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Full Length Research Paper

Pitfalls using tributyrin agar screening to detect lipolytic activity in metagenomic studies

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The metagenomics approach is an efficient method for obtaining novel biocatalysts and useful genes from uncultured microorganisms within diverse environments. In this study, we constructed a metagenomic library using a South African deep mine biofilm sample. The library was screened for lipolytic activity using LB Tributyrin (TLB). Although we were able to identify 3 diverse esterase enzymes, we found that 70% of the obtained sequence data revealed the presence of enzymes and genes completely unrelated to that of lipolytic enzymes thereby highlighting the limitation of screening with TLB.

Key words: False positives, tributyrin, lipolytic activity, metagenome.

INTRODUCTION

The microbial world seems to offer the greatest natural resource of molecular diversity. The upper 4 km of the earth's crust contains about 9.5 million cubic kilometers of ground water, 56% of which lies below 0.75 km in depth. Due to the enormous sub-terrestrial volume which may be amenable to life, the majority of the earth's prokaryote population is believed to reside here and it may even rival the flora and fauna on the surface (Moser et al., 2005). Reports of microbial life in the deep subsurface of South African gold mines indicate that specialist communities can be found at depths of up to 5 km (Moser et al., 2005). The classical approach of cultivating and characterizing isolates on the strain level prior to screening and gene isolation is valid and powerful yet severely restricted in scope (Lorenz and Schleper, 2002) because the majority of microbial life forms have been found to be uncultivatable (Yun and Ryu, 2005). Since its introduction, metagenomics has identified a significant number of novel genes encoding for biocatalysts or molecules with high potential for use in pharmaceutical products or production processes from previously uncultured microorganisms (Streit and Schmitz, 2004). Microorganisms serve as a potential source for new biocatalysts, as they have adapted to a wide range of different environmental conditions based on the development of highly optimized enzymes, which are suited to perform optimally under the physiochemical conditions of their habitats (Lämmle et al., 2007). Many lipolytic enzymes, including lipases, esterases and various types of phospholipases have been found in a wide range of organisms from bacteria to humans (Rhee et al., 2005). Lipolytic enzymes catalyze both the hydrolysis and synthesis of acylglycerides and other fatty acid esters.

The true lipases differ from the carboxylesterases in their maximal activity on water insoluble long chain esters (Lee et al., 2004). Functional lipolytic genes have been previously identified from metagenomic libraries of soil, hot spring sediments and alkaline soda lakes (Ranjan et al., 2005). Functional screening for enzymatic activities can however be a daunting task as thousands of clones needs to be screened to effect adequate coverage of a library. A rapid and cheap screen which does not give false positives or negatives is therefore the ideal. In this study, we constructed a metagenome library from biofilm obtained from the Beatrix gold mine. Several clones with lipolytic activity were detected by screening on TLB agar plates. However, subsequent sequence analysis revealed that 70% of the clones displaying activity on the screening media did not harbour genes responsible for lipolytic activity.

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MATERIALS AND METHODS

Sample, strains and culture conditions

Biofilm was collected from the Beatrix Gold Mine (Gold Fields Limited), from a depth of 808 m below the surface in a standing pool of water (temperature = 33°C, conductivity = 5.0 mS/cm and pH 7.14). Beatrix is situated at latitude 28°15'S and longitude 26°47'E near the towns of Welkom and Virginia about 240 km southwest of Johannesburg, in the Free State Province of South Africa. Biofilm was kept cold and processed immediately on arrival at the laboratory. *Escherichia coli* TOP10 was used as the host for cloning and was provided with the pZER0-2 Background cloning kit (Invitrogen). The *E. coli* cells were grown in Luria-Bertani (LB) liquid medium or on LB agar plates at 37°C. The media was supplemented with 50 ug/ml kanamycin.

