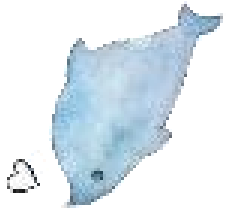

读书报告

汇报人：赵卓丽　汇报日期：2018-4-14

Metagenomics of an Alkaline Hot Spring in Galicia (Spain): Microbial Diversity Analysis and Screening for Novel Lipolytic Enzymes

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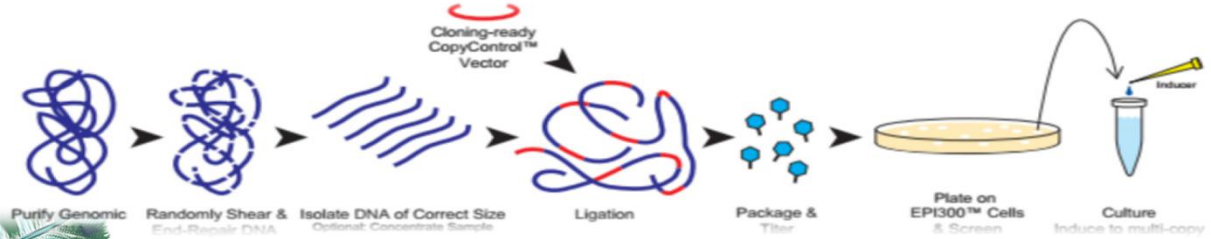
01 Abstract

研究对象：Lobios hot spring, Ourense, Galiza, Spain. (76℃, pH = 8.2)

Fosmid library: Metagenomic DNA(assemble 9722contigs: 500-56,677bp; >18Mbp; 23207个ORF.)



Biodiversity

Predominant: bacteria; less abundant: archaea

Taxonomic classification

six most abundant bacterial phyla

Deinococcus-Thermus, Proteobacteria, Firmicutes, Acidobacteria, Aquificae, and *Chloroflexi.*

栖热菌门、变形菌门、厚壁菌门、酸杆菌门、产水菌门、绿弯菌门

archaea

the phylum *Thaumarchaeota* was predominant with the dominant species "*Candidatus Caldiarchaeum subterraneum*".

奇古菌门

Genes

most abundant: one-carbon metabolism

Functional classification

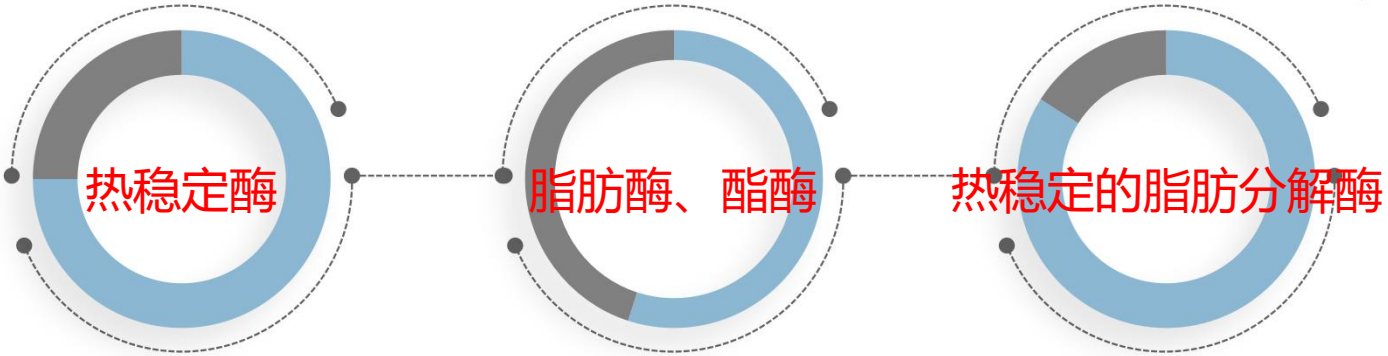
Both taxonomic and functional classifications showed a mixture of different microbial metabolic patterns: aerobic and anaerobic, chemoorganotrophic and chemolithotrophic, autotrophic and heterotrophic. Remarkably, the presence of genes encoding enzymes with potential biotechnological interest, such as xylanases, galactosidases, proteases, and lipases, was also revealed in the metagenomic library.

Functional screening of this library was subsequently done looking for genes encoding lipolytic enzymes. Six genes conferring lipolytic activity were identified and one was cloned and characterized. This gene was named LOB4Est and it was expressed in a yeast mesophilic host.

LOB4Est codes for a novel esterase of family VIII, with sequence similarity to β-lactamases, but with unusual wide substrate specificity. When the enzyme was purified from the mesophilic host it showed half-life of 1 h and 43 min at 50°C, and maximal activity at 40°C and pH 7.5 with p-nitrophenyl-laurate as substrate. Interestingly, the enzyme retained more than 80% of maximal activity in a broad range of pH from 6.5 to 8.

VIII家族新型酯酶 β-内酰胺酶 对硝基苯基月桂酸盐

02 Introduction



应用于食品加工、化妆品工业、精细化学合成、
废物处理、洗衣业等。

反应温度>45℃

Metagenomics

enzymes from microorganisms that have not efficient conditions of culture in laboratory (99% of total; Amann et al., 1995) cannot be identified through other methods.

打破实验室纯培养的限制

Hot spring

Hot springs are natural habitats of thermophilic microorganisms, producing thermostable enzymes with catalytic activity at high temperatures. These features, in addition to the intrinsic resistance of thermophilic lipases and esterases to the presence of organic solvents or extreme pH values (Bornscheuer, 2002) highlight them as robust and versatile biocatalysts for industrial applications.

耐高温酶

研究对象：Lobios hot spring, Ourense,Galiza,Spain. (76◦C, pH = 8.2)

新型耐高温、耐碱性酶

Fosmid Pcc1fos:
sequenced - assembled - analyzed to draw a picture of the microbial diversity captured into the Lobios library.

subjected to functional screening for lipolytic activity using tributyrin（三丁酸甘油酯）agar plates.

