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Full Length Research Paper

Bacterial diversity of lipase-producing strains in different soils in southwest of China and characteristics of lipase

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Due to the rapid development observed in industries, environmental pollution became more and more critical. Lipase-producing strains played a key role in the enzymological remediation of polluted soils. Diversity of culturable lipase-producing bacteria in the southwest of China was studied with 198 samples collected from different points, along the transect from Tibetan Plateau-Ya An–Sichuan Basin. The enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) technology was used to amplify the isolated lipolytic producer's DNA samples and the amplified results were analyzed by NTSYS-pc2.1 to identify the similarity of isolates. A total of 54 bacterial strains were isolated from samples: 21 strains from Sichuan Basin (38.9%), 15 strains from Ya An (27.8%) and 18 strains from Tibetan Plateau (33.3%). The result of enzyme activity analysis showed that intracellular enzyme activity was nearly three folds than extracellular enzyme activity, and the intracellular enzyme activity was 6.79, 6.23 and 5.59 U/ml from Sichuan Basin, Ya An and Tibetan Plateau, respectively. Based on 16S rRNA gene sequence analysis, 12 strains were identified and belonged to *Bacillus, Klebsiella, Pseudomonas* and *Enterobacter.* The results showed that the *Bacillus* strains were the preponderant strains in the Tibetan Plateau and the *Klebsiella* strains were predominant isolates in Ya An. It suggested that the strains of *Klebsiella* were the transitional lipase-producing strains from Tibetan Plateau to Sichuan Basin. It could be concluded that the diversity of lipase-producing strains showed a transitional tendency: diversity of lipase-producing strains in Sichuan Basin were significantly higher than that of other two places and the diversity decreased gradually from plain region to Plateau region.

Key words: Enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR), lipase-producing microorganisms, diversity, Tibetan Plateau.

INTRODUCTION

Lipase (triacylglycerol acylhydrolase EC 3.1.1.3) is a class of hydrolase which catalyzes the hydrolysis of triglycerides to glycerol and free fatting acids over an oil-water interface (Cooper and Morgan, 1981). Furthermore, lipases can also catalyze the hydrolysis and esterification of other esters as well as the transesterification, acidolysis and ammonolysis of the esters. The food, detergent, cosmetic, organic synthesis and pharmaceutical industries have gotten special interest in the microbial lipase (Gupta et al., 2004; Davies, 1954). Lipases have emerged as one of the leading biocatalysts with proven potential for contributing to multibillion dollar underexploited lipid technology bio-industry and have been used *in situ* lipid metabolism and *ex situ* multifaceted industrial applications.

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Lipases are ubiquitous in nature and are produced by several plants, animals and microorganisms. Study on lipase can be traced back to 100 years ago, while the lipases from microbe have gained enough attention in the last decades (Ciafardini et al., 2006). In recent years, the screening of lipase producer has been fixed at two aspects: on one hand, some scientists have devoted themselves to detecting some lipases with novel and specific properties such as alkalophile, halophile, psychrophile and thermophile from some adverse environments (Klibanov, 2001; Joseph et al., 2008; Sorokin and Jones, 2009); on the other hand, some microbiologists have paid enough attention to screening some productive strains from some oil sludge areas or industrial waste (Ertugrul et al., 2007; Kiran et al., 2008).

The articles about strains that can produce lipases with some specific properties can be observed by a great number of published papers in latest decades (Ito et al., 1998; Kulkarni and Gadre, 2002; Mourey and Kilbertus, 1976), while there are a few papers about the distribution or the component of the micro-flora in different soils. The challenge for the soil microbial ecologist is to identify the populations and guilds of microorganisms that have key functional roles in specific soil processes. Polymerase chain reaction (PCR) amplification of 16S rDNA genes (Weisburg et al., 1991; Stackebrandt and Goebel, 1994) and enterobacterial repetitive intergenic consensus (ERIC)-PCR amplification (Hulton et al., 1991; Bruijn, 1992; Gillings and Holle, 1997) using consensus bacterial primers and separation of the resultant PCR amplify-cations by agarose gel electrophoresis (AGE) constitutes one of the popular techniques, which is used to describe soil bacterial ecology (Versalovic et al., 1991; Cullen and Hirsch, 1998; Pennanen et al., 2001). Bands on the gel can be sequenced and the resultant information can be used to infer something about the diversity of the original sample. There is a proliferation of these studies applied to soils, as molecular techniques have been systematically applied to many diverse environments. The diversity of Dhapa landfill at the East Kolkata (India) is investigated in 2007 with the usage of PCR amplification of 16S rDNA genes (Ghosh et al., 2007).