Metagenomic DNA isolation

DNA was extracted using a combination of 3 extraction methods (Dong et al., 2006; Labuschagne and Albertyn, 2007; Towner, 1991). Prior to DNA extraction, the sample was treated with aluminum sulfate according to Dong et al. (2006) to remove inhibitors that may have been present. The treatment involved mixing 2 g of the biofilm sample with 300 µl phosphate buffer (0.1 M NaH2PO4-Na2HPO4; pH 6), followed by the addition of 100 µl 100 mM aluminum sulfate. The pH of the sample was adjusted to 8 by the addition of 1 M sodium hydroxide. Following treatment, DNA extraction was performed according to Labushagne and Albertyn (2007). The sample was mixed with 500 µl DNA isolation buffer [100 mM Tris-HCl (pH 8); 50 mM EDTA; 1% SDS] and vortexed. To the suspension, 200 µl glass beads were added and the sample was vortexed for 4 min with immediate cooling on ice thereafter. After cooling, 275 µl 7 M ammonium acetate (pH 7) was added, incubated for 5 min at 65°C and followed by cooling on ice for 5 min. Addition of 500 µl chloroform, vortexing and centrifugation at 14000 rpm for 5 min at 4°C followed. The supernatant was removed and precipitated overnight with an equal volume of isopropanol at -20°C. Following precipitation, the DNA was spooled and centrifuged for 1 min at 4°C. The pellet was washed with 70% ethanol and centrifuged at 14000 rpm for 5 min at 4°C, dried and dissolved in 50 µl sterile water containing RNase A and stored at 4°C.

Metagenomic library construction

The metagenomic DNA was partially digested with 1 U *Bam*HI (New England Biolabs) for 1 h at 37°C and size fractionated between 2 - 6 kb, stained with SYBR Gold, excised from the gel, purified using the GFX Gel Purification Kit (Amersham Biosciences) and ligated into the pZERO-2 suicide vector (Invitrogen). The ligation mixture was used to transform *E. coli* TOP10 host cells by heat shock according to Sambrook et al. (1989). Plates were incubated overnight at 37°C.

DNA manipulation techniques

Plasmid DNA extractions and restriction digestions were done according to standard procedures described by Sambrook et al. (1989).

Library screening for lipolytic activity

Lipolytic activity was measured by the tributyrin plate halo assay. The plate assay was performed using an adapted protocol described by Ro et al. (2004). Clones were replica plated onto LB tribuytrin (TLB) plates. Plates were incubated for 3 days at 37°C. Plasmids were isolated from lipolytic clones and re-transformed into *E. coli* TOP10. Positive clones displaying large zones of clearance, after the secondary screening, were selected for sequence analysis.

DNA sequencing and analysis

The recombinant clones were sequenced by primer walking using an ABI 3130XL genetic analyzer (Applied Biosystems, Foster City, CA) by the incorporation of the ABI Big Dye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems, Foster City, CA). Sequencing was performed by Inqaba Biotechnological Industries Pty. Ltd., South Africa. Electropherograms of the sequences generated were inspected with FinchTV software (Geospiza) and assembled with Vector NTI (Invitrogen). Translated ORFS were compared to known sequences deposited in the non-redundant protein databases of the National Centre for Biotech-nology Information (NCBI, USA) using standard protein-protein BLAST (*blastp*) (Altschul et al., 1997).

RESULTS AND DISCUSSION

Metagenomic library construction

The constructed library contained ~10 000 clones. Analysis of the insert fragments generated by *Xho*I and *Hin*dIII restriction digestion of 20 recombinant plasmids was used to estimate an average insert size of ~4 kb for the library (Figure 1). The restriction patterns obtained indicated that the plasmid clones potentially possessed a rich diversity of genes from organisms that may be present in the metagenome.

Screening the metagenomic library for lipolytic clones

The initial library screen identified 44 positive clones on TLB, which all showed the characteristic clear halo on the screening plate. After re-transformation and re-screening, 18 clones were active. The 18 clones differed in their restriction patterns and clearance zones. The zone of hydrolysis of tribuytrin by different clones varied from 5 to 13 mm in diameter after 3 days of incubation at 37°C. To further confirm that the observed phenotype could be attributed to the metagenomic DNA insert, recombinant plasmid DNA was isolated and the presence of an insert was confirmed by restriction analysis for all clones isolated. Based on zone of diameters, 10 clones (large clearance zones) were sequenced (data not shown). The insert size in these plasmids (pNS1-pNS10) varied from 1.5 to 5 kb. The sizes of the inserts after re-transformation were consistent with the previous restriction analysis of the respective plasmids. As no IPTG was added to the screening plates, the activity obtained was from the native promoter in the clones. In addition, the clones were plated out onto LB olive oil and rhodamine B containing agar plates; however, none of the clones

