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Characterization of Short-Chains Hydrocarbons Produced by *Arthrobacter Nitroguajacolius* Strain IHBB9963 Isolated from Shule River Soil

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Abstract: ***The main objective of this study was to characterize short-chain hydrocarbons produced by arthrobacter Nitroguajacolius strain IHBB9963 isolated from Shule river soil in Gansu province-China. Results showed that in a total of 6strains that grown well on CMC-Agar medium with colony diameter ranged from3mm-4mm only 1strain was of greater hydrocarbons production ability. Chemical properties of extracted biofuel were analysed by Gas chromatography Mass Spectrometry .Among 41 identified short-chains hydrocarbons 5were detected at highest peaks and they contained carbon number in range of C_{16}-C_{22}. Belong to Alkane, Alkene and Alcohol. This strain is of a big importance on our society as it should be used in industries to produce energy that should solve a part of world energy problems.***

Keywords: *Arthrobacter Nitroguajacolius,* short-chain hydrocarbons, Shule River

1. Introduction

Recently extensive investigations of microbial hydrocarbons are greatly enhanced by the development of new analytical techniques, in particular, gas–liquid chromatography. By the60th years of the last century, a lot of information appeared on the intracellular hydrocarbons of the representatives of different systematic groups of microorganisms and mechanisms of the hydrocarbon biosynthesis. In contrast to higher organisms, microbial forms could be cultivated in reactors that allow the industrial production of hydrocarbons to be developed[1].Currently, there is industrial interest in nongaseous microbial hydrocarbons for specialty chemical applications and, more recently, as high-energy biofuels .Microbes produce hydrocarbons of different types, for example, aliphatic isoprenoid compounds and alkanes from fatty aldehyde decarbonylation. Fatty aldehyde decarbonylation is not well understood but offers a clean route to diesel fuels from fatty acids. Certain microbes also make a distinctly different class of long-chain hydrocarbons, generally C_{25} to C_{33}in chain length, that contain a double bond near the middle of the chain (1, 3, 5, 15, 30, 31, 33, 34). These long-chain olefinic hydrocarbons are thought to derive from processes different than isoprene condensation and decarbonylation mechanisms. This class of hydrocarbons has been shown by carbon-14-labeling studies to derive from fatty acids. The process, described in 1929 Buchannan and Chibnall has become known as head-to-head hydrocarbon biosynthesis. Albro and Ditmar defined the head-to-head condensation as coupling of the head (C_1) and the carbon (C_2) of two fatty acids with decarboxylation, a reaction that should not be confused with an acyloin-like carboxyl carbon-to-carboxyl carbon coupling. Products of the head-to-head mechanism have been identified in Gram-positive bacteria such as *Micrococcus luteus* and *Arthrobacteraurescens* and in Gram-negative bacteria such as *Stenotrophomonasmaltophilia Micrococcus* and *Arthrobacter* strains produce fatty acids that are methyl branched terminally and subterminally. The long-chain olefinichydrocarbons from those strains similarly contain a mixture of terminal and subterminal methyl group branching [2]. Aliphatic hydrocarbons are favorable targets for advanced cellulosic biofuels, as they are already predominant components of petroleum-based gasoline and diesel fuels and thus would be compatible with existing engines and fuel distribution systems. Certain bacteria are promising sources of the enzymes necessary for conversion of saccharification products such as glucose to aliphatic hydrocarbons, as a number of strains capable of aliphatic hydrocarbon production have been reported[3].

