Isolation and Identification of Thermophilic Bacteria of Guar Gum Biodegradation for Potential Use in Enzyme Breaker

Xin Ma[1,a], Lei Guang lun[1,b], Yao Chuanjin[1.c], Li Zi[1,d], Wang Zhihui[1,e], Cheng Mingming[1,f]

[1]Petroleum Engineering Department, University of Petroleum, Qingdao Shandong, China 266580

[a]maxin4561@163.com, [b]leiglun@163.com, [c]ycj860714@163.com, [d]liziisbest@yahoo.com, e17806286309@163.com, [f]Rachel19cheng@163.com

Keywords: thermophilic bacteria; enzyme breaker; guar gum; biodegradation

Abstract. Enzyme breakers have many significant advantages compared to the traditional oxidizer chemical breakers because of their substrate specificity, high efficiency and non-toxicity, thus enzyme have been widely used in the water-based fracturing fluids as guar gel breaker. However, there is an industry-wide demand for enzyme breakers that can function under high temperature of formation. The purpose of this paper is to develop the thermostable enzyme through the isolation and identification of thermophilic bacteria.

Analysis of 16S rDNA sequence is used to identify the bacteria and viscosity of guar gun aqueous solution is used to assess the biodegradation ability of bacteria. There is 16 bacteria isolated from water samples from oil field and one of them is identified as *Bacillus licheniformis* . *Bacillus licheniformis* are efficient in degrading the guar gum at high temperature and have the great potential to extract the thermophilic enzyme, as enzyme breakers, for application in hydraulic fracturing. The degradation curve of guar gum aqueous solution shows the degradation process: keeping flat, then rapidly decreasing, finally fluctuate at a stable value, which can support the hypothesis that induced enzyme plays the role in biodegradation.

Introduction

Hydraulic fracturing is a proved stimulation method used for optimizing the production of hydrocarbon reservoirs. It is a key technology in the oil and gas development of low permeability and tight formation. Generally, high viscosity fracturing fluid with solid proppant is pumped into the well at sufficient pressure to fracture the subterranean formation. Then breakers are used to reduce the viscosity of fluid by reducing the molecular weight of the polymers. The fluid flows back and the proppant is settled into fracture. The fracture then becomes a high permeability conduit for hydrocarbons to be produced back to the surface (Akash Tayal, 1997). Usually, most of hydraulic fracturing fluids are water-based gels, which means that guar gum or its derivative is commonly used in the fracturing fluid as viscosifying agent. Guar gel breakers generally be the key factor to limit the effectiveness of the fracturing operation. Guar gum is composed of a (1–4)-linked β- D -mannose backbone with (1–6)-linked α-D-galactose (Moreira, L. R.2008), which is natural polymer extracted from guar beans and makes it possible for the enzyme breaker development because the guar gum can be easily biodegraded.

Currently, breakers are either oxidizer or enzymes. Compared to the oxidative breaker, enzymes are more attractive with the inherent advantages of natural catalyst and substrate specificity. First, the enzyme breaks long–clain guar gum polymers specifically without undesirable reactions to the wellbore, formation, or fracturing equipment. Second, enzyme are not consumed in the reaction and can contimue working on other guar polymers during their liftime, thus providing an extended and controlled breaking profile. Third, the enzmy can break guar gum into much smaller fragments thus providing a more complete guar break with fewer residues.(Zhang B, 2012).

However, the limit factor of enzyme breakers application is the temperature of oil formation because of the temperature sensitivity of enzymes as proteins. In this study, therefore, the screening of

thermophilic bacteria will be the main task, these bacteria not only have the biodegradability of guar gum but also can adapt to the simulated formation temperature and high salinity. This bacteria will be the most important microbial resources of the novel enzyme breaker development.