选择一个阳性克隆，使用酿酒酵母BJ3505作为异源宿主、质粒YEpFLAG-1作为表达载体克隆，表达、表征重组酶。

03 Materials and methods

Sampling

样点：Lobios hot spring, Ourense, Galician, Spain. (GPS 41.86113, −8.1062)

样品：钻孔取25L地下热泉水(temperature >76◦C, pH > 8.2)于瓶子中（70%乙醇洗涤，热泉泉水润洗)

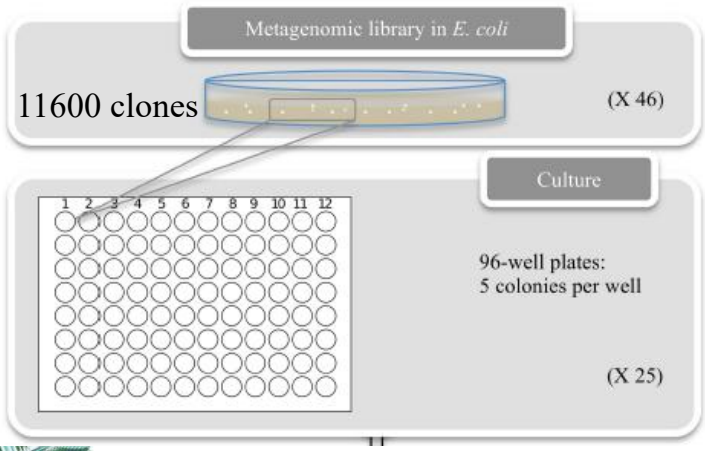
样品处理：室温储存；第二天使用0.2μm的硝酸纤维素滤膜过滤，4℃储存。

Construction of Metagenomic Library

试剂盒：Metagenomic DNA Isolation Kit for Water（Epicentre Biotechnologies）

Fosmid文库：Copy Control Fosmid Library Production kit (Epicentre Biotechnologies)

pCC1FOS Metagenomic Fosmid Library　　*Escherichia coli* EPI300-T1

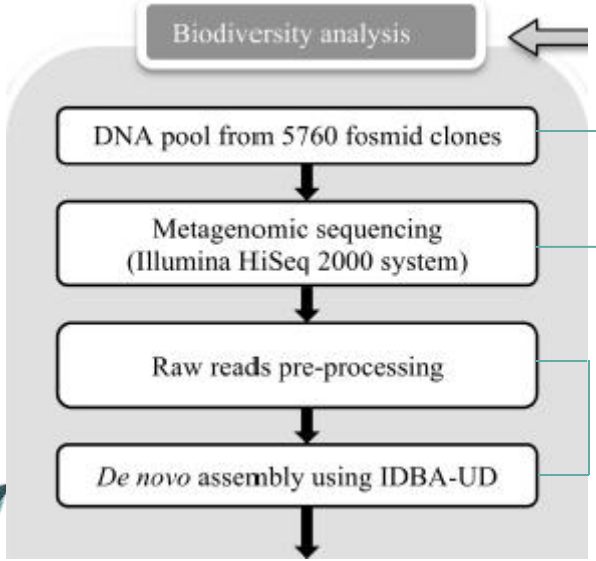


5*96*25

0.2mL LB
(12.5mg/mL氯霉素)

37°C培养24h
-80°C储存

Metagenomic Library Sequencing



5*96*12

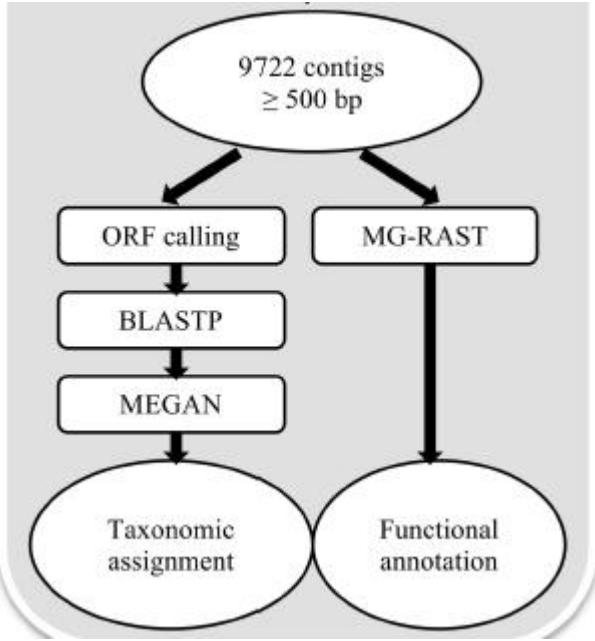
2ul菌液接种于5mL LB(12.5 μg/mL 氯霉素、1X CopyControl Induction Solution)，37℃过夜培养，诱导高拷贝。

FosmidMAX™ DNA Purification Kit (Epicentre)
混合每次提取的DNA量-5μg Fosmid DNA
A total of 11982436 reads with a read size of 100 bp were generated.

Sequence Pre-processing and Assembly

9722 sequences

Sequence Annotation and Lipolytic Genes Screening

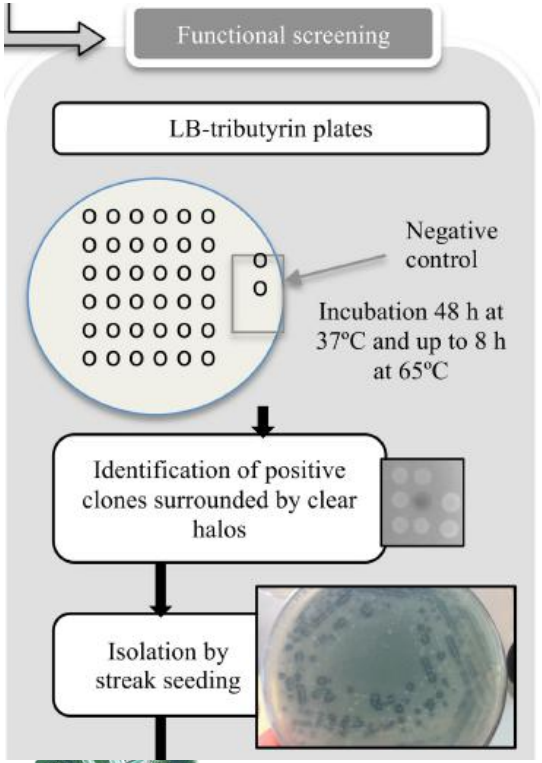


分类学分析与功能注释

Alpha多样性计算(MG-RAST)