2. Materials and Methods

Shule River description and geography

Shule River flows generally westwards through the Tibetan Plateau and the desert regions of northwestern China. It starts in the Qilian Mountains and stretches for some 900 kilometers (560 mi); however, the river dries up about two-thirds of the way along its course and is lost in the Kumtag Desert, an endorheic basin, meaning its water never reaches the sea. Historically, the river reached farther west to Lop Nur, an intermittent salt lake in the Taklimakan Desert. It rises as several streams in a valley on the northern side of the Qilian Mountains or Nan Shan, on the Tibetan Plateau of extreme northern Qinghai province. In its upper 310 kilometers (190 mi) the river flows generally NNW through deep, glacier-carved valleys and precipitous gorges into Gansu Province, and is known as the Changma. It comes out of the mountains in Guazhou County, and spreads onto a massive inland delta or alluvial fan. The Shule's drainage basin covers about 102,300 square kilometers (39,500 sq. mi) of land, but the contributing portions total only 20,197 square kilometers (7,798 sq. mi). There are many other valleys and basins that would drain into the river during extreme floods, but because of the aridity of the region, high flow events are rare. The average precipitation in the watershed is 200 to 300 millimeters (7.9 to 11.8 in) per year. In the high mountain areas it can be up to 800 millimeters (31 in), mostly in the form of snow. In the deserts to the north annual precipitation is usually below 100 millimeters (3.9 in) and in some years, no rain falls at all[4].