Tibetan Plateau, Ya An and Sichuan Basin are located in the southwest of China. These areas seem to be a ladder in topographic and the Tibetan Plateau is on the top of the ladder, and the bottom of the ladder is Sichuan Basin (ladder areas). The climate in Ya An is a transitional climate between Tibetan Plateau and Sichuan Basin. The mean altitude in Tibetan Plateau is up to 4000 m and the ultraviolet radiation here is much stronger than that of most places in the world. From the plain to plateau, the complex and varied terrains and landforms as well as the unique type of ecological system have created a natural paradise for biodiversity. However, there are few papers about the diversity of lipase-producing microorganisms in these ladder areas but some papers about Tibetan Plateau. Zhang et al. (2007) reported one psychrotolerant strain isolated from Qinghai-Tibet Plateau permafrost region. Lin et al. (2003) analyzed molecular mechanism of cold adaption of polar microorganisms.

In our present study, we investigated the diversity of lipase-producing strains in the Plateau-Ya An-Sichuan Basin with the usage of ERIC-PCR amplification and PCR amplification of 16S rRNA genes, in order to make sense of the composition cultivable lipase-producing strains in the south of China.

MATERIALS AND METHODS

Sampling site selection

Samples were collected from the southwest in China, including Tibetan Plateau (25°~40°N, 74°~104°E), Ya An (28°~30°N, 101°~103°E) and Sichuan Basin (30°~31° N, 102°~104°E).

Sample collection

One hundred and ninety-eight (198) samples were collected from the top 13 to 15 cm from each site and stored temporarily at 4°C in ice bag with well-labeled, autoclaved, sealed polythene ice bag. In addition, some water samples were collected directly into sterilized polythene tubes (Potumarthi et al., 2008; Ghosh et al., 2007).

Isolation and culturing of bacteria

Approximately 1 g sample was suspended in 9 ml of sterile water and then subjected to shaking for 1 h at 37°C. Two (2) ml of the supernatant was poured into 20 ml enrichment medium (Kulkarni and Gadre, 2002; Hasan et al., 2009) [yeast extract 0.02% (w/v), Na_2HPO_4 0.35% (w/v), KH_2PO_4 0.15% (w/v), $MgSO_4 \cdot 7H_2O$ 0.05% (w/v), NaCl 0.05% (w/v), olive oil emulsion 4% (w/w)] and subjected to shaking for 2 days at 30°C. Then, the supernatant was serially diluted and 200 µl was placed on pre-screening medium (Kulkarni and Gadre, 2002; Hasan et al., 2009) [yeast extract 0.5% (w/v), peptone 1.0% (w/v), NaCl 0.5% (w/v), glucose 0.3% (w/v), olive oil emulsion 4% (v/v), bromcresol purple 0.4% (v/v)]at 30°C for 3 to 7 days generally, but sometimes for 20 days. Isolation of the lipase-producing strains was detected by the clearance zones on the pre-screening medium. Based on the zones of clearing, pure colonies were selected and used for further studies. All the isolates were stored as glycerol stock in -80°C freezer in Sichuan Typical Culture Collection Center (SCTCC).

Detection of the lipase activity

In our study, we adopted the acid-base titration (Peralta et al., 2009) and the lipase activities of the extracellular and intracellular lipase were taken into account (Lee et al., 2004; DS et al., 2007). All the isolates were incubated for 48 h at 30°C in 4 ml fermentation medium (Kulkarni and Gadre, 2002; Hasan et al., 2009) [peptone 0.2% (w/v), sucrose 0.5% (w/v), $(NH_4)_2SO_4$ 0.1% (w/v), $MgSO_4 \cdot 7H_2O$ 0.05% (w/v), K_2HPO_4 0.1% (w/v), olive oil emulsion 4% (v/v)]. Two (2) ml fermentation medium broth was removed and centrifuged at 12000 rpm for 2 min at 4°C. The supernatant was used for detecting the lipase activity in extracellular, while the cell pellet was re-suspended in 500 µl phosphate buffer and treated with Ultrasonic cell disruption system (Chang Zhou, China). Then the suspension was centrifuged at 12000 rpm for 2 min at 4°C. Next, the supernatant was used for detecting the intracellular lipase

activity.

Extraction of bacterial genomic DNA for PCR assays

All the isolates were cultured in Luria-Bertani (LB) medium at 37°C for 24 h. DNA was prepared from overnight culture by sodium dodecyl sulfate-proteinase k-cetyltrimethylammonium bromide (CTAB) method (Stewart and Laura, 1993; Yuan et al., 2010). All DNA preparations were treated with RNase A and DNA concentration were estimated by visual examination of ethidium bromide (EB)-stained agarose gels as well as spectrophotometrical examination.