提取与脂解基因相关的序列
鉴定脂肪酶、酯酶基因
(http://www.uniprot.org/)

Functional Screening



培养基：LB+1%三丁酸甘油酯

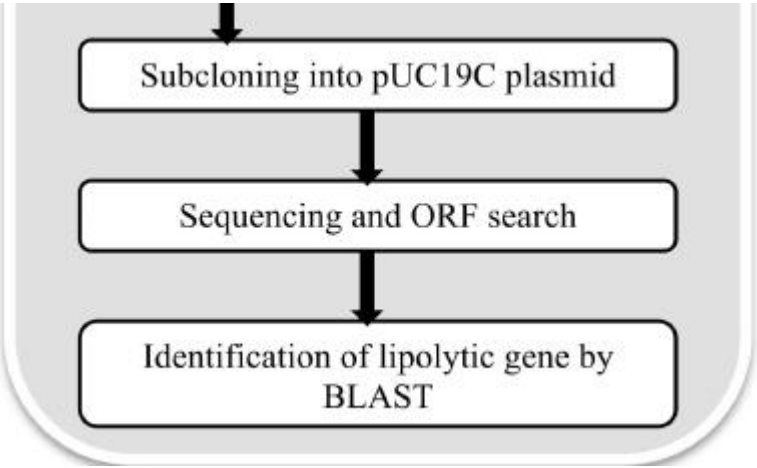
接种量：每孔5μl；共25个96孔板

培养：37℃，2d；65℃，8h

筛选：透明晕圈

选择：明显晕圈的单菌落（划线分离）

Subcloning and Sequence Analysis



阳性克隆：FOS4

提取DNA：FosmidMAX DNA purification kit (Epicentre Biotechnologies)

限制性位点：SphI EcoRI

质粒：pUC19

涂布：100μg/mL氨苄青霉素-LB-三丁酸甘油酯平板

测序-预测ORF-BLASTP-ORF3-Clustal W(5个最相似序列、28个已有脂肪分解酶序列)-MEGA 6(p-distance model)-ORF3为酯酶-提交其编码的酯酶序列

Subcloning and Sequence Analysis

ClustalW-ORF3编码的蛋白质与VIII家族酯酶多重序列比对

↓

Signal P 4.0-信号肽搜索

↓

ProtParam-在线预测蛋白质的物理化学参数

Subcloning and Sequence Analysis

LOB4Est

引物
YFF4A3F AAAAGAGACTACAAGGATGACGATGACAAGagccgcccgcgtaccg
YFF4A3R TGGGACGCTCGACGGATCAGCGGCCGCTTAggcgcagccgagttcctcgc

PCR：95℃, 5min; 30X (95℃, 30s; 64℃, 30s; 72℃, 70s); 72℃,7min
Pfu DNA聚合酶（Thermo Scientific）和含有4%DMSO的PCR缓冲液

质粒：YEpFLAG-1　　宿主：*S.cerevisiae* BJ3505
用EcoRI和SalI消化线性化-乙酸锂方法(lithium acetate procedure)与PCR产物、EpFLAG-1质粒共转化-涂布在无色氨酸完全培养基（用于选择含有重组质粒YEpFLAG1-LOB4Est的转化体）-筛选验证重组质粒

Culture Conditions

Sc-LOB4Est

YPHSM培养基(w/v)

8%细菌蛋白胨

1%酵母提取物

3%甘油

1%右旋糖

培养：2mL YPHSM, 30℃，200rpm，3d
50mL 螺旋盖玻璃管

扩大：20%体积 YPHSM, 30℃，200rpm，3d
接种量 1:20　锥形瓶

Cell Fractionation

验证重组菌株分泌的酯酶的正确性

Esterase Activity

使用对硝基苯月桂酸酯作为底物
通过分光光度法测定酯酶活性

Biochemical Characterization of the Recombinant Enzyme

底物偏好性、动力学参数、温度、pH

β-lactamase Activity

使用硝基头孢烯作为底物
通过分光光度法测定β-内酰胺酶活性

Polyacrylamide Gel Electrophoresis and Western Blotting

04 Results and discussion

温度与微生物多样性程度成反比

TABLE 1 | Alpha diversity estimates for various hot springs around the world.

Sample location	T(°C)	pH	MG-RAST accession no	α-Diversity*	Reference
China	65	7.0	4530144.3	457.729	Menzel et al., 2015
Russia	61–64	5.8–6.0	4544453.3	615.968	
Italy	76	3.0	4529716.3	86.121	
Iceland	85–90	5.0	4530143.3	196.142	
USA	92	3.0–4.0	4529719.3	117.640	
Colombia	29	2.7	4449206.3	467.609	Jiménez et al., 2012
Spain	76	8.2	4570559.3	330.865	This study

Alpha diversity, number of distinct species in a sample, was estimated using the MG-RAST server (Meyer et al., 2008).