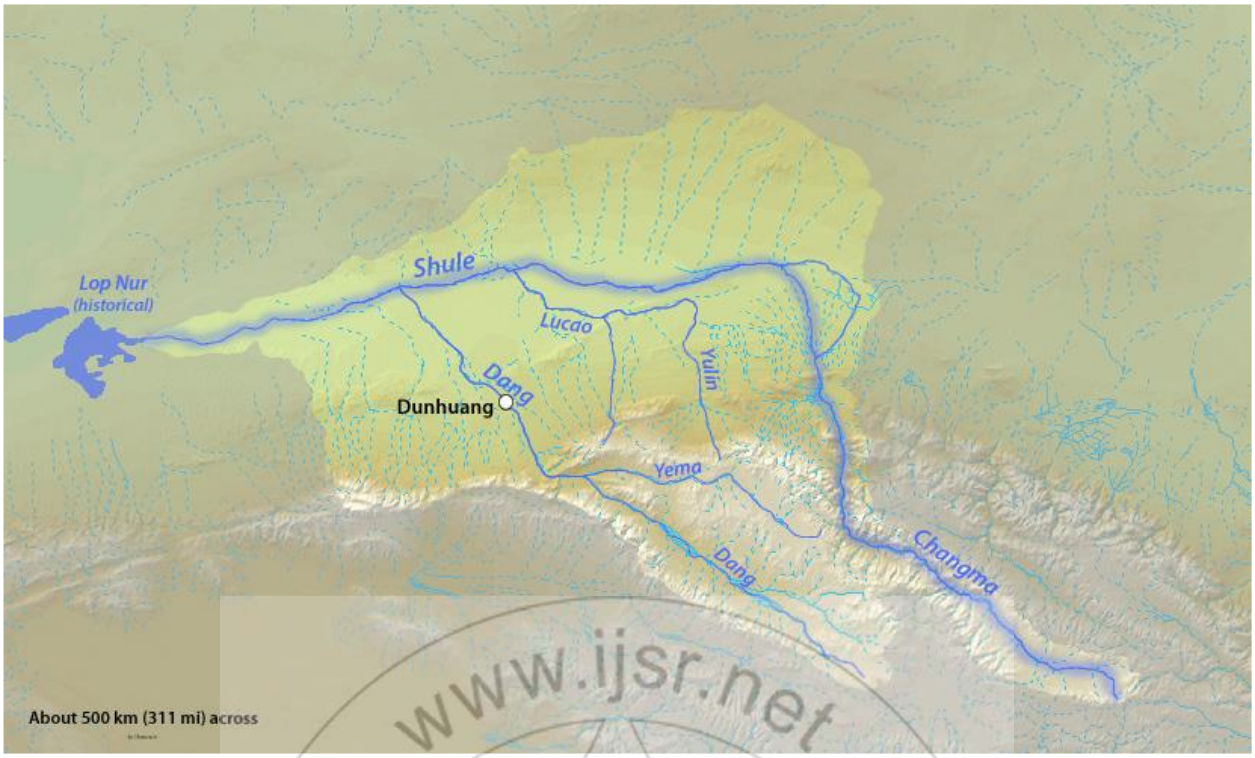

Figure1: Map of the Shule River drainage basin

3. Strains Isolation

Strains were isolated from different soil samples collected from Shule River .After collection all samples were stored at 4^0c in sterile containers. For each sample 0.5 gram was taken and diluted six-fold in 1000µL of distilled water. From each serial dilution 150µL were spread on plate containing (g/L) 10 CMC, 15 Agar,1 KNO_3, 0.5 NaCl, 0.5 $K_2HPO_4.3H_2O$, O.5g Mg $SO_4.7H_2O$, 0.01g$FeSO_4$, pH of 6.8-7.2, 1000µL of nalidixic acid in order to let only ***arthrobacter*** strains be isolated from the soils. The plates were incubated at 30ºC, after 10days strains were grown up my colonies on most of plates.

Evaluation of cellulose degradation ability by strains

A qualitative assay method was used to evaluate strains ability to degrade cellulose. All32 isolated strains were used to evaluate cellulose degradation ability. By using an inoculating needle, a small amount of strain was taken colony by colony and suspended in10µL of distilled water.1µL of the suspension for each strain was used culture at least 15 strains on plate made by previous CMC solid medium and soluble starch solid medium containing (g/L) 15 Agar, 1 KNO_3, 0.5 NaCl, 0.5 $K_2HPO_4.3H_2O$, 0.5g Mg $SO_4.7H_2O$, 0.01g$FeSO_4$

Investigation on hydrocarbons production ability of strains

A quantitatively assay was used to evaluate all strains ability to produce hydrocarbons by mean of quantity in grams of extracted hydrocarbons in 1gram of wet weight for each strain. Strains that showed good growth ability on both CMC-Agar medium and soluble starch-Agar (SS-Agar) medium were used. For each strain a small amount was suspended in 1000µL of distilled water, 200µL of the suspension was spread on 8plates:4LB-Agar plates and 4SS-Agar plates and incubated at25^{0}C.LB-Aagar medium was prepared by using (g/L) 10NaCl, 10Pryptone, 5Yeast-Extrat, 15Agar.After 5days of incubation, strains were grown up on all plates; they were collected in sterile containers plate by plate. For each strain, hydrocarbons were extracted by using methanol and hexane in proportion1:5. Reagents were added to all strain containers and centrifuged at 8000rpm for 15minutes.The hexane layer was then removed for soxhlet extraction.

Characterization of extracted hydrocarbons

Extracted hydrocarbons were characterized on basis their chemical properties with Gas Chromatograph Mass Spectrometry in the laboratory of Chinese Academy of Science in Gansu province-China.

16S rRNA Gene sequencing and phylogenetic analysis

When strains what strongly produce hydrocarbons were isolated, their chromosomal DNA were extracted with E.Z.N.A Bacteria DNA Kitthen DNA fragment of rRNA gene were amplified [5].The PCR products were sent to Beijing Liu He Huada gene technology Ltd to be cloned. The clones were sequenced and the sequences were aligned with 26 Fasta. Phylogenetic analysis was done by MEGA5.5[6].

Statistical analysis

Data were analysed using Microsoft excel. The t-test was performed for comparing mean values of individual variable for each strain between the two conditions at 99% significance level. When comparing data of each variable among 6 strains, one-way analysis of variance (one-way ANOVA) was applied. The difference of considered variable was estimated by Duncan's multiple range tests according to α-risk of 5%.

4. Results and Discussion

Strains ability to degrade cellulose

Among 32 strains which morphologically belong to arthrobacter species, a qualitative assay of cellulose degradation revealed that 26 strains showed a good growth and soluble starch-Agar medium and a poor growth on CMC-Agar medium whereas 6strain showed a good growth on both medium as showed in Venn diagram below.