Materials and Methods

Media and samples. Enrichment medium contained (L^{-1}): 2.0 g $NaNO_3$, 2.0 g KH_2PO_4, 1.0 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 6.0 g guar gum(Zhengzhou, Henan, China) and pH 7.0. After enrichment cultivation at 55℃, the enrichment cultures were diluted and coated repeatedly on isolation medium. The isolation medium (L^{-1}) was prepared with 2.0 g $NaNO_3$, 2.0 g KH_2PO_4, 1.0 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 4.0 g guar gum and 10.0 g ager. The purification and preservation medium was beef extract peptone ager medium, including 3.0 g beef extract, 10.0 g peptone, 5.0 g NaCl, 10.0 g ager (Cheng LJ,2000).Simulated formation water (SFW) in experiment contained (L^{-1}) : 21.0 g $CaCl_2$, 12.9 g NaCl, 0.65 g $BaCl_2$, 0.08 g $NaHCO_3$, 0.08 g $MgCl_2$ and pH 6.8, and the ion composition of which was showed in table. 1.

Table. 1 Simulated formation water (SFW) salinity

pH	Analysis item (ppm)									
	CO_3^{2-}	HCO_3^-	Cl^-	SO_4^{2-}	Ba^{2+}	Ca^{2+}	Mg^{2+}	Na^+	Salinity	Water type
6.8	0	80	56380	0	650	21000	80	12980	112920	$CaCl_2$

The screening medium which used to observe the effect of SFW on the growth of bacteria contained: 3.0 g beef extract, 10.0 g peptone, 10.0 g ager, 1L SFW.

Samples were obtained from the production water of the oil production wells, in Gudong-Xintan reservoir, Shengli oilfield, Shandong Province, China, in 2015. Temperature in the reservoir is approximately 53°C and well depth reaches 1000-1100 m. The temperature of samples were collected from wellhead is 50℃, and stored in plastic buckets at room temperature.

All reagents were of analytical grade.The main instruments of the experiment are 101 electric heating forced air drying incubator, Brookfield DV-III viscometer, NDJ-1B-1 viscometer.

Strain screening. Optimum growth temperature can be showed in the growth of bacteria lawn at different temperature (Cheng Lijuan, 2000). The purified bacteria were inoculated into isolation medium using the plate smearing method, then after sealed with plastic membrane, the plates were cultured at 40, 50, 55, 60 and 70℃ respectively for 3 days. Finally, the growth of bacteria lawn at different temperature were observed every 8 hours. All the experiments were done in triplicate.

Strain identification. Observe the colony characteristics and analysis of 16S rDNA sequence. Samples were sequenced in the BGI-Huada Genomics Institute in Shenzhen and the results were similarity searched in the NCBI though Blast program. Finally, the phylogenetic tree was constructed with the strains of high sequence similarity using the Neighbour-joining methods in the Mega 4.0.2.

Guar gum biodegradation. The guar gum solution (4.0 g/L guar gum, 2.0 g/L $NaNO_3$, 2.0 g/L KH_2PO_4, 1.0 g/L $(NH_4)_2SO_4$, 0.5 g/L $MgSO_4 \cdot 7H_2O$) was inoculated with strains. Then they was cultured at 50℃, 120r/min for 3 days. The rheological curves of guar gum solution before or after biodegradation were determined with different shear rate using the Brookfield DV-III viscometer. Viscosity at different time was assayed on a viscometer (NDJ-1B-1, Shanghai Jichang Instrument Co.,Ltd.) at 60 r/min. All the measuring temperature was 25℃.

Result and Discussion

Isolation, Purification and Screening.

Table.2 Isolation and purification result (40ºC) of bacteria for guar gum biodegradation

Strain number	Colony characteristics					Strain number	Colony characteristics				
	Shape	Color	Wettability	Transparency	Size(mm)		Shape	Color	Wettability	Transparency	Size(mm)
X7	Circular	Slightly yellow	Moist	Translucent	3.0	X2	Circular	White	Dry	Opaque	3.0
X10	Circular	White	Moist	Opaque	2.0	X3	Circular	White	Dry	Opaque	5.0
X11	Circular	Colourless	Moist	Opaque	1.0	X4	Circular	White	Moist	Opaque	4.0
X13	Circular	White	Moist	Opaque	3.0	XH	Circular	Dark yellow	Moist	Opaque	3.0
S3-1	Irregular	White	Dry	Opaque	3.0	G40	Circular	white	Moist	Opaque	6.0
G32	Irregular	White	Dry	Opaque	4.0	G50	Circular	White	Dry	Opaque	3.0
S2	Circular	White	Dry	Opaque	2.0	G502	Irregular	White	Moist	Opaque	4.0
X1	Circular	white	Dry	Opaque	4.0	X8	Circular	Yellow	Moist	Opaque	3.0