中温

高温

低温

分类多样性

宏基因组序列分配：

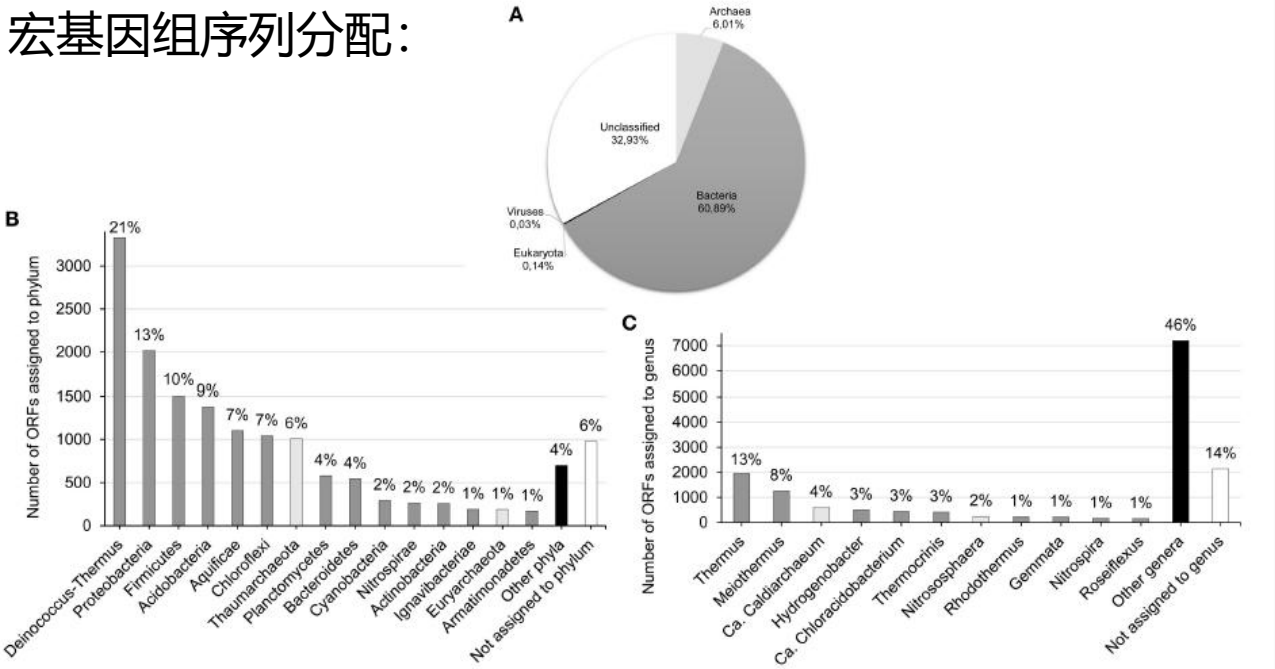


FIGURE 2 | Taxonomic classification of ORFs predicted from contigs. (A) All ORFs ($n = 23.207$) predicted from contigs (≥500 bp) at the superkingdom level. Predicted ORFs were compared using BLAST to the NCBI protein database and assigned to taxa using MEGAN with a minimum bit-score of 100 and e-value 1e-05. Over 67% (15,565) ORFs were assigned to Bacteria, Eukaryota, Achaea, and Viruses, while <33% remained unclassified. **(B)** ORFs assigned at the phylum level. Of 15,557 ORFs assigned by MEGAN to Bacteria, Eukaryota, and Achaea, 14,577 (94%) could be classified at the phylum level. Bacterial phyla are colored in dark gray and achaeal are represented by light gray bars. Phyla with abundance <1% were collapsed into the "Other phyla" category (black bar). **(C)** ORFs assigned at the genus level. Of 15,557 ORFs assigned by MEGAN to Bacteria, Eukaryota, and Achaea, 13,419 (86%) could be classified at the genus level. Bacterial genera are colored in dark gray and archaeal are represented by light gray bars. Genera with abundance less <1% were collapsed into the "Other genera" category (black bar).

功能多样性

碳水化合物
蛋白质代谢
氨基酸及衍生物
辅酶因子、维生素、色素
RNA代谢
DNA代谢
呼吸作用
膜转运
细胞膜、囊
核苷酸
脂肪酸、脂质
毒性、疾病、防御
应激响应
细胞分裂、细胞周期
氮代谢
硫代谢
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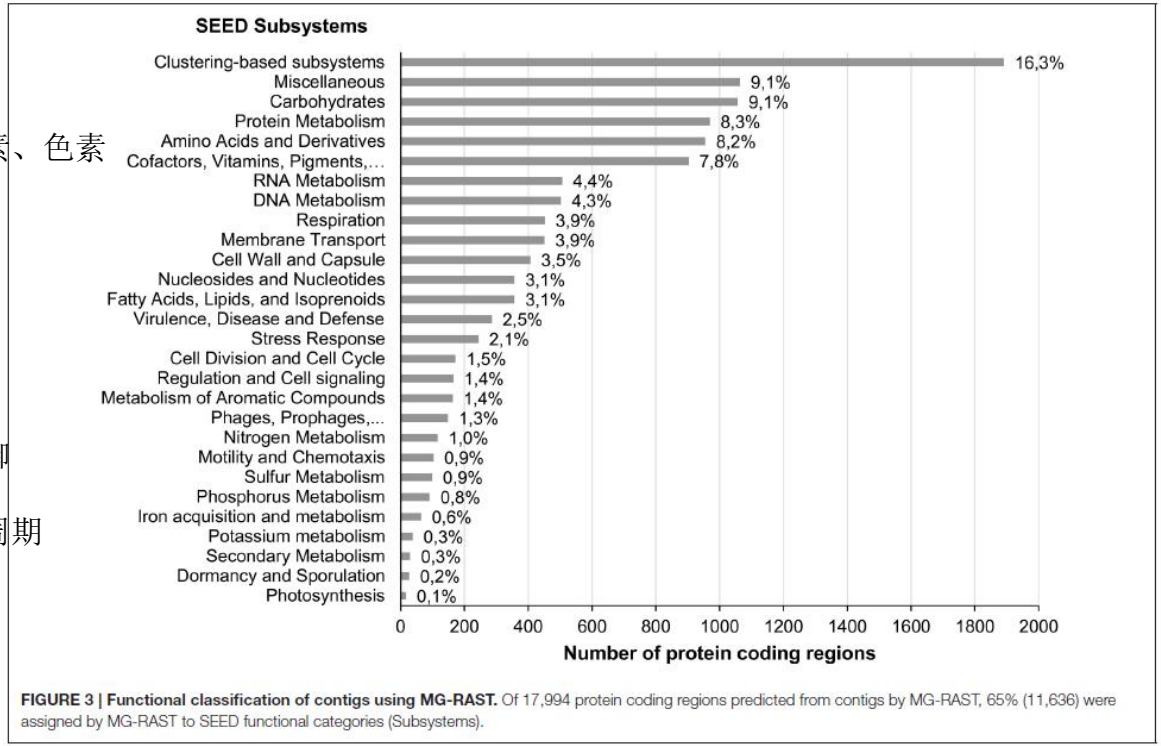
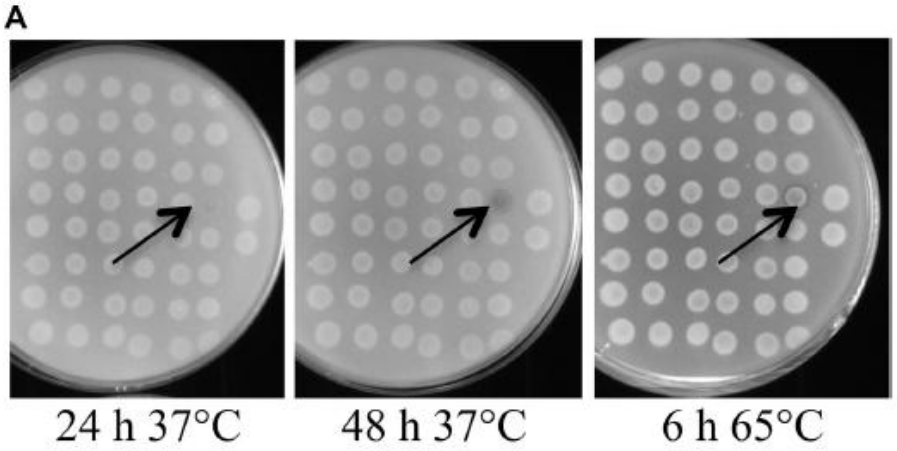