ERIC-PCR fingerprints analysis

The primers ERIC1 (5′ ATGTAAGCTCCTGGGGATTCAC3′) and ERIC2 (5′AAGTAAGTGACTGGGGTGAGCG3′) (Versalovic et al., 1991) were synthesized by Shanghai Biological Engineering Technology and Services Co., Ltd. The PCR (25 µl) contained 10×buffer 2.5 µl, 0.2 mM deoxynucleotide triphosphates (dNTPs), 0.5 U TaqDNA polymerase, 2 mM $Mgcl_2$, primers 400 pM each (10 µM) and template DNA 3 µl (Duan et al., 2009; Yuan et al., 2010). The amplification was carried out by incubation of the mixture for 7 min at 95°C for pre-denaturation, followed by 30 cycles of denaturation at 94°C for 1 min annealing at 52°C for 1 min and extension at 65°C for 8 min. A final extension was performed at 65°C for 10 min. The PCR products were identified by 0.8 to 1.5% AGE (0.5×TBE, EB, 1 to 1.5 h, electrophoresis at 3 vcm^{-1}).

ERIC-PCR fingerprints of amplified DNA fragments obtained from AGE were recorded. The observed bands in the gels were evaluated based on the presence (encode 1) or absence (encode 0) of polymorphic fragments for the ERIC primers. Cluster analysis was performed with NTSYS-pc 2.1 (Rohlf, 2000), a numerical taxonomy and multivariate analysis software package, based on Dice's similarity coefficient (SD) with a 1% position tolerance and the unweighted pair group method using arithmetic averages (UPGMA). In addition, each isolate was considered as an operational taxonomic unit (OUT). In order to reduce the number of OUTs in dendrogram and facilitate interpretation, isolates of more than 90% similarity were treated as the same isolate (Borges et al., 2003).

Amplification of 16s DNA

The primers 27F (5′ AGAGTTTGATC(AC)TGGCTCAG′) and 1492R (5′ TACGGYTACCTTGTTACGACTT′) (Macrae, 2000) were synthesized by Shanghai Biological Engineering Technology and Services Co., Ltd. Partial amplification of the 16s rDNA gene was performed with the thermal cycler. The PCR of the genomics DNA of 12 isolates (Table 4), which were grouped into five clusters in the dendrogram at a coefficient level of 0.012, were conducted in a final volume of 50 µl. In order to examine whether strains grouped into one cluster shared the same genus, one more strains from clusters II and IV were sent to be sequenced (Table 3). The reaction mixture included 30 to 40 ng of isolated genomic DNA, 2 U Taq-ploymerase (Invitrogen, China), 1×PCR buffer with 1.5 mM $Mgcl_2$, 200 µM each dNTP and 10 pM of each primer (Invitrogen). The amplification was carried out by incubation of the mixture for 1 min at 94°C for pre-denaturation, followed by 30 cycle of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 90 s (Yuan et al., 2010). The samples were stored at 4°C until analysis by AGE electrophoresis. The amplified and gel-eluted PCR fragments were purified using purification kit (TianGen, China), and then cloned into PMD19-T vector, according to the manufacturer's instructions (Invitrogen, China) for sequencing.

Statistical analysis

Data obtained from the experiment including the amounts of lipase-producing strains which were isolated from different areas and the altitude of the sampling site were taken into account to analyze the distribution of lipase-producing strains in different altitude. In this study, statistical analysis was performed by employing Minitab 15.0 (Minitab Inc., Pennsylvania, USA).

Accession numbers

The sequence were submitted to GenBank and accession number were assigned: HQ622335(7), HQ622336(8), HQ622337(9B), HQ622338(14H), HQ622339(18), HQ622340(22), HQ622341(29-5), HQ622342(T38), HQ622343(47-1), HQ622344(T53), HQ622346(T12) and HQ622348(×10).

RESULTS

Isolation and characterization of lipase-producing strains in different regions and the regression analysis between rate of lipase-producing strains and the different altitude

Fifty-four (54) pure lipase-producing strains were isolated from 198 samples from the southwest area. Based on the zone of clearing on the prescreening medium, all the isolates were functionally screened and were used for further study. Ninety-eight percent (98%) of the isolates were acid lipase-producing microorganisms, only 1 in 54 microorganisms was alkaline lipase-producing strain during microbiological and enzymatic studies. The alkaline lipase-producing microorganism was predicated to be *Enterobacter*. Twenty-one (21) strains were isolated from Sichuan Basin, 19 isolates from Ya An and 14 strains from Tibetan Plateau, with the percentage of 38.9, 35.19 and 25.93% in total, respectively (Table 1a). Strains isolated from Sichuan Basin grew obviously more quickly than those from Ya An or Tibetan Plateau. Only when microorganisms from Tibetan Plateau were cultured at 20°C and cultured on the medium with 0.5% agar; they could produce clear clearance zones (Figure 1). Strains from Sichuan Basin and Ya An could grow at 20°C, but they would only produce clearance zones at 30°C. The regression equation was obtained based on the Minitab 15.0:

$$C1 = 0.474 - 0.000105\ C2$$

C1, The rate of lipase producing strains; C2, the different altitude (Table 1b).