There are 16 strains bacteria (**Table. 2**) isolated from samples using the isolation medium. All the bacteria can grow well on the ager plate with the guar gum and inorganic nitrogen source at 40ºC.

Table. 3 Effect of temperature and simulated formation water (SFW) on the growth of bacteria strains

Strain number	Temperature (ºC)					SFW	Strain number	Temperature (ºC)					SFW
	70	60	55	50	40			70	60	55	50	40	
X7	-	-	-	-	+++	+++	X2	-	-	-	++	+++	+++
X10	-	-	-	-	+++	+++	X3	-	-	+	++	+++	+++
X11	-	-	-	+	+++	+++	X4	-	-	-	++	+++	++
X13	-	-	-	-	+++	+++	XH	-	-	-	+	+++	++
S3-1	-	-	-	+	+++	+++	G40	-	-	-	++	+++	+++
G32	-	+	++	+++	+++	+++	G50	-	-	-	+	+++	+++
S2	-	-	-	+	+++	+++	G502	-	-	-	++	+++	+++
X1	-	-	+	+++	+++	+++	X8	-	-	-	+	+++	+++

Figure.1 Photo showing the bacteria lawn of G32 on the ager plate with guar gum at 55ºC.

The growth of bacteria on the screening ager plates is got by the observation of bacteria lawn. "-", "+", "++" and "+++" (**Table.3**) mean that None growth, Growth, better growth and Best growth respectively. Simulated formation water (SFW) with high salinity hardly influences the growth of all the strains. That also means that the simulated formation water have little influence on the enzyme activity in the biodegradation, which is the necessary condition for enzyme breaker to decrease guar gel viscosity in the formation environment. From **Table. 3** and **Figure. 1**, G32 performs better than other bacteria, which is the preferred strain for further research.

Identification. Phylogenetic analysis based on 16S rDNA gene sequence analysis indicated that strain G32 is closely related to *Bacillus licheniformis* ATCC 14580(T) (Chester.1901) (98.86% gene sequence similarity) and *Bacillus aerius* 24K(T) (Shivaji S,2006) (98.79 % gene sequence similarity). The neighbour-joining phylogenetic tree further confirmed that the strains are phylogenetically related

to species of *Bacillus* and that G32 forms a clade with *Bacillus aerius* 24K(T). *Bacillus aerius* was discovered by Shivaji S in 2006, in the air samples from high altitudes (Shivaji S,2006), however, its growth occurs at 8-37 ºC, but not at 40ºC, which is not consistent with G32.Thus, based on colony characteristics and 16S rDNA sequence analysis, Strains G32 is identified as *Bacillus licheniformis*. *Bacillus licheniformis* was discovered by Chester in 2006. The rapid growth of *Bacillus licheniformis* occurs at 15-30ºCand it have been proved that it can produce β-mannanase which is thought as the main enzyme to degrading guar gum, but in this paper, more attention will be focus on the thermozymes above 50ºC.