FIGURE 3 | Functional classification of contigs using MG-RAST. Of 17,994 protein coding regions predicted from contigs by MG-RAST, 65% (11,636) were assigned by MG-RAST to SEED functional categories (Subsystems).

单碳代谢（4.1%,最丰富）、甲烷生成、TCA循环…

基于序列筛选脂肪分解酶基因

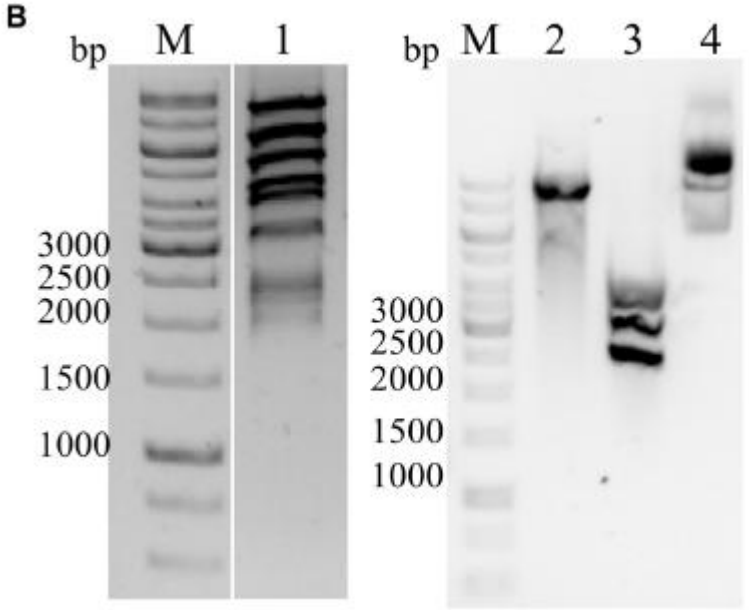


LB-三丁酸甘油酯平板筛选：

6个阳性克隆

选择在最短培养时间内具有明显透明晕圈的克隆FOS4进行下一步实验。

脂肪分解酶ORF鉴定

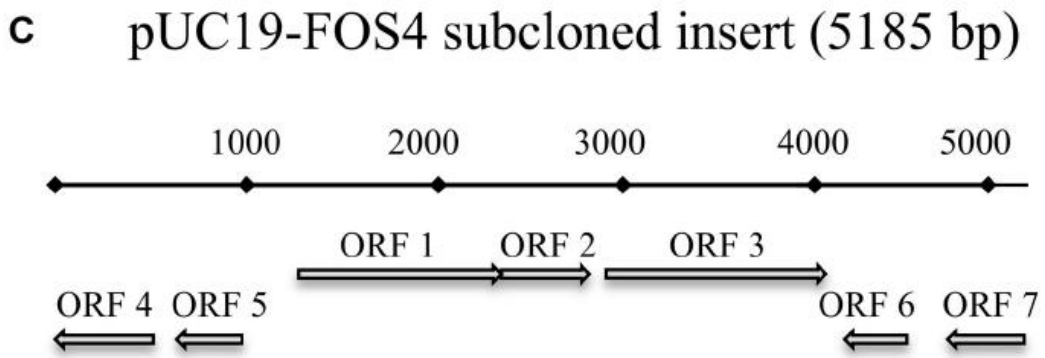


FOS4 after incubation for 24 and 48 h at 37°C, and 6 h at 65°C. **(B)** Analysis by electrophoresis in agarose gels (0.8%) of: 1- digestion by SphI and EcoRI of fosmid FOS4 (2686 bp); 2- digestion by EcoRI of subclone in plasmid pUC19 (2628 bp); 3- digestion by SphI of subclone in plasmid pUC19; 4- subclone in plasmid pUC19 undigested. M: GeneRuler 1kb DNA Ladder (Fermentas). GelGreen stain. **(C)** Physical map of the insert of FOS4A subcloned into

M: GeneRuler 1kb DNA Ladder
1: SphI、EcoRI 消化 FOS4 (2686bp)
2:EcoRI消化pUC19中的亚克隆(2628bp)
3:SphI消化pUC19中的亚克隆
4:未消化的pUC19中的亚克隆

脂解活性的亚克隆被测序，大小约为5.2kb

脂肪分解酶ORF鉴定



从插入序列预测到七个ORF。其中三种与梭状芽胞杆菌相匹配，表明克隆的插入物可能源自尚未报道的梭菌属成员。

脂肪分解酶ORF鉴定

TABLE 2 | Best hits by BLASTP search against non-redundant protein (nr) database for ORFs detected in the insert from FOS4A subcloned in pUC19.

