ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 14 (4): 194-199, 2015
ISSN 1682-296X

RESEARCH ARTICLE OPEN ACCESS

DOI: 10.3923/biotech.2015.194.199

Primers and Probe Design for Identification and Analysis of Oil Degradation Bacteria (*Bacillus licheniformis*) with Quantitative PCR Technology

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ARTICLE INFO
Article History:
Received: April 14, 2015
Accepted: June 11, 2015

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ABSTRACT
Oil degradation bacteria (*Bacillus licheniformis*) were isolated and identified by using quantitative PCR-DGGE technology. The *Bacillus licheniformis* quantity was evaluated by its DNA content and its DNA content was measured by amplification of the DNA target gene. So the specific primers and probe of target gene were designed. Then, the difference was analyzed between using single *Bacillus licheniformis* bacteria and using *Bacillus licheniformis* compounded with other bacteria to degrade oil. The results showed that after 60 days cultivation, the oil degradation rate was only 5.6% by using single *Bacillus licheniformis* bacteria and DNA content of the single bacteria is 1.3×10^6 copies. When the single bacteria were mixed with other bacteria, the oil degradation rate was 30.7% and DNA content of the *Bacillus licheniformis* bacteria was 3.3×10^6. But, when the *Bacillus licheniformis* bacteria were removed, the oil degradation rate of mix bacteria was 21.3% and the DNA content of *Bacillus licheniformis* bacteria was 0. It showed the primers and the probe had good specificity. The oil degradation rate and DNA content of single *Bacillus licheniformis* bacteria were both 0 in the sterilization control group. Compared with traditional technology, the quantitative PCR-DGGE technology could accurately analyze the role of single bacteria in compound bacteria community.

Key words: Quantitative PCR-DGGE, primers and probe, oil degradation, *Bacillus licheniformis*

INTRODUCTION

When the oil pollution bioremediation, oil biodegradation and wastewater biodegradation were conducted, they need to constantly monitor the content of bacteria, especially for the specific functional bacteria, so as to identify the best degradation bacteria. But the oil degradation bacteria had great diversity (Li *et al*., 2012; Zhang *et al*., 2012). So the traditional technology did not meet this requirement, especially for the research of some specific functional degradation bacteria, which were difficult to be cultivated (Zhang *et al*., 2010).

In recent years, PCR-DGGE (Polymerase Chain Reaction-denaturing gradient gel electrophoresis) technology has been developed rapidly. But it is mainly used for general research and analysis of bacteria community and it is difficult to study the specific functional bacteria. While the quantitative PCR-DGGE technology has provided a new way to quantitative study the specific functional bacteria (Sun *et al*., 2013; Geets *et al*., 2007; Limpiyakorn *et al*., 2006; Lim *et al*., 2011). Because, the specific primers and probe was designed so the specific functional bacteria could be accurate analyzed by the quantitative PCR-DGGE technology (Geets *et al*., 2007; Limpiyakorn *et al*., 2006; Lim *et al*., 2011; Nakagawa *et al*., 2007; Wang *et al*., 2012; Song *et al*., 2010). Thus, the specific functional bacteria could be researched from oil field such as nitrate and sulfate reducing bacteria, iron, manganese reducing bacteria, methane-producing archaea bacteria (Roling *et al*., 2003; Gieg *et al*., 2008; Jones *et al*., 2008; Townsend *et al*., 2003; Khelifi *et al*., 2010; Aktas *et al*., 2010; Yang *et al*., 2008) and it is also used for oil pollution bioremediation, oil biodegradation, wastewater biodegradation (Li *et al*.,

2012; Zhang *et al*., 2012, 2010; Sun *et al*., 2013). In this study, the specific primers and probe were designed and using the quantitative PCR- DGGE technique to identify and analyze the oil degradation bacteria.

MATERIALS AND METHODS

Bacteria isolation and cultivation: The samples were from oil field. Then, they were sent to the laboratory immediately for enrichment culture (about 50 L).

Preliminary cultivation: Hundred milliliter water samples, 100 mL enrichment medium and 5 g oil were added into the bottle. Thirty days later, when the solution of bottle bottom turned dark, take supernatant from it and repeat the process 4 or 5 times, get bacteria solution, take the 0.6 mL bacteria solution, use sterile water to dilute it with the 10^{-1}-10^{-7} gradient dilution and then take 30 μL to coat on the solid medium (add 1.5% agar into enrichment medium) and cultivation in 30°C. A week later, check the bacteria and pick well-grown single strain bacteria and add it into selective medium, then repeat above operation twice in order to ensure the purity of bacteria.