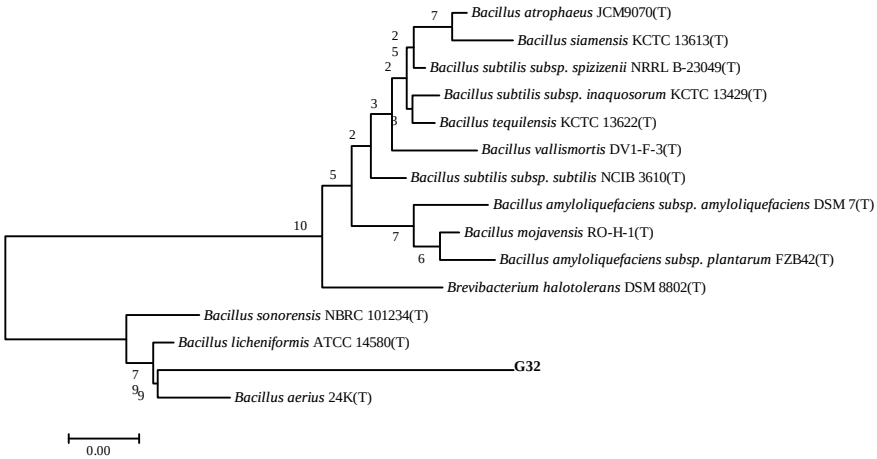

Figure 2. Phylogenetic tree constructed by the neighbour-joining method using 16S rDNA sequences of one bacteria culture obtained in this study and their close relatives retrieved from the GenBank database. Bootstrap values shown at nodes for frequencies at or above a 50% threshold (1000 bootstrap re-sampling).

Effect of bacteria on viscosity of guar gum.

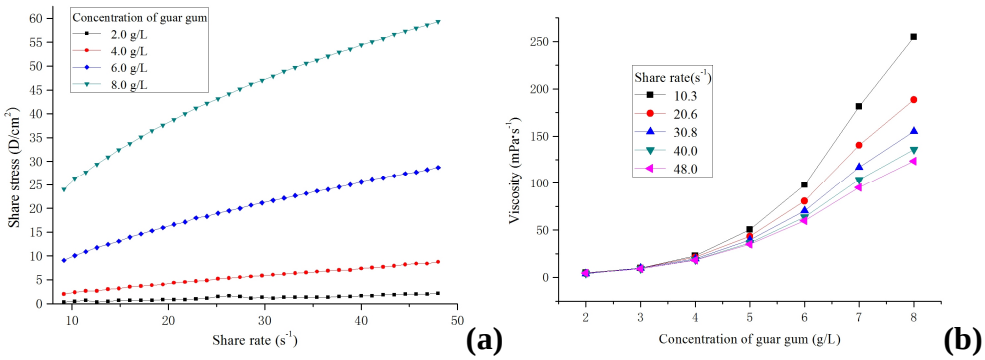

Figure.3 The viscosity of guar gum solution at different concentration and share rate

The rheology curves of guar gum solution were measured by Brookfield DV-III viscometer. It can be seen in the **Figure.3** that when the concentration of the guar gum in the solution was less than or equal to 4.0 g/L guar gum, the viscosity (μ) of the guar gum solution does not change with the change of share rate (γ), so solution between 2.0 g/L to 4.0g/L can be seen as the Newtonian fluid ($\mu=\tau/\gamma$, τ is shear stress, γ is shear stress, μ is a constant).

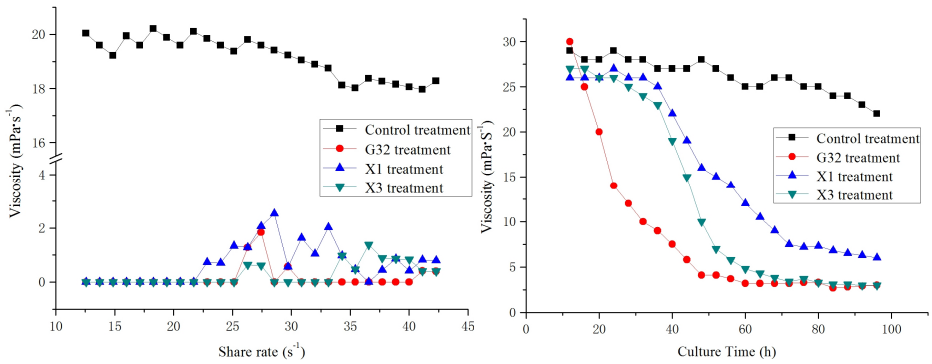

Figure.4 Effect of bacteria treatment on rheological curve of 4.0 guar gum solution