ORF (bp)	Possible function and microorganism	Accession number	% Query coverage	% Identity
ORF 1 (1254)	hypothetical protein, *Desulfotomaculum* sp. BIC-A1/1_c6	WP_034102674	84	44
ORF 2 (459)	alkyl hydroperoxide reductase, *Cyanothece* sp. PCC 7425	WP_012629091	80	48
ORF 3 (1149)	esterase Est8, uncultured organism from soil	AEM45116	99	64
ORF 4 (513)	O-phosphoserine sulfhydrylase, *Longispora albida*	WP_018348134	100	54
ORF 5 (339)	ModE family transcriptional regulator, *Acetohalobium arabaticum*	WP_013278309	98	43
ORF 6 (327)	hypothetical protein, *Porphyromonas* sp. COT-290_OH3588CRE	KGN97422	56	32
ORF 7 (402)	hypothetical protein, *Clostridiales* bacterium VE202-07	WP_024726181	100	55

ORF3显示与德国土壤功能性宏基因组学分离的酯酶Est8具有最高的氨基酸相似性（99%的查询覆盖率和64%的相同性），命名为LOB4Est。

脂肪分解酶ORF鉴定

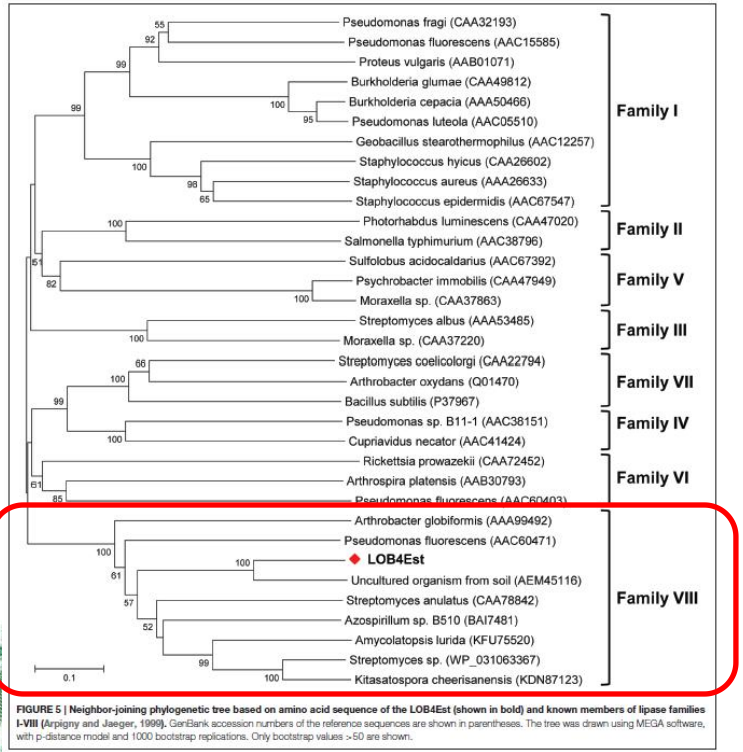


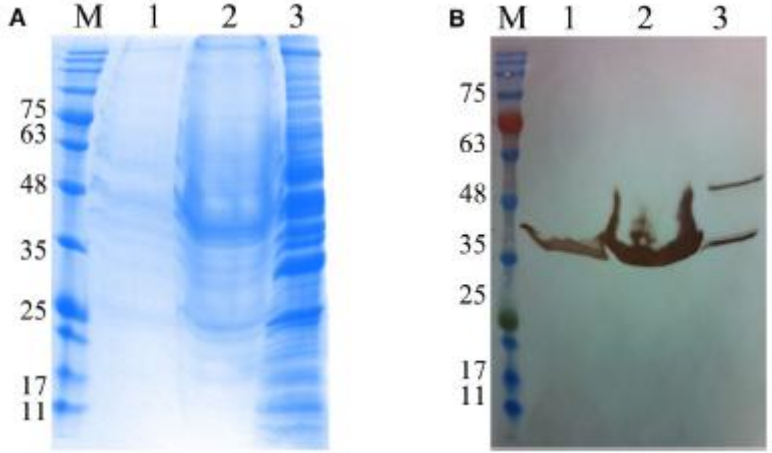
FIGURE 5 | Neighbor-joining phylogenetic tree based on amino acid sequence of the LOB4Est (shown in bold) and known members of lipase families I-VIII (Arpigny and Jaeger, 1999). GenBank accession numbers of the reference sequences are shown in parentheses. The tree was drawn using MEGA software, with p-distance model and 1000 bootstrap replications. Only bootstrap values >50 are shown.

系统发育分析确定LOB4Est编码的蛋白属于酯酶家族VIII；

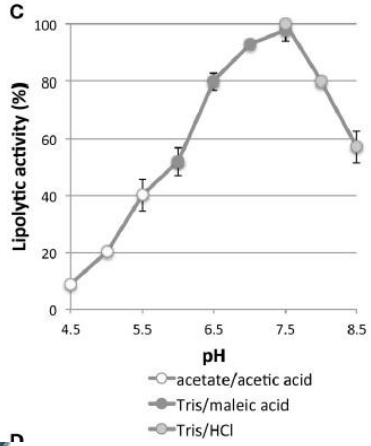
VIII族的典型酯酶长约380个残基，大小为40 kDa，与C类β-内酰胺酶有很高的相似性。基因LOB4Est编码382个残基和40.47 kDa的蛋白质，预测pI为5.58。BLAST结果显示与酯酶和β-内酰胺酶的序列相似性。