Then the single bacteria solution and compound bacteria solution was prepared and bacteria content was adjusted to 1.0×10^{8} CFU mL^{-1} (Li *et al*., 2012). Then use the single bacteria solution and compound bacteria solution to research oil degradation rate. The bacteria solution was 5% and oil was 1%, then adding the selective medium. Meanwhile, the sterilization control group was done. After 60 days, using infrared spectrophotometry to calculate oil degradation rate (Zhao *et al*., 2011).

Selective medium KH_2PO_4 5.0 g, K_2HPO_4 5.0 g, NH_4Cl 5.0 g, NaCl 1.0 g, $MgCl_2$ 2.0 g, $CaCl_2$ 0.1 g, L-cysteine hydrochloride 0.5 g, ammonium ferrous sulfate 0.1 g, deionized water 1 L, pH:7.0-7.2. Enriched medium KH_2PO_4 5.0 g, K_2HPO_4 5.0 g, NH_4Cl 5.0 g, NaCl 1.0 g, $MgCl_2$ 2.0 g, $CaCl_2$ 0.1 g, yeast 1.0 g, L-cysteine hydrochloride 0.5 g, deionized Water 1 L and pH:7.0-7.2.

PCR-DGGE analysis and bacteria identification: After the DNA was extracted from single bacteria, then conduct PCR amplification to 16 s rDNA and the primers were 341 f and 534 r. Then, the DNA sequence of objective bacteria was analyzed and the similar species were identified in the international authoritative NCBI website (Wilms *et al*., 2006). 16S rDNA-DGGE (Denaturing Gradient Gel Electrophoresis) was performed using the DCode System (Universal Mutation Detection System, BIO-RAD).

Primers and probe design of qPCR and standard plasmid construction: According to authoritative GenBank database, the specific primers and the probe were designed for quantitative PCR. In order to test the primers and probe specificity of the *Bacillus licheniformis*, the control group was designed with the same primers and probe (the *Bacillus licheniformis* was removed).

The quantitative PCR reaction system were total 25 μL, including, DNA polymerase mixture 12.5 μL, primers 0.5 μL, probe solution 1 μL, the DNA template solution 0.5 μL. The standard plasmid was built with above primers and probe. Then standard curve was built with the standard plasmid to measure the DNA content (Geets *et al*., 2007; Limpiyakorn *et al*., 2006; Lim *et al*., 2011; Nakagawa *et al*., 2007).

Oil degradation rate measure: After degradation the extract after being diluted to a definite factor was analyzed by infrared spectroscopy to measure the hydrocarbon content (GB/T16488-1996, China) and the oil degradation rate was calculated (Zhao *et al*., 2011; Hu *et al*., 2015).

Oil saturated hydrocarbon gas chromatography-mass spectrometer: Oil saturated hydrocarbon was analyzed (according to SY/T 5779-1995). Instrument: Agilent 6890N GC/5975i mass spectrometry.

Inlet temperature: 300°C; the transmission line temperature: 280°C; Chromatographic carrier gas: 99.999% helium; Column: HP-5M Selastic quartz capillary column (60 m×0.25 mm×0.25 μm); Column temperature (temperature-programmed): initial temperature was 50°C min^{-1} and then up to 120°C according 20°C min^{-1} and up to 250°C according to 4°C min^{-1}, then up to 310°C according 3°C min^{-1}, keep 310°C for 30 min, carrier gas flow rate: 1 mL min^{-1} (McFarlin *et al*., 2014).

Data analysis: The data were mapped using origin 8.0 and all of the statistical analyses were performed using SPSS statistical software (SPSS Inc., Chicago, IL). The Standard Deviation (SD) and Least Significant Difference (LSD) were calculated to compare the treatment means.

RESULTS AND DISCUSSION

Identification of oil degradation bacteria: Using primers (341f and 534 r) to analyze the compound bacteria (PCR-DGGE analysis) and then the DNA sequence of objective bacteria were analyzed and the similar species were identified (Table 1). Through comparison in the NCBI database, there were two strains bacteria. They were similar to oil degradation bacteria (*Arthrobacter* sp. and *Acinetobacter* sp.) and the similarity degree was 95 and 94%, respectively and then, the Uncultured bacterium clone bacteria were compared and the similarity degree was 96%. The three single bacteria were not identified, so they need to be further researched.

