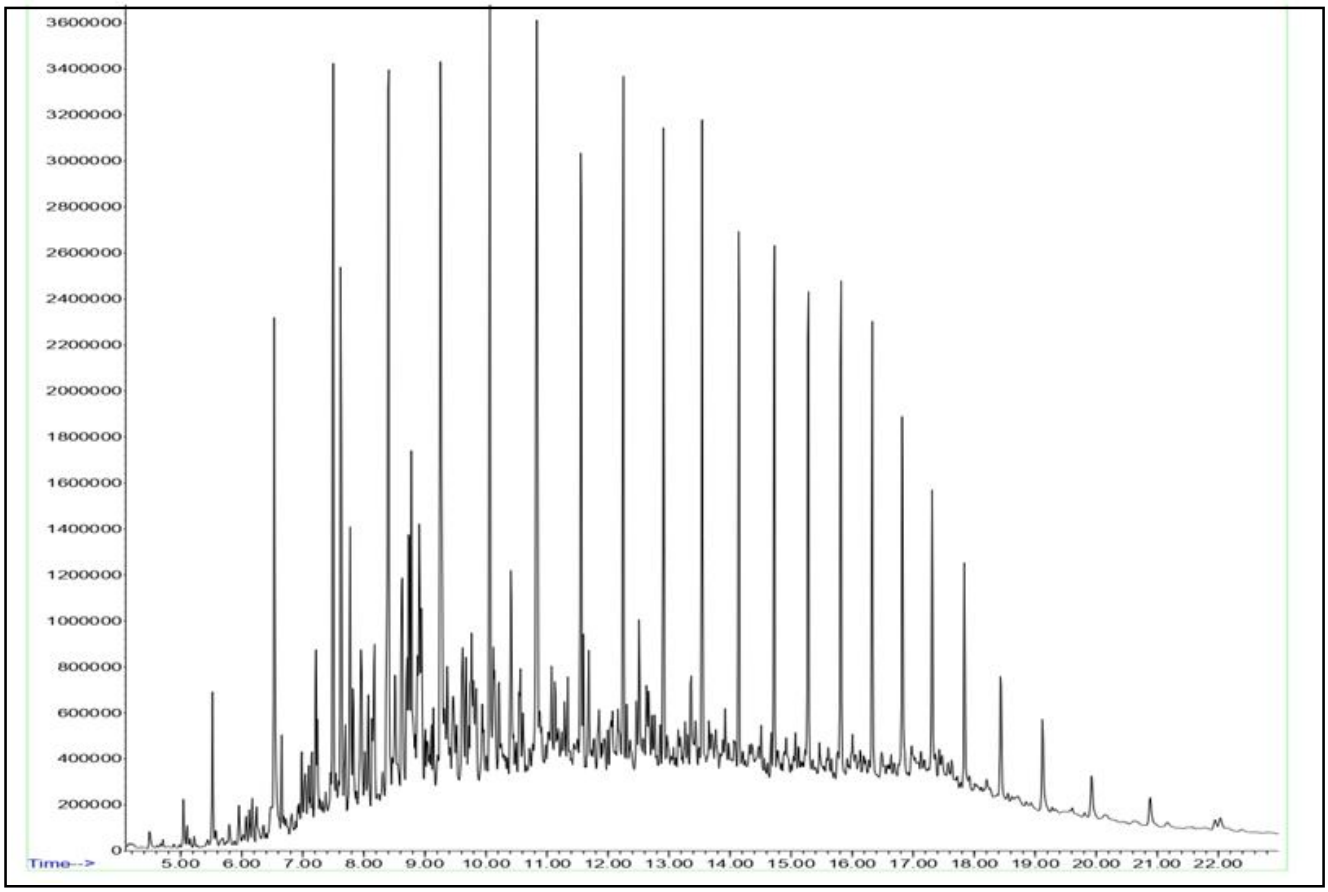


Figure 8: GC-MS spectrum of PS6-treated crude oil under optimized parameters.