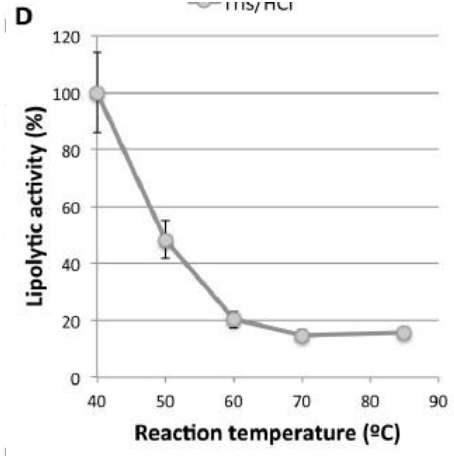
克隆，生化表征和异源表达

重组质粒：YEpFLAG1-LOB4Est　重组酵母菌株：Sc-LOB4Est



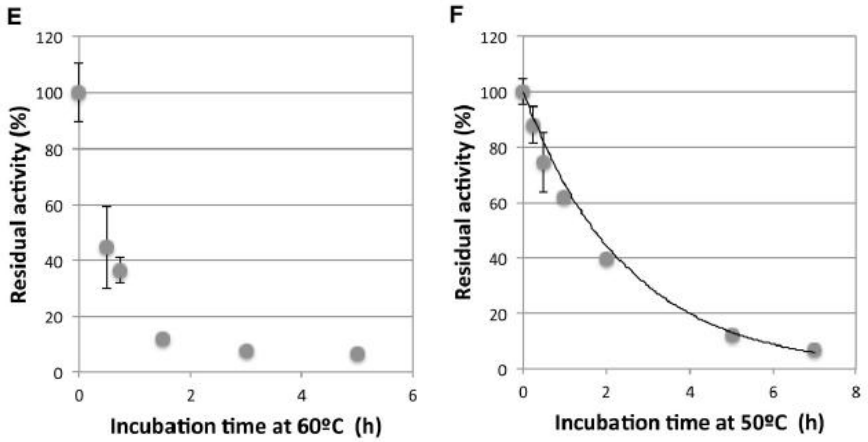
验证重组菌株产生和分泌酯酶：
在2mL YPHSM中进行培养；
通过SDS-PAGE和Western印迹分析上清液和粗提取物；
在两个样品中检测到大约40kDa的条带，与重组蛋白质的预测分子量一致。