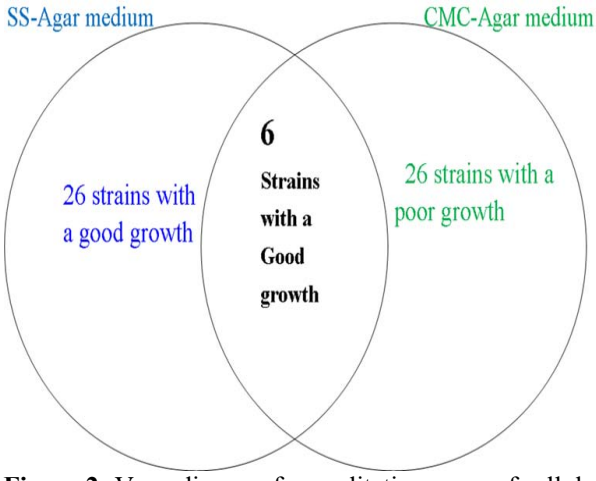

Figure 2: Venn diagram for qualitative assay of cellulose degradation

As shown in the figure2 6strains that grown well on CMC-Agar and SS-Agar medium were isolated from a total of 32 strains.

Figure 3: Growth ability of strains on CMC-Agar and soluble starch (SS)-Agar medium

Figure 3 is showing strains ability to grow on CMC-Agar and SS-Agar medium, on SS –Agar plate all strains showed a good growth whereas on CMC-Agar plate only few strains showed a good growth.

Table 1: Strains morphological characterization

Strain	Morphological characterization	Colony diameter in mm
SLP4(10^{-2})a	rod-coccus ,V-shape	3.5mm
SLP9(10^{-4})c	rod-coccus,V-shape	4mm
SLP8(10^{-3})a	rod-coccus,V-shape	3mm
SLP2(10^{-4})c	rod-coccus,V-shape	3.5mm
SLP1(10^{-2})a	rod-coccus,V-shape	3mm
SLP4(10^{-4})c	rod-coccus,V-shape	4mm

As given in Table1morphological characteristic and colony diameter of the 6strains that showed a good growth on CMC-Agar and SS-Agar medium .In this study, qualitative assay of cellulose degradation only 6strains with colony diameter comprise between 3mm-4mm showed a good growth on CMC-Agar medium and they were used for hydrocarbons production assay. In recent studies many other bacteria have been reported by other authors as cellulose degrading bacteria, their potential to degrade cellulose was qualitatively and quantitatively analysed[7].Cellulose degradation study by Bacillus Brevis was shown by formation of clear zone on culture medium that indicated cellulose degradation[8].Other cellulose degradation bacteria were isolated from Mangrove forest .Using CMC-Agar medium ,cellulose degradation was indicated by formation of halo zone on culture medium[9].

Hydrocarbons production

Results from quantitative assay of hydrocarbons production showed that among the 6stains which grow well on CMC-Agar and SS-Agar plates, only one strain was of a greater hydrocarbon production.