ERIC-PCR analysis

The electrophoretic profiles of the ERIC-PCR products were determined for the lipase-producing strains isolates screened from Tibetan Plateau, Ya An and Sichuan Basin. Simultaneous use of ERIC1 and ERIC2 primers

Table 1a. The number of samples and strains detected in different areas.

Sampling site	Amounts of samples	Lipase-producing strains	L/S	Rate (%)
Tibetan Plateau	95	14	14/95	25.93
Ya An	50	19	19/50	35.19
Sichuan Basin	53	21	21/53	38.89

L/S, The number of the isolates/the number of the samples; Rate, the number of the isolates in each area/the total isolates.

Table 1b. Results of regression analysis between different altitude and amounts of lipase producing strains.

Term	Coefficient	Coefficient standard error	T-test	P-value
Constant	0.47434	0.05100	9.30	0.068
C2	-0.00010498	0.00002682	-3.91	0.159

S, 0.0487471; R^2, 93.9%; R^2 (adjust), 87.7%; C2, different altitude.

Figure 1. Isolation of lipase-producing strains based on the clearance zone on the plate. Strains from Tibetan Plateau can be detected through the clearance zone with the 0.5% agar.

yielded strain-specific complex banding pattern of about 10 bands in the range of 300 to 4200 bp. The data matrices based on the DNA fragments and the dendrogram using the NTSYS-pc2.1 software were constructed, dividing all the isolates obtained in this study into different clusters or branches based on the ERIC-PCR pattern similarity.

As shown in Figure 2, the dendrogram of the total 54 strains, the isolates could be categorized into different subgroups, which were vital to analyze the distribution of different strains in one area, based on the ERIC-PCR fingerprints. Five clusters were indentified at a coefficient level of 0.012 in the UPGMA dendrogram based on the ERIC-PCR fingerprints and strains in each cluster were thought to share the same genus (Bruijn, 1992; Gillings and Holle, 1997). Despite the fact that some bands were common to several isolates, highly polymorphic fingerprints were obtained. There were only 2 strains, 3 and T65, sharing 100% similarity in all strains, which indicated that strains 3 and T65 was the same strains. There was also 95% similarity between 15 and 5-2LI, and there was just 80% similarity between 56-1 and N5 and between 40 and 2. The others shared low similarity, for instance, T6 and 38-1 just shared 56% similarity.

All the isolates could be categorized to four genera: *Klebsiella, Bacillus*, *Pseudomonas* and *Enterobacter* based on morphological, physiological and biochemical tests with reference to Bergey's Manual of Determinative Bacteriology combined with 16S rDNA sequence analysis. Especially, strains 9B, 14H and 22 were selected out II cluster and they were identified to be *Bacillus* sp., *Bacillus cereus* and *Bacillus subtilis* based on 16S rDNA sequence analysis. Strains T53, 18, 8, 29-5, 7 and T12 were randomly selected out and sequenced; the results suggested that they were *Klebsiella,* which supported that the strains in each cluster shared the same genus (Table 4). There were 12 *Bacillus* strains, 25 *Klebsiella* strains, 4 *Enterobacter* strains and 13 *Pseudomonas* strains. 67.77% of *Bacillus* strains were isolated from Tibetan Plateau and 76% *Klebsiella* strains were isolated from the plain or low altitude areas.

Extracellular and intracellular enzyme activity analysis

All 54 strains could produce extracellular and intracellular lipases and the intracellular enzyme activity was much higher than the extracellular enzyme activity (Tables 2a, b and c). It indicated that all these isolates were the intracellular lipase producers rather than extracellular enzyme producers. For different regions, the characteristics of lipase displayed diversity. For Sichuan Basin, extracellular lipase activity was 2.92 U/ml and the intracellular lipase activity was 6.79 U/ml; for Ya An mountain area, the extracellular enzyme activity was 2.53