4. Discussion

Remediation is a field of study where bacteria have a major role in utilizing hydrocarbons as the sole form of carbon. Hydrocarbon contamination was found to be increasingly widespread in everyday life causing serious hazardous problems for living things. In the environment, bacteria are considered as the major agent in hydrocarbon degradation which are ubiquitous and free living in nature (Dasgupta *et al*., 2013). Parallel to the results obtained in the current study, both the types that include Gram-positive and Gram-negative rods were isolated in their study (Gupte and Sonawdekar, 2015) was in evidenced. According to a study in crude oil degradation, 44% of isolates were identified as Gram-positive and 32% of isolates were Gram-negative bacteria, they also reported in their study that Gram-positive bacteria dominate in oil-contaminated soils as Gram-positive bacteria have stronger envelopes when compared to Gram-negative bacteria and they can tolerate to a higher concentration of hydrocarbons as they posses resistance endospores (Jayanthi and Hemashenpagam, 2015). A controversial result was reported in a work where they got a higher proportion of Gram-negative bacteria from oil-contaminated sites (Subathra *et al*., 2013). Some studies focus on fungal degradation of crude oil. AI-Dhabaan (2021), chosen fungal strains includes *A. niger*, *A. spelaeus*, *A. polyporicola* for determining crude oil degradation, in that *A. niger* has higher degradation efficiency when compared with other two strains. In another study, they isolated *M. irregularis* and *A. oryzae* from oil spillage sites degrades engine oil (Asemoloye *et al*., 2020).

In the same way, a study isolated BaP degrading *B. velezensis* from fermented food and mentioned that *B. velezensis* completely degraded the benzo(a)pyrene BaP in broth and produced a clear zone on bushnell haas agar supplemented with BaP (Sultana *et al*., 2021). In a study on the biodegradation of diesel reported that *Bacillus* degraded diesel at a maximal level up to 80% (Nwaogu *et al*., 2008). The previous report revealed that isolated crude oil-degrading bacteria such as *Pseudomonas*, *Bacillus*, *Alcaligens* from the crude oil contaminated site (Das and Chandran, 2011).

Few studies have reported on the roles of *B. velezensis* that this bacterium is able to degrade hydrocarbon in soil. Even in one more study reported that *B. velezensis*, *B. flexus*, *P. brenneri,* and *P. azotoforman* comprise high crude oil degrading ability through GC-MS analysis, similar to our present study, they isolated 36 strains from that they selected *B. velezensis* which had high OD value when compared to other isolates, so this single strain was utilized for their further studies (Ullah *et al*., 2021). Crude oil contaminations can radically decline the soil bacterial diversity (Deng *et al*., 2020).

According to a study, four potential hydrocarbon degraders such as *Bacillus*, *Pseudoxanthomonas moxicana*, *Halomonas daqingensis,* and *Parapuillimonas granuli* yeast extract and peptone served as efficient carbon and nitrogen sources and the optimum pH was 7 (Preeti Singh *et al*., 2015). Also, the authors discussed that higher pH has a negative effect on the hydrocarbon degrading efficiency of the organisms (Rahman *et al*., 2002; Meredith *et al*., 2000). Similarly, the author reported that the inhibition of oil biodegradation was

found in light acidic pH (5) and alkaline pH (10) conditions (Kao *et al*., 2005). Similarly, the results from a study indicated that a higher concentration of crude oil concentration results in lowering the degradation rates (El-Liethy *et al*., 2017). Also in a work, they concluded that 750 µl of inoculum favors growth with sufficient oxygen, a low amount of inoculum leads to insufficient microbial cells whereas a higher amount leads to oxygen deficiency (Janaki *et al*., 2016). From the current study, the bacteria efficiently degrade the crude oil, so the study will focus on the isolation of particular gene alkB responsible for the oil degradation for further analysis and improvisation.

5. Conclusion

The outcome of the present research indicates that the isolated strain *B. velezensis* has the efficiency to breakdown several components of crude oil. The strain degradation efficiency is increased by optimizing its growth parameters such as nitrogen source availability, and various concentrations of carbon sources. The isolated strain *B. velezensis* can be utilized for the bioremediation process of controlling oil spillage. Furthermore, genetic engineering technology can improve the significant degradation rate and breakdown of various components of crude oil.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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