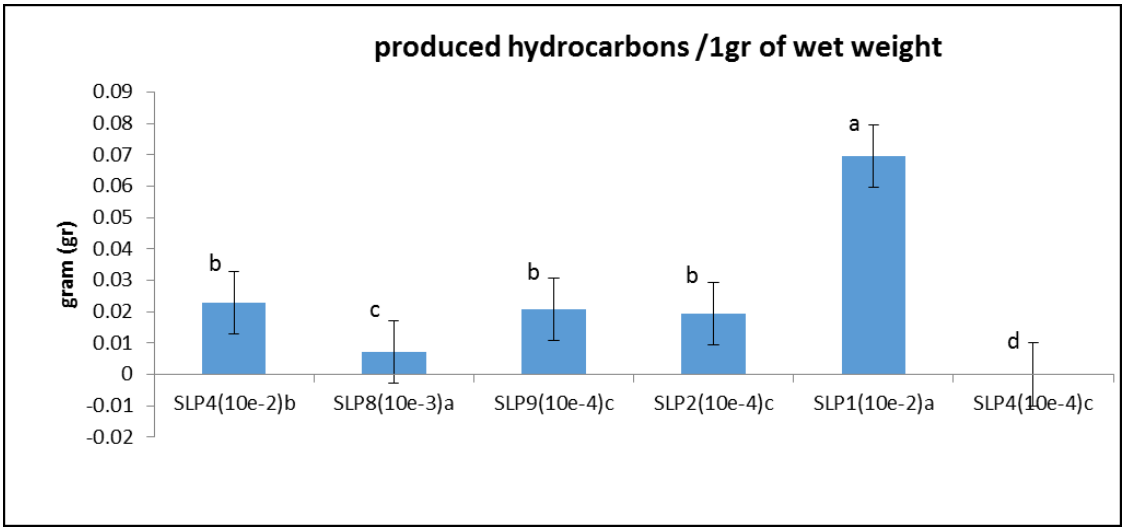

Figure 4

In this figure all the 6strains hydrocarbons production ability was analysed. Post-test analysis were done by Duncan's multiple range test ($p<0.01$) using Microsoft-Excel; same letter indicate the same significance.

The analysis of variance (ANOVA) of the model for hydrocarbons production ability is shown in the figure4.The predicted models were fitted significantly ($p<0.01$).The figure4 was used to analyze the significant interaction effect of independent variables on 6strains hydrocarbons production ability. The strain IHBB9963isolated for the name of SLP1 (10^{-2})a was identified as more hydrocarbon producer.

Table 2 Gas Chromatography Mass Spectrometry analysis report of extracted hydrocarbons from the strain IHBB996

	Detected hydrocarbons	Signal strength	Retention time in min
1	Dodecane Heneicosane Hexadecane	0.98	9.296
2	2-Bromododecane Tridecane 1-iodo-Hexadecane	3.68	9.73
3	2,4-bis(1,1-dimethylethyl)Phenol, 2,4-bis(1,1-dimethylethyl) Phenol, 2,4-bis(1,1-dimethylethyl)	2.8	10.029
4	2-Bromo dodecane Heneicosane Tridecane, 1-iodo-	1.3	10.267
5	2-Bromotetradecane Heneicosane Tetratriacontane	1.04	11.373
6	Heptadecane, 3-methyl- Octadecane Pentadecane, 2,6,10-trimethyl-	1.03	11.509
7	Heptadecane, 9-octyl-Octadecane Octadecane	1.46	11.944
8	Heptadecane Heptadecane Heptadecane	3.04	12.086
9	Heneicosane 2-Bromotetradecane	4.66	12.229
	Heptadecane		
10	Heneicosane Heneicosane Heptadecane	1.46	12.69
11	1-Octadecene 1-Nonadecene Pentadecyl -trifluoroacetate	7.85	13.111
12	Octadecane Octadecane Octadecane	1.59	13.166
13	Heneicosane Pentacosane	3.49	13.695
14	Eicosane, 10-methyl-Heneicosane Octadecane	1.32	14.089
15	Heptadecane, 3-methyl-Triacontane Tetracosane	1.12	14.13
16	Pentadecane, 8-hexyl-Eicosane, 10-methyl-Octadecane	1.88	14.197
17	Octadecane Octadecane, 1-iodo-Eicosane	0.9	14.387
18	Heneicosane Octacosane Dodecane, 2,6,11-trimethyl-	5.76	14.476
19	Octadecane Heneicosane Pentacosane	1.9	14.89
20	5-Eicosene, (E)- 3-Eicosene, (E)- 1-Docosene	1.8	15.127
21	Eicosane Eicosane Eicosane	0.96	15.182
22	Heptadecane, 3-methyl-Heneicosane Tetratriacontane	1.08	15.786
23	Tetratriacontane Tetratriacontane Hentriacontane	1.22	16.057
24	Heneicosane Heneicosane Heneicosane	2.2	16.125
25	Tetratetracontane Tetratriacontane	1.11	16.248