Table 1: Analysis and identification of oil degradation bacteria

Name of similar bacteria	No. of NCBI database	Similarity (%)
Arthrobacter sp.	JF775581	95
Acinetobacter sp.	HQ670708	94
Uncultured bacterium clone	AB518653	96
Bacillus licheniformis strain	F3EU256500.1	99

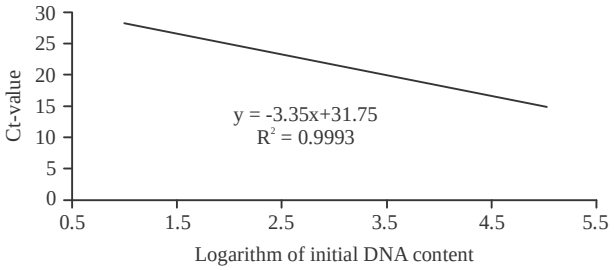

Fig. 1: Standard curve of plasmid DNA

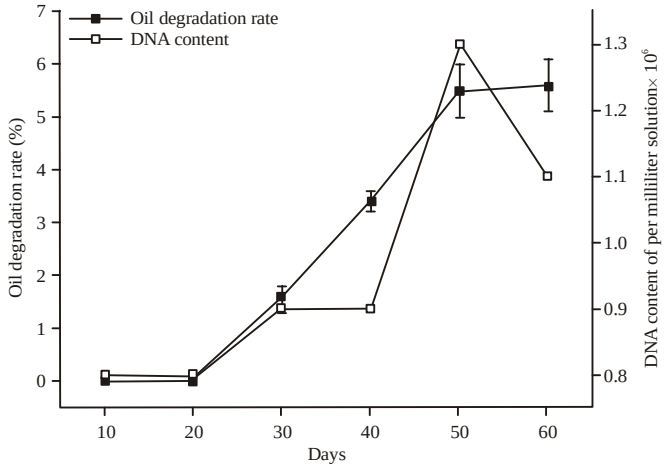

Fig. 2: Oil degradation rate and DNA content of the single *Bacillus licheniformis* bacteria

Table 2: Primers and probe of qPCR

Bacteria genus	Sequence of primers and probe	Target gene
Bacillus licheniformis genus	Primers:	DNA gyrase
	F460:AAAGCTGATTTGAAAGTCATTGGAGAT	gyrB gene
	R577: GAGTGGCGAGCGTATCATAGTC	
	Probe (with the report and quenching group):	
	ACGGGAACGACCACACACTTCAAGCCT	

At last, the bacteria belong to *Bacillus licheniformis* genus and the similarity degree was 99% to *Bacillus licheniformis* strain F3. And through single bacteria analysis, it was isolated and identified.

qPCR analysis of *Bacillus licheniformis:* The specific functional bacteria quantity was evaluated by their DNA content. And their DNA content was measured by amplification of the DNA target gene. So, in the quantitative PCR, the target gene sequence of *Bacillus licheniformis* was amplified by specific primers and probe (with the report and quenching group). Meanwhile, the fluorescent signal was collected, then, when the fluorescent signal reached a threshold, the PCR cycle times were recorded, so it was the Ct value. It was linear inverse proportion with the logarithm of initial DNA content (Geets *et al*., 2007; Limpiyakorn *et al*., 2006; Lim *et al*., 2011; Nakagawa *et al*., 2007; Wang *et al*., 2012; Song *et al*., 2010). The correlation was good when the R^2 value was closed to 1. But, when the probe was integrity, report and quenching group were very close and the report group was stimulated, then it transmitted energy to quenching group, so there was no fluorescent signal (Geets *et al*., 2007; Lim *et al*., 2011). In this study, the DNA polymerase could cut the report group and then the report group was not stimulated, so, there was fluorescent signal and the more initial DNA content, the stronger the fluorescent signal. Thus, the key oil degradation bacteria were identified in the biodegradation process.

The gyrB gene was amplified by the specific primers and probe of *bacillus licheniformis* genus (Table 2). So the DNA content of single *bacillus licheniformis* bacteria was measured, then the degradation status of the single *Bacillus licheniformis* bacteria was identified. Specifically, the DNA content of single *Bacillus licheniformis* bacteria was computed by standard curve and the standard curve was built by plasmid DNA (Fig. 1).