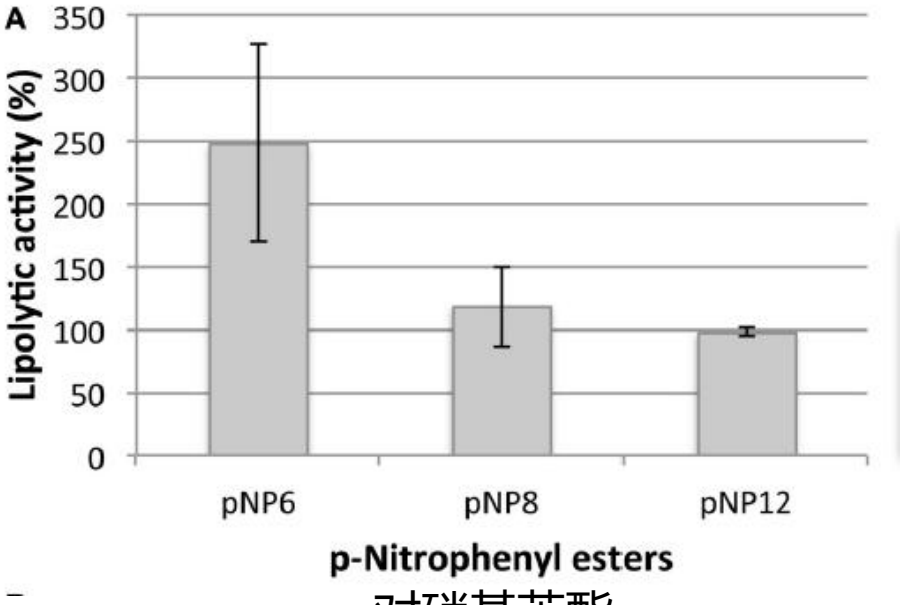


最适pH=7.5 最适温度=40°C

在pH6.5-8范围内保持高度活性（活性高于80%）
在6-8.5范围内保持中等活性（高于50%活性）
嗜碱性偏好

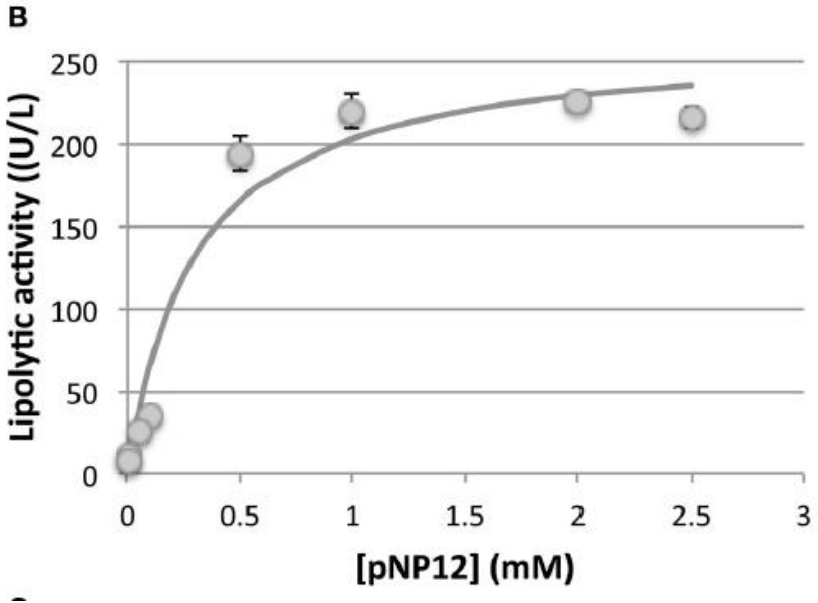


热稳定性：
在60℃温育45和90分钟后，重组酶分别保留了36%和10%的初始活性，而在50℃孵育2小时后，重组酶仍保持超过44%的初始活性。

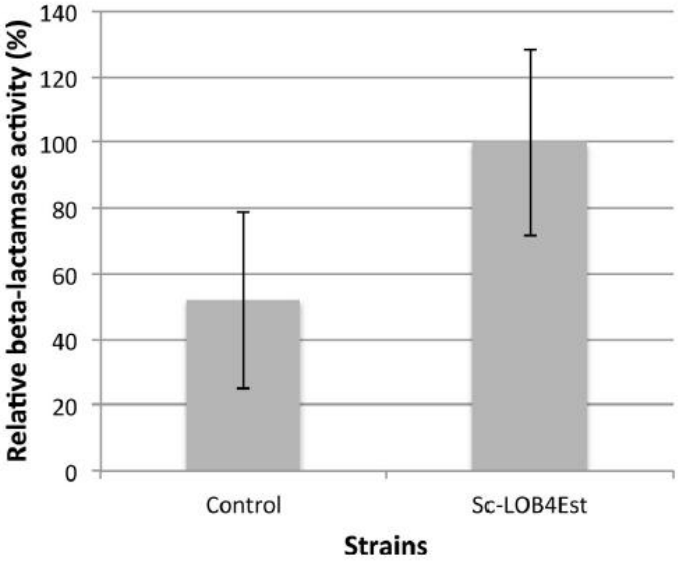


对硝基苯酯

广泛的底物偏好；更偏向于短链酰基底物

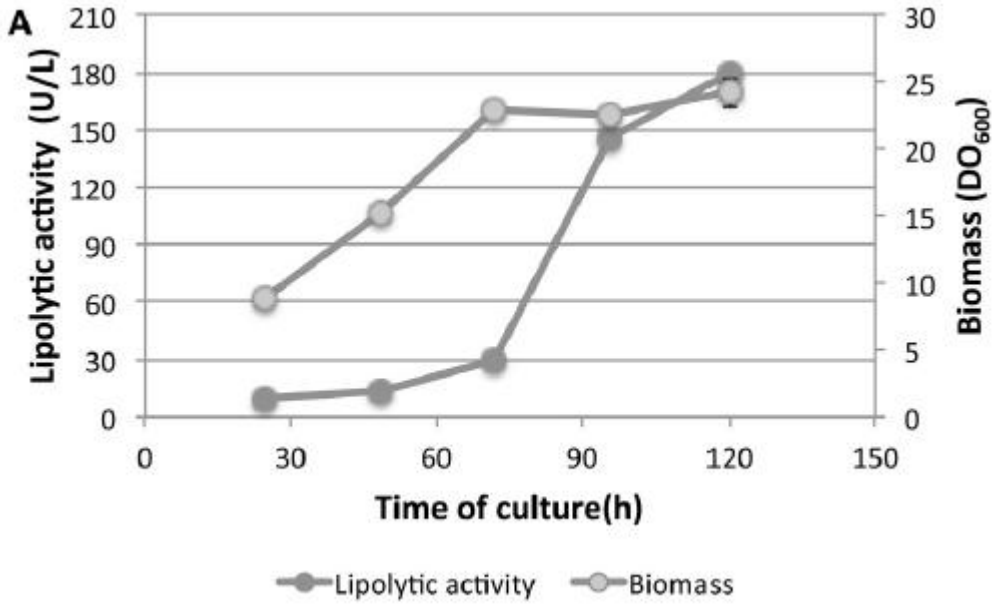


在不同浓度的pNP12下测量重组酶的初始反应速率，并将曲线调整为米氏曲线，鉴定为酯酶。

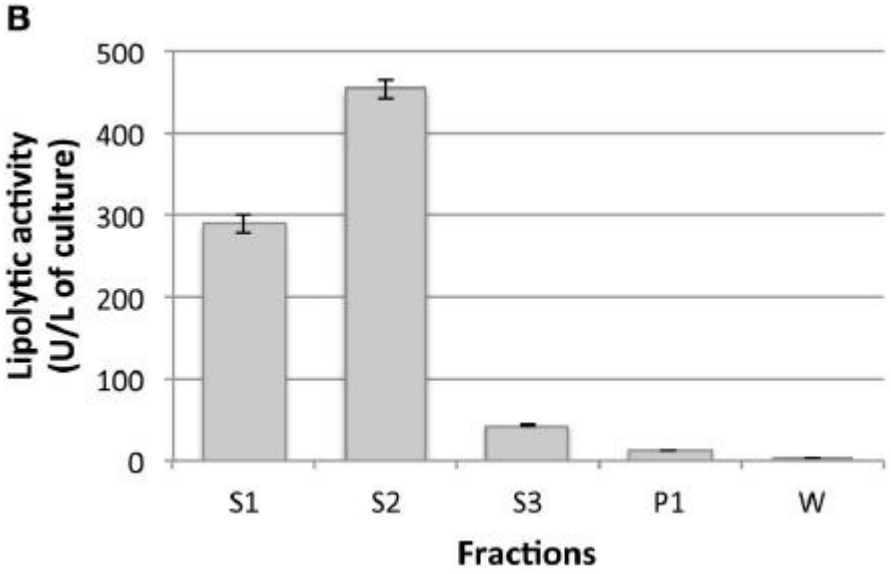


活性水平都非常低，无统计学的显著差异性。

虽然酯酶显示与β-内酰胺酶的序列相似性，但大多数不具β-内酰胺酶活性。



培养76小时后达到稳定期，直到培养96小时，细胞外活性显著增加。



S1：细胞外，S2：周质，S3：细胞质，P1：细胞碎片，W：洗涤。

分泌56%重组蛋白，但保留在周质水平，36%分泌到细胞外培养基中。与已有报导的*T.myophilus* HB27 菌株产生的酯酶（20%的蛋白质分泌到细胞外培养基）相比，具有更高的产量。

05 Conclusion

Metagenomic sequencing of the fosmid library constructed in this work from the thermal water of the alkaline hot spring at Lobios (Ourense, Spain) demonstrated the predominance of Bacteria over Archaea, being Deinococcus-Thermus the most abundant phylum, followed by other phyla that are also common in other thermal environments around the world.

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