	Octacosan		
26	Octadecane, 1-iodo-Hexacosane Heptadecane, 2-methyl-	2.2	16.397
27	Heptacosane Heneicosane Hexadecane, 2-methyl-	4.52	16.506
28	Tetratriacontane Heptacosane Hentriacontane	2.09	16.791
29	Octadecane, 1-iodo-Hentriacontane Octacosane	1.31	16.872
30	Behenic alcohol 1-Heneicosanol Pentadecylpentafluoropropionate	10.86	17.001
31	Docosane Hexadecane, 2,6,10,14-tetramethyl- Docosane	2.55	17.035
32	Heneicosane Octadecane Octacosane	1.73	18.311
33	Hexadecane, 2-methyl- Tetratriacontane Octadecane, 1-iodo-	2.35	18.359
34	4-([(Furan-2-ylmethyl)-amino]-methyl)-3,5-dimethyl-1H-pyrrole-2-carboxylic acid ethyl ester Benzeneethanamine, N[(pentafluorophenyl)methylene]-4-[(trimethylsil yl)oxy]- Benzo[h]isoquinoline	1.85	18.427
35	9-Octadecenamide, (Z)- 9-Octadecenamide, (Z)- 9-Octadecenamide, (Z)-	2.94	18.569
36	Heptadecane Octadecane, 3-methyl-Octadecane, 5,14-dibutyl-	1.15	19.391
37	Nonadecane Tricosane Heptadecane, 2-methyl-	1.13	19.52
38	Hentriacontane Octadecane, 1-iodo-Octacosane	2.05	20.002
39	Octacosane Hentriacontane Hentriacontane	0.89	20.056
40	Octadecane Hentriacontane Heptadecane, 3-methyl-	1.24	21.848
41	1-Docosanol, methyl ether Eicosane, 9-cyclohexyl-Hexatriacontyl pentafluoropropionate	5.52	22.33

Figure 5: Chemical structures of detected hydrocarbons at big peak

Peak number	Retention time	Signal strength	
9	12.229	4.66	
11	13.111	7.85	
18	14.476	5.76	
30	17.001	10.86	
41	22.330	5.52	

As seen from the [figure5], in this study five short-chains of hydrocarbons were detected at highest peaks eluted in time distance of 12,229-22,330 min from C_{16}-C_{22} belonging to Alkane, n-Alkene, Alcohol. Different authors have studied about hydrocarbon synthesis from many other bacteria. In gram-positive bacteria of genus *Clostridium*, which growth is based on fermentative process, produced intracellular hydrocarbons from C_{18}-C_{27} or long-chain n-alkanes C_{25}-C_{35}. In yeast, hydrocarbon synthesis depended considerably on the growth condition[1].Long-chain monoalkenes predominantly cis-3,25-dimethyl -13-heptacosene were produced by *arthrobacter* species[10].

Strain morphology and phylogenetic analysis

Morphological characteristics of the isolate IHBB9963 were analysed through optical microscope observation as it is presented in Table1 which showed that this isolate had the general features of genera *Athrobacter*. The identification of the isolate IHBB9963 was further corroborated by its 16S rRNA gene sequence. A phylogenetic tree was constructed through 16SrRNA gene as indicate in [figure6].The results showed that the isolate IHBB9963 was phylogenetically related to the members of the genus *Arthrobacter*. Hence this isolate was referred as to ***Athrobacternitroguajacolicus***