Through 60 days cultivation of single *Bacillus licheniformis* bacteria, the oil degradation rate was 5.6% Fig. 2). And the DNA content of *Bacillus licheniformis* bacteria was highest 1.3×10^6 copies at 50 days. In the sterilization control group, the oil degradation rate was 0 and DNA content of *Bacillus licheniformis* bacteria was

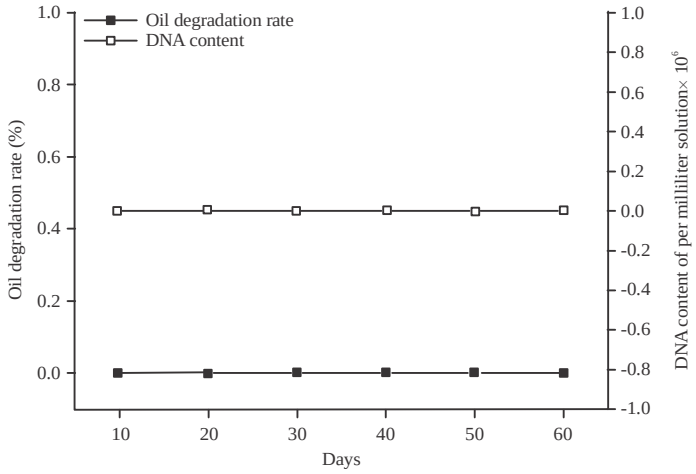

Fig. 3: Sterilization control group

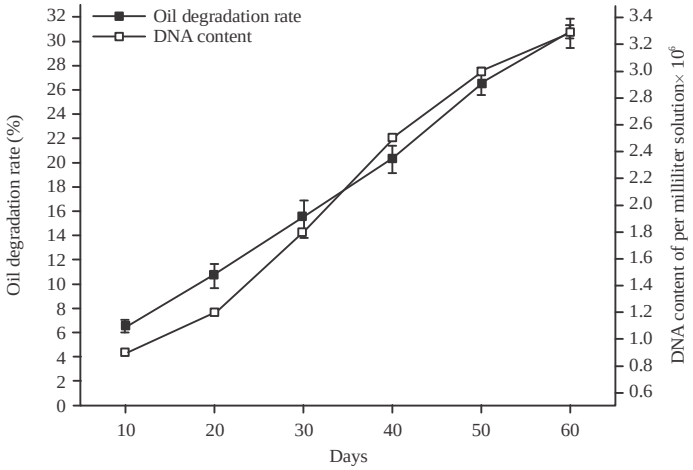

Fig. 4: Oil degradation rate of compound bacteria four strains bacteria and the DNA content of *Bacillus licheniformis* bacteria

also 0 (Fig. 3) and when the *Bacillus licheniformis* bacteria were removed, the oil degradation rate was 21.3% and the DNA content of *Bacillus licheniformis* bacteria was 0. It showed the primers and the probe had good specificity. The gyrB and rpoB gene of *Bacillus licheniformis* were utilized to design specific primers and probe and it had a good effect on specificity and accuracy (Renouf *et al*., 2006).

Analysis of oil degradation bacteriafoi: When the single bacteria mixed with other bacteria, the oil degradation rate was 30.7% and DNA content of the *Bacillus licheniformis* bacteria was 3.3×10^6 at 60 days as shown in Fig. 4. They were greater than the oil degradation rate and DNA content of using the single *Bacillus licheniformis* bacteria and when the *Bacillus licheniformis* bacteria were removed, the oil degradation rate of three strains bacteria was 21.3% at 60 days as shown in Fig. 5 and it was less than oil degradation rate of compound bacteria (four strains bacteria).

It showed that the *Bacillus licheniformis* bacteria could increase the oil degradation rate of compound bacteria and the *Bacillus licheniformis* bacteria had important role in compound bacteria community. But, when the bacteria were single researched, there was no significance and its oil degradation rate was very low (Fig. 2). By the quantitative PCR-DGGE technology, the bacteria role could be researched in compound bacteria community.

GC-MS analysis of oil saturated hydrocarbon degradation: The oil saturated hydrocarbon was fewer degraded by single *Bacillus licheniformis* bacteria (Fig. 6b) and when the *Bacillus licheniformis* bacteria were removed, the oil saturated hydrocarbon was well degraded (Fig. 6c). The long-chain saturated hydrocarbon was best degraded by compound bacteria (four strains bacteria) and the result was the best as shown in Fig. 6d. This once again showed that the *Bacillus licheniformis* had important role in the compound bacteria community and when the bacteria were single researched, there was no significance.