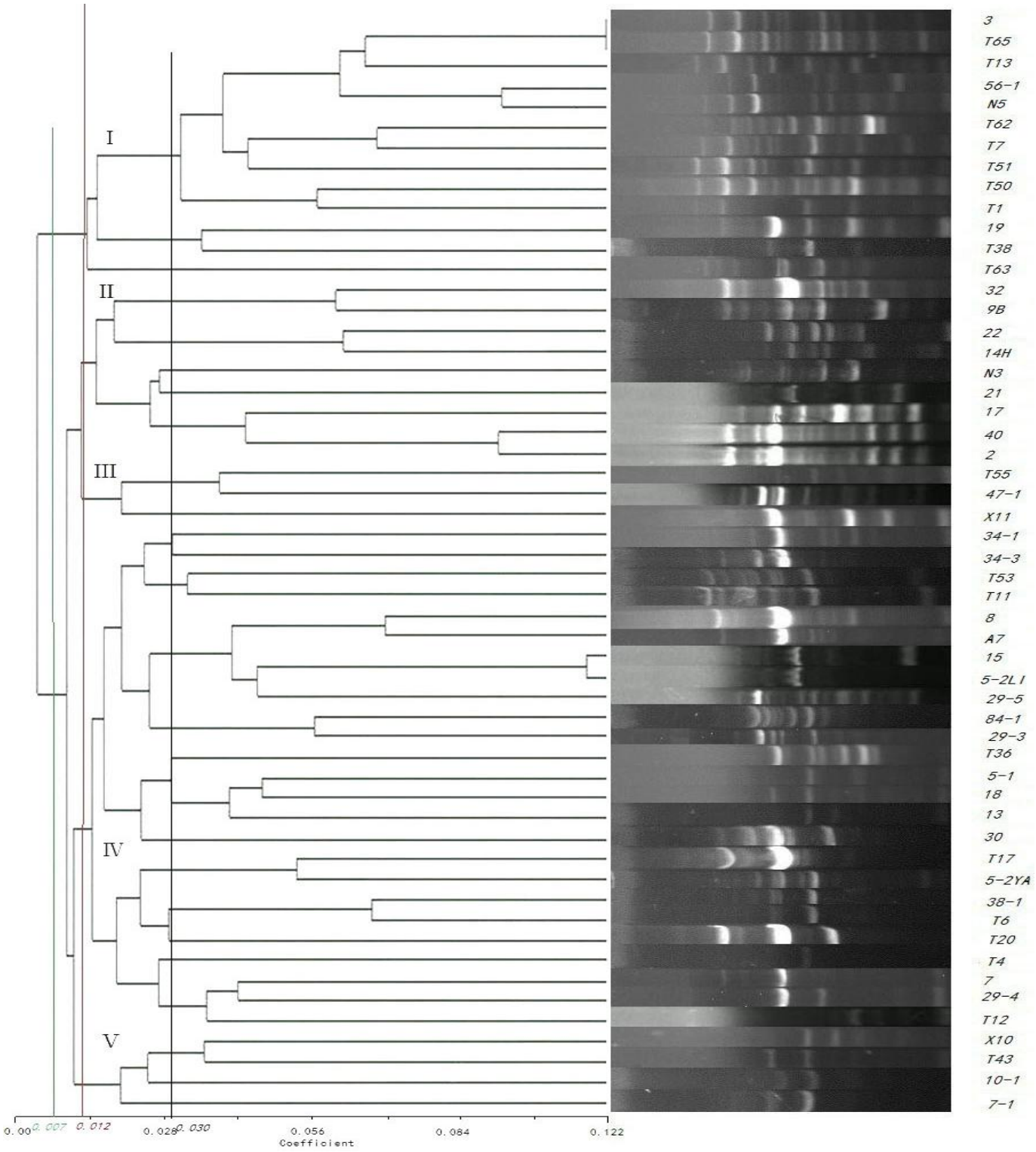

Figure 2. Dendrogram of 54 lipase-producing strains based on ERIC-PCR fingerprints. All the isolates were grouped into five clusters: I, II, III, IV and V when they are at a coefficient level of 0.012.

U/ml and the intracellular lipase activity was 6.23 U/ml; for Tibetan Plateau, the enzyme activity was 2.20 and 5.59 U/ml in average, respectively. We obtained 9 strains, whose intracellular enzyme activity was over 10 U/ml.

Table 2a. Lipase producing strains isolated from Ya An and the analysis of lipase activity.