Figure.5 Viscosity decrease as a function of time, at different bacteria treatment of G32, X1, X3

It can be seen in the **Figure. 4** that the viscosity of control group keeps around 19.0 mPa·S^{-1} (3.5 g/L guar gum), while the solution with G32,X1 and X3 bacteria treatment for 3 days fluctuates at 1.0 mPa·S^{-1}, approaching the viscosity of water. The viscosity of guar gum solution decreased by approximately 94.7% ,which shows that the G32,X1,X3 can efficiently degrade the guar gum. These data support the hypothesis that microbes growing in the agar plate with guar gum can decrease the viscosity of guar gum solution.

The viscosity of solutions in the **Figure. 5** was determined using the NDJ-1B-1 viscometer at 60 r/min at 25°C with the guar gum concentration of 4.0 g/L. From the X1 treatment and X3 treatment in **Figure. 5**, the viscosity decrease of solution is observed after cultured 32 h. Then viscosity of the solution rapidly reduces from 40 h to 50 h, and the decreasing rate is slowed down from 50 h to 60 h. Finally, the viscosity of the solution approaches a stable value below 7.0 mPa·s^{-1}. However, the viscosity sharp decrease of G32 treatment begins before 16 h, then the decreasing rate begins to reduce from 28 h and finally tends to be stable at 50 h. In brief, the solution viscosity of Control treatment, G32 treatment, X1 treatment and X3 treatment decrease by 24.1%, 90.0%,76.9% and 91.4% respectively in 96 h.

The results also suggest that the enzyme have the most possibility to be extracellular induced enzyme. This enzyme is produced by bacteria in the presence of high concentrations of its substrate or a structurally similar substance. When G31, X1, X3 are inoculated in the guar gum solution, the guar gum as the substrate induces the bacteria to produce the relevant enzyme to degrade the guar gum. The time spent on the inducing process dependent on many parameters. Especially, the physiological and biochemical features in different microbial species maybe play an important role in the process.

Conclusions

(1) On the basis of the result, it can be concluded that strains G32, indentified as Bacillus licheniformis, are efficient in degrading the guar gum at high temperature and have the great potential to extract the thermophilic enzyme, as enzyme breakers, for application in hydraulic fracturing.
(2) Guar gum aqueous solution can be seen as the Newtonian fluid with the concentration less than 4.0g/L.
(3) Guar gum in the aqueous solution can be adequate degraded by Bacillus licheniformis with the viscosity of solution close to water.
(4) The degradation curve of guar gum aqueous solution shows the degradation process: keeping flat, then rapidly decreasing, finally fluctuate at a stable value, which can support the hypothesis that induced enzyme plays the role in biodegradation.

Acknowledgements

This work was financially supported by the Innovative team Yangtze scholars and development plans (IRT1294).

References

[1] Tayal A, Kelly R M, Khan S A. Viscosity Reduction Of Hydraulic Fracturing Fluids Through Enzymatic Hydrolysis. Spe Journal, Vol 2, 2013, 204-212.
[2] Zhang B, Huston A, Whipple L, et al. A Superior, High-Performance Enzyme for Breaking Borate Crosslinked Fracturing Fluids Under Extreme Well Conditions. Spe Production & Operations, Vol. 28 (2012), 210-216.
[3]Cheng Lijuan,. Microbiology laboratory technology. (World Pubishing, China 2000).
[4] Moreira, L. R., & Filho, E. X. Applied Microbiology and Biotechnology, Vol.79(2008)., 165–178
[5] Chester FD (1901). A manual of determinative bacteriology. New York: The Macmillan Co.
[6] Shivaji S, Chaturvedi P, Suresh K, Reddy GS, Dutt CB, Wainwright M, Narlikar JV, Bhargava PM. Bacillus aerius sp. nov., Bacillus aerophilus sp. nov., Bacillus stratosphericus sp. nov. and Bacillus altitudinis sp. nov., isolated from cryogenic tubes used for collecting air samples from high altitudes. Int. J. Syst. Evol. Microbiol. 56(7) (2006), 1465-73.