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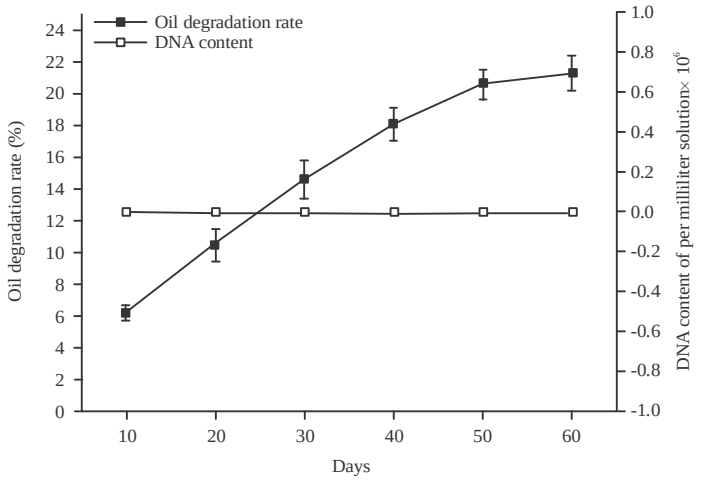

Fig. 5: Oil degradation rate of three strains compound bacteria (*Bacillus licheniformis*) were removed and the DNA content of *Bacillus licheniformis* bacteria

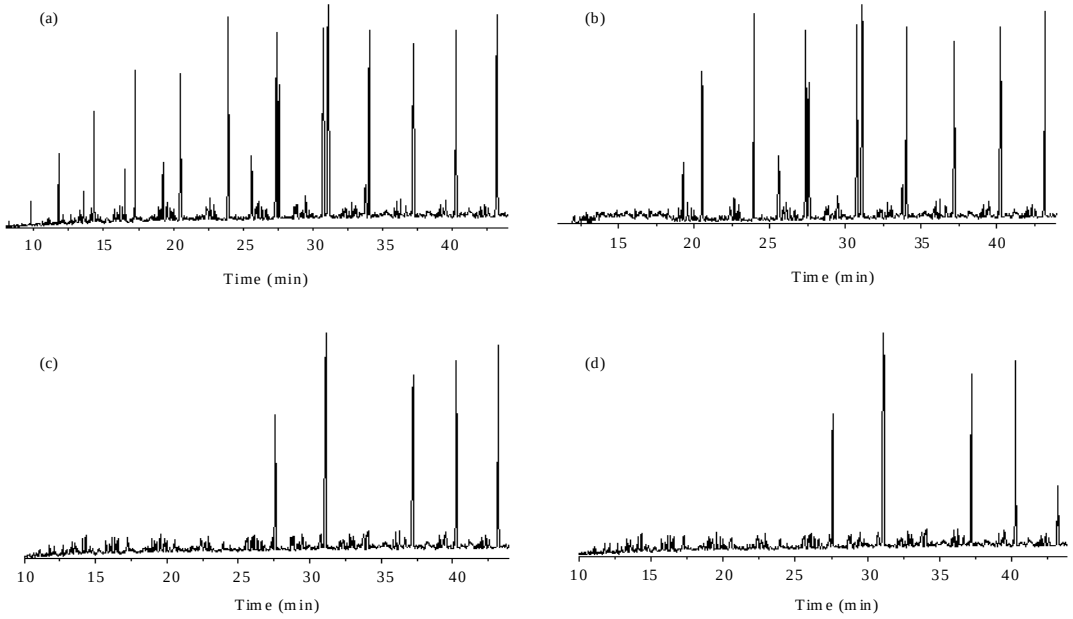

Fig. 6(a-d): GC-MS analysis of oil saturated hydrocarbon degradation, (a) Initial oil saturated hydrocarbon, (b) Oil saturated hydrocarbon was degraded by single *Bacillus licheniformis* bacteria, (c) Oil saturated hydrocarbon was degraded by three strains bacteria (*Bacillus licheniformis*) was removed and (d) Oil saturated hydrocarbon was degraded by compound bacteria

CONCLUSION

By the quantitative PCR-DGGE technique, the compound bacteria and single bacteria were analyzed and there were four strains single bacteria which were isolated. Then, the *Bacillus licheniformis* bacteria were identified. The specific primers and probe of *Bacillus licheniformis* bacteria were designed for accurate analysis and there were the following conclusions:

- Compared with traditional technology, the quantitative PCR-DGGE technology could accurately analyze the role of single bacteria in compound bacteria. And the important oil degradation bacteria were identified in complex bacteria community
- The oil degradation rate could be improved by the *Bacillus licheniformis* mixed with other bacteria. Meanwhile, the *Bacillus licheniformis* bacteria have an important effect on oil degradation in compound bacteria

- In this study, the specific primers and probe could be used to analyze *Bacillus licheniformis* bacteria which degrade oil

ACKNOWLEDGMENTS

The authors are graceful to the financial supports provided by China University of Petroleum and Science Research and Technology Developing Program, CNPC (2008D-4704-2).

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