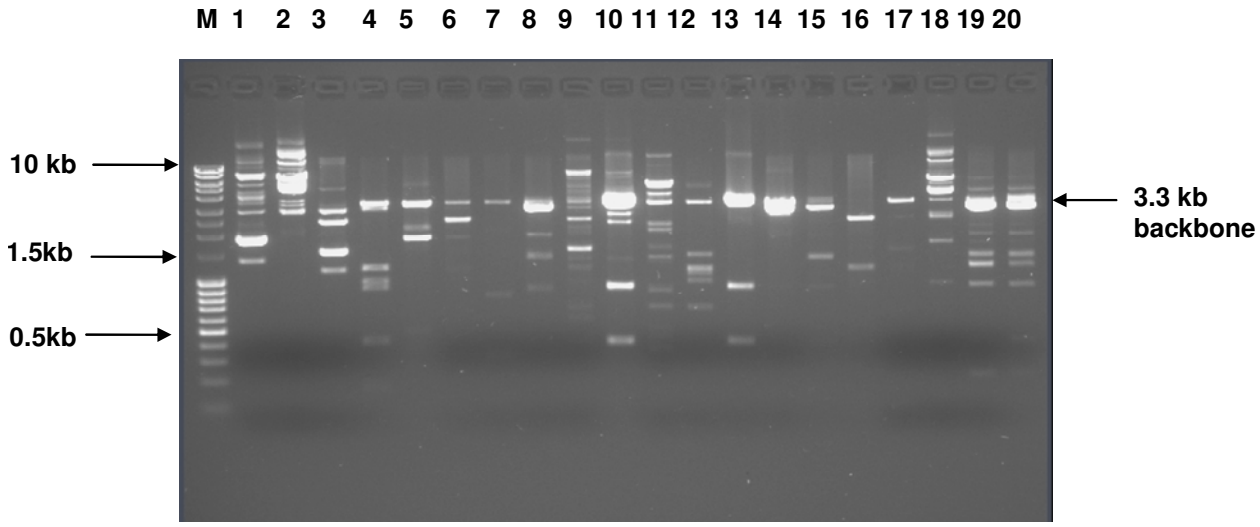

Figure 1. Restriction analysis of clones from the Beatrix metagenomic library. M: Mass ruler DNA ladder (SM # 0403-Fermentas); lanes: 1 - 20: randomly selected clones.

Table 1. Sequenced plasmids showing esterolytic activity and their closest hits.

Plasmids	**Function and organism**	**% Similarity**	**E value**	**Accession No.**
pNS1	Isochorismatase family protein, *Geobacter sulfurreducens* PCA	92	3e-73	NP_953629
pNS2	Sulfatase, *Parvibaculum lavamentivorans* DS-1	74	2e-52	YP_001414401
pNS3	Molybdopterin oxidoreductase Fe4S4, *Alcanivorax sp.*	45	2e-149	DX90729
pNS4	Type I restriction-modification system, M subunit, *Brucella ovis*	47	7e-04	ABQ62383
pNS5	DNA-dependent ATPase, SNF2 family protein, *Chlorobium tepidum* TLS	87	8e-153	NP_661799
pNS6	Phospholipase, patatin family protein, *Plesiocystis pacifica* SIR-1	55	2e-119	ZP_01911272
pNS7	Primosomal protein, *Acidovorax sp.* JS42	66	1e-96	YP_988045
pNS8	Oxidoreductase, *Anaeromyxobacter dehalogenans* 2CP-1	57	3e-41	ZP_02325211
pNS9	Formate dehydrogenase *Rhodococcus sp.* RHA1	49	1e-107	YP_700405
pNS10	Putative RHS-related transmembrane protein, *Ralstonia solanacearum*	50	5e-31	NP_523144

Plasmids marked in bold were identified to be esterolytic enzymes and possibly responsible for the observed enzymatic activity.

produced any fluorescent halo thereby indicating that the lipolytic enzymes produced by the clones were probably esterases due to their inability to hydrolyze long carbon chain fatty acid esters (Ro et al., 2004).

Sequence analysis of selected clones

Analysis of the sequencing data revealed the presence of 3 unique esterase type enzymes (pNS1, pNS2 and pNS6) in the metagenome (Table 1). However, the remaining clones showed hits of enzymes completely unrelated to that of lipolytic enzymes highlighting the possible drawback of screening with tribuytrin because, of its inability to specifically detect lipolytic activity. Domain searches were performed on the clones in order to detect the classical lipase/esterase motif (G-X-S-X-G). The motif was only identified in pNS6, the patatin protein. Although isochoristmatases and sulfatases are different types of esterases, the catalytic residues responsible for activity are different from those of lipolytic enzymes

The reason for the presence of false positives is still unclear; there was no loss of the insert during the re-transformation and re-screening. This was confirmed by performing restriction digestion of selected clones before and after re-transformation and re-screening, the digestion patterns were consistent. The lack of correlation between halo diameter and esterolytic activity during screening of a metagenomic library using TLB was also reported by Jung et al. (2003), who observed no close correlation between halo formation and whole cell enzyme activity. In the future, it will be feasible to implement an alternative media that can be used in conjunction with TLB for screening metagenomic libraries for lipolytic activity thereby reducing the number of false positives that could be obtained.