Strain No.	Altitude (m)	Habitat	Extracellular activity (U/ml)	Intracellular activity (U/ml)	Genus
N5	853	Forest land	1.85	4.74	*Pseudomonas*
29-3	934	Forest land	2.83	4.43	*Klebsiella*
34-1	934	Forest land	2.83	9.34	*Klebsiella*
56-1	1097	Forest land	2.90	7.84	*Pseudomonas*
8	1104	Forest land	3.25	8.76	*Klebsiella*
29-4	1117	Forest land	5.93	13.89	*Klebsiella*
10-1	1128	Forest land	1.45	6.78	*Enterobacter*
5-2YA	1211	Forest land	1.36	2.13	*Klebsiella*
29-5	1222	Forest land	4.66	10.99	*Klebsiella*
5-1	1229	Forest land	1.19	4.35	*Klebsiella*
13	1307	Forest land	0.98	2.36	*Klebsiella*
T4	1334	Forest land	5.32	9.39	*Klebsiella*
19	1334	Forest land	3.67	8.39	*Pseudomonas*
T11	1341	Forest land	2.38	5.99	*Klebsiella*
17	1462	Forest land	1.37	3.11	*Bacillus*
7	1583	Forest land	1.52	4.38	*Klebsiella*
84-1	1609	Forest land	1.76	4.34	*Klebsiella*
9B	1649	Forest land	1.41	3.53	*Bacillus*
7-1	1675	Forest land	1.39	3.73	*Enterobacter*

Table 2b. Lipase-producing strains isolated from Sichuan Basin and the analysis of lipase activity.

Strain No.	Altitude (m)	habitat	Extracellular activity (U/ml)	Intracellular activity (U/ml)	Genus
3	482	Soil	1.09	1.35	*Pseudomonas*
T63	483	Soil	4.29	11.86	*Pseudomonas*
T53	483	Soil	1.88	4.47	*Klebsiella*
T17	485	Farm land	0.65	3.35	*Klebsiella*
T36	485	Waste water	3.73	10.54	*Klebsiella*
T6	486	Polluted water	1.98	5.32	*Klebsiella*
X11	486	Polluted soil	6.36	13.81	*Bacillus*
T65	488	Polluted soil	5.36	7.38	*Pseudomonas*
T55	488	Polluted lake	2.53	7.08	*Bacillus*
T7	488	Polluted soil	3.59	11.13	*Pseudomonas*
18	488	Farm land	2.93	7.34	*Klebsiella*
A7	490	Polluted water	4.52	10.53	*Klebsiella*
X10	491	Soil	2.54	6.73	*Enterobacter*
T13	492	Soil	1.09	3.72	*Pseudomonas*
T38	492	Polluted water	1.64	3.15	*Pseudomonas*
T50	493	Polluted lake	1.58	3.25	*Pseudomonas*
T12	493	Farm land	2.45	5.30	*Klebsiella*
T43	494	Polluted water	3.28	8.49	*Enterobacter*
T1	495	Soil	3.65	6.37	*Pseudomonas*
T62	498	Polluted water	3.58	6.95	*Pseudomonas*
T51	506	Soil	2.58	4.77	*Pseudomonas*

45% were *Klebsiella* strains and 30% were *Bacillus* strains. More than half of the strains with high enzyme activity were isolated from Sichuan Basin. Although as the altitude changed, the characteristics and type of lipase-producing strains would change, but there was no positive correlation between the enzyme activity and

Table 2c. Lipase-producing strains screened from Tibetan Plateau and the analysis of lipase activity.

Strain No.	Altitude (m)	Habitat	Extracellular activity (U/ml)	Intracellular activity (U/ml)	Genus
38-1	2245	Farm land	1.48	3.35	*Klebsiella*
T20	2367	Farm land	1.97	4.85	*Klebsiella*
30	2413	Farm land	1.98	6.51	*Klebsiella*
34-3	2413	Alkaline land	1.69	4.03	*Klebsiella*
N3	3120	Grass land	4.72	10.54	*Bacillus*
15	3126	Grass land	1.05	2.46	*Klebsiella*
2	3134	Alkaline land	0.93	1.89	*Bacillus*
22	3197	Grass land	3.25	7.14	*Bacillus*
32	3200	Alkaline land	1.95	6.78	*Bacillus*
47-1	3249	Qinghai lake	3.25	8.24	*Bacillus*
5-2LI	3300	Grass land	1.28	3.75	*Klebsiella*
14H	3321	Farm land	4.24	10.86	*Bacillus*
40	3441	Alkaline land	1.28	2.54	*Bacillus*
21	3495	Alkaline land	2.58	5.38	*Bacillus*