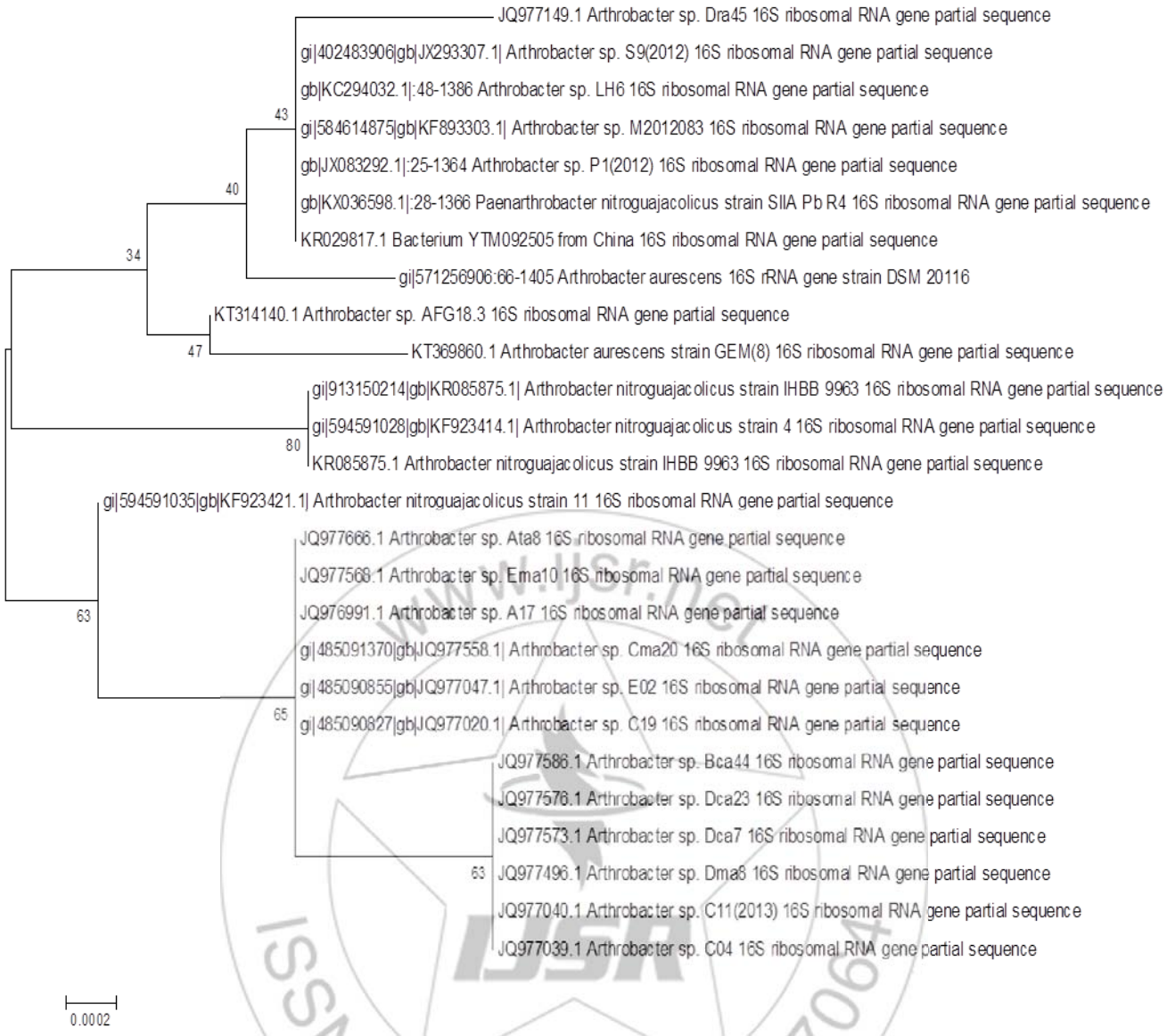

Figure 6: Neighbour-joining tree based on the 16S rRNA gene sequence of the strain IHBB9963 and the sequences of representative strains from Gene Bank. The bar represents 0.0002 substitutions per site respectively

5. Conclusion

From this study it can be concluded that ***Athrobacternitroguajacolicus* strain IHBB9963** isolated from Shule river of Gansu province could efficiently produce short-chain hydrocarbons which could probably be used as energy sources and it could also be used in some industries to solve world energy problem.

6. Acknowledgement

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