Conclusion

Metagenomics affords researchers the opportunity to search for novel bioactive agents and functions from uncultured organisms (Jones et al., 2007). In this study, we highlighted a negative aspect using TLB media for the screening of lipolytic enzymes due to the large number of false positives obtained. This becomes a practical problem especially when a large clone library is obtained. In order to identify clones expressing a desired trait within the library, robust functional screens are required, otherwise valuable time and resources will be wasted characterizing false positive clones (Jones et al., 2007). Presently, tributyrin is the most widely used substrate for the screening of lipolytic activity, however, our results together with previous literature highlights the drawback of this substrate for screening as there is no apparent relationship between the size of the hydrolysis halo and the enzyme activity. Therefore, finding an alternative substrate to tributyrin could present a possible solution. The alternative substrate will have to be a triacylglyceride with a medium length carbon chain (≤ 10 carbons) so that it can select for both lipases and esterases. Substrates that can be used are the triglycerides tricaproin (TC6:0), tricaprylin (TC8:0) and tricaprin (TC10:0), which to our knowledge has not yet been tested.

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REFERENCES

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25: 3389-3402.

Dong D, Yan A, Liu H, Zhang X, Xu Y (2006). Removal of humic substances from soil using aluminium sulfate. J. Microbiol. Methods., 66: 217-222.

Jones BV, Sun F, Marchesi JR (2007). Using skimmed milk agar to functionally screen a gut metagenomic library for protease may lead to false positives. Letters in Appl. Microbiol., 45: 418-420.

Jung HC, Ko S, Ju SJ, Kim EJ, Kim M, K, Pan JG (2003). Bacterial cell surface display of lipase and its randomly mutated library facilitates high-throuput screening of mutants showing higher specific activities. J. Mol. Catalysis B: Enzy., 26: 177-184.

Labuschagne M, Albertyn J (2007). Cloning of an epoxide hydrolase-encoding gene from *Rhodotorula mucilaginosa* and functional expression in *Yarrowia lipolytica*. Yeast. 24: 69-78.

Lämmle K, Zipper H, Breuer M, Hauer B, Buta C, Brunner H, Rupp S (2007). Identification of novel enzymes with different hydrolytic activities by metagenome expression cloning. J. Biotechnol., 127: 575-592.

Lee SW, Won K, Lim HK, Kim JC, Choi GJ, Cho KY (2004). Screening for novel lipolytic enzymes from uncultured soil microorganisms. Appl. Microbiol. Biotechnol., 65: 720-726.

Lorenz P, Schleper C (2002). Metagenome a challenging source of enzyme discovery. J. Mol. Catalysis B. 19(20): 13-19.

Moser DP, Gihring TM, Brockman FJ, Fredrickson JK, Balkwill DL, Dollhopf ME, Lollar BS, Pratt LM, Boice E, Southam G, Wanger G, Baker BJ, Pfiffner SM, Lin LH, Onstott TC (2005). *Desulfotomaculum* and *Methanobacterium* spp. dominate a 4- to 5-kilometer-deep fault. Appl. Environ. Microbiol., 71: 8773-8783.

Ranjan R, Grover A, Karardar RK, Sharma R (2005). Isolation of novel lipolytic genes from uncultured bacteria of pond water. Biochem. Biophy. Res. Commun., 335: 57-65.

Rhee JK, Ahn DG, Kim YG, Oh JW (2005). New thermophilic and thermostable esterase with sequence similarity to the hormone sensitive lipase family, cloned from a metagenomic library. Appl. Eniviron. Microbiol., 71(2): 817-825.

Ro HS, Hong HP, Kho BH, Kim S, Chung BH (2004). Genome-wide cloning and characterization of microbial esterases. FEMS Microbiol Lett 233: 97-105.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular Cloning*:* A Laboratory Manual. Vol 2, Cold Spring Harbor Laboratory Press. Plainview, New York.

Streit WR, Schmitz RA (2004). Metagenomics- the key to the uncultured microbes. Curr. Opinion in Microbiol., 7: 492-498.

Towner P (1991). Isolation of DNA by SDS-proteinase K treatment. In: TA Brown (ed.) Essential molecular biology, A practical approach, Oxford University Press, UK, 1: 52-53.

Yun J, Ryu S (2005). Screening for novel enzymes from metagenome and SIGEX, as a way to improve it. Microbial Cell Factories. 4(8): 1-5.