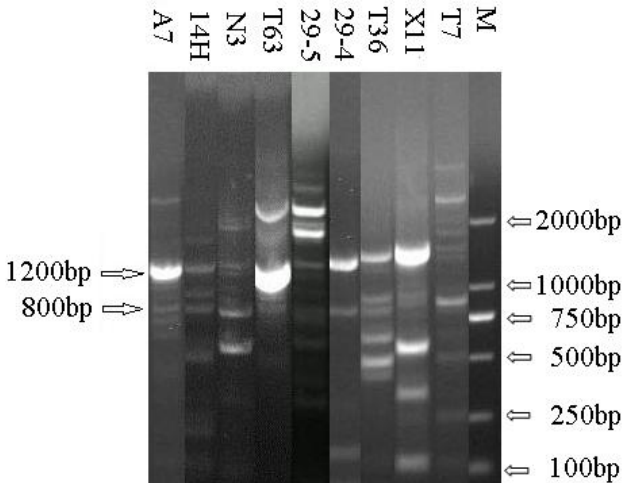

Figure 3. ERIC-PCR fingerprints of high lipase activity-producing strains. M, Maker.

altitude. The results indicated that lipase-producing strains isolated from Tibetan Plateau preferred to produce low temperature lipase other than normal temperature lipase as microorganisms from other two places did. Two consensus fragments were observed in these 9 isolates (Figure 3), which might be related to their higher enzyme activity.

DISCUSSION

For three sample sites: Sichuan Basin, Ya An and Tibetan Plateau located in the southwest in China varied with terrain from basin to plateau as altitude changed. The climate and composition of the soil varies a lot as the topography changed (Xie et al., 2003). There are 21 strains isolated from 53 samples in Sichuan Basin, 19 isolates from 50 samples in Ya An and 14 microorganisms from 95 samples of Tibetan Plateau. As shown in Table 1a, the L/S in Sichuan Basin is 39.62% twice than that in Tibetan Plateau. As shown in Table 1b, the rate of lipase-producing strains in total strains is decreasing as the altitude varied from low to high elevations. So there is more widespread distribution of lipase-producing microorganisms in Sichuan Basin than that in Tibetan Plateau, while Ya An shows a tendency of transition between the other two regions. It can be inferred that the climate in Sichuan Basin is more suitable for microorganisms than other two districts. The results suggest that bacteria diversity in Sichuan Basin is higher than the others.

Table 3. Strains selected out from five clusters for 16S rDNA sequencing.

Group	Strain No.
I	T38
II	9B, 14H, 22
III	47-1
IV	T53, 18, 8, 29-5, 7, T12
V	X10

In the process of culturing, an interesting phenomenon is observed. On one hand, strains isolated from Tibet Plateau grew too slower to be detected in the screening plate and the colonies from Sichuan Basin or Ya An were always able to be detected during 24 to 48 h in the plate. Microorganisms from Tibetan Plateau could produce clearance zones, only when they were cultured at 20°C on the medium with 0.5% agar. It can be concluded that strains from Tibetan Plateau produced some lipase different from others' and produced lipase complexes with high molecular mass that cannot diffuse into

Table 4. Identification of twelve lipase-producing strains.

Strain No.	Strains	Similarity (%)	Blast hit	Sampling site
7	*Klebsiella* sp.	98	DQ277701.1	Ya An
8	*Klebsiella* sp.	99	AY363386.2	Ya An
9B	*Bacillus* sp.	99	GQ281063.1	Ya An
14H	*Bacillus cereus*	98	JF833090.1	Ya An
18	*Klebsiella pneumoniae*	98	AP006725.1	Sichuan Basin
22	*Bacillus subtilis*	99	FR729926.1	Tibetan Plateau
29-5	*K. pneumoniae*	99	EU078620.1	Tibetan Plateau
T38	*Pseudomonas* sp.	99	FJ211165.1	Tibetan Plateau
47-1	*Bacillus firmus*	99	EF032672.1	Tibetan Plateau
T53	*K. pneumoniae*	99	CP000964.1	Sichuan Basin
T12	*Klebsiella variicola*	99	CP001891.1	Sichuan Basin
X10	*Escherichia coli*	99	AP012030.1	Sichuan Basin

concentrated agar, while most strains can produced the clearance zones on the medium with 1.5 to 2% agar (Hasan et al., 2009). While strains isolated from plateau soil produce lipase different from plain, soil is still an enigma to us and there is a need to study in detail. The reason why growth characteristics and amounts of lipase-producing strains shows a tendency of a steady decrease as the topography changing is that, most of lipase-producing strains cannot adapt to the atrocious weather in Tibetan Plateau and climate offers a selective pressure on the distribution of lipase-producing microorganisms in plain soil or plateau soil. Thus, there are kinds of lipase-producing microorganisms settling in Sichuan Basin with a mild climate and the isolates from Sichuan Basin could grow more quickly in contrast to strains from Ya An and Tibetan Plateau. Ya An is the transitional zone between Sichuan Basin and Tibet Plateau, so the isolates from this region appear dualism: the amounts of the strains producing lipase occupies the middle between the Sichuan Basin and Tibet Plateau; the speed of strains growth also shows transitivity.

When the lipase enzyme activity from isolated strains is analyzed, the results (Tables 2a, b and c) show that the intracellular lipase activity of most isolates is nearly three times than extracellular lipase activity. Intracellular lipase activity and extracellular lipase activity of strains T65, X11 and T4 are remarkably higher than other 51 strains and they may play a great role in the enzymological remediation of polluted soils. Strains T65, X11 and T4 are predicted to be *Pseudomonas, Bacillus* and *Klebsiella*, respectively, which indicated that there is significant difference in the ratio of intracellular and extracellular lipase activity among strains from different genera. Despite that, characteristics of the environment also can have a correction with the ratio. As shown in Table 2, extracellular enzyme activity from Tibet Plateau is lower than that of other two place and extracellular enzyme activity from Sichuan Basin is highest of all, which may have a close correction with their completely different habitats: such as high altitude, cold weather and heavy radiation in Tibet Plateau; low altitude, warm, moist and fertile soil in Sichuan Basin. It indicates that lipase is mostly stored in intracellular when climate is vile; and lipase is tended to be secreted out to extracellular when the climate is warm and moist like that of Sichuan Basin. Strains isolated from polluted soil or farm land and forest land show higher lipase activity than that isolated from alkaline or poor soil. It can be concluded that strains with higher lipase activity always prefer to fertile soil rather than poor soil and low-temperature is good for lipase stored in the cell and high temperature is propitious for lipase to secrete out from cell.

The ERIC-PCR is an accurate and reliable molecular identification technology for the study on microorganisms' diversity and genetic similarity (Bruijn, 1992; Gillings and Holle, 1997). It has been reported that the ERIC-PCR method is a faster, resolution-higher and more cost-effective than pulsed-field gel electrophoresis (PFGE) or multilocus sequencing for generating information about the genetic similarity of bacterial strains (Bruijn, 1992).

As shown in Figure 2, total 54 strains are categorized into five clusters at a coefficient level of 0.012 in the UPGMA dendrogram based on the ERIC-PCR fingerprints as previously stated and strains in each subgroup are considered to share the same genus (Rodriguez-Barradas et al., 1995; Ye et al., 2009). Sequencing and blast suggests strains of *Bacillus* were predominant in I, II and III branches, while in the IV and V branches are dominated by strains of *Klebsiella* and *Enterobacter,* respectively. There is an interesting phenomenon that strains isolated from Sichuan Basin include four genera: *Bacillus*, *Enterobacter*, *Klebsiella* and *Pseudomonas* and only two different genera: *Klebsiella* and *Bacillus* in Tibetan Plateau and Ya An based on the analysis of the UPGMA dendrogram (Figure 2) (Burr et al., 1998). Comparing strains of *Bacillus* with *Klebsiella* in the Tibetan Plateau and Ya An, it is clear that the 57.14% strains are *Bacillus* in Tibetan Plateau,

63.16% strains in Ya An are *Klebsiella.* It can be inferred that *Bacillus* is the preponderant strains other than *Klebsiella* stains in the plateau soil, while the *Klebsiella* may be the preponderant strains in plain soil rather than plateau soil.

It can be concluded that the diversity of lipase-producing strains in Sichuan Basin are significantly higher than that of other two places and the diversity decreases gradually from plain region to plateau region and the dominant strains in each region are varied, too. Thereby, we presume that different environments provided a certain selection pressure on the type and diversity of native lipase-producing strains in different regions. Sichuan Basin has a comfortable climate and provides little selection pressure, so it is suitable for many kinds of strains. However, Tibetan Plateau with high altitude and radiation provides a great selection pressure; so, there are just relatively fewer lipase-producing strains. Since *Klebsiella* and *Bacillus* strains in turn become the main lipase-producing strains of Ya An and Tibetan Plateau, it can be concluded that strains of *Klebsiella* is probably the transitional ones from low-altitude, warm regions to high-altitude, cold regions, while strains of *Bacillus* dominate the cold regions with high altitude due to their excellent viability in adverse surroundings.

In conclusion, *Bacillus* sp*.* may specially restore the polluted soil where is cold and high radiant, other lipase-producing strains may be effective in remediation of contaminated soil where is warm and moist. In the further study, on one hand, we will pay attention to the organic tolerability of each isolates in order to screen out some strains which can be utilized to produce biodiesel conveniently, and on the other hand, we will sequence the bands which were a symbol of lipolytic microorganisms with the purpose for making sense of the difference of sequence in different regions.

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