SCREENING OF PROTEASE ENZYME BY CONSTRUCTION OF METAGENOMIC LIBRARY FROM MARINE SOIL SEDIMENTS

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Abstract

Nonetheless, the cultivable microorganisms constituting these resources correspond to only a small fraction of the microbial diversity less than 1% of the microorganisms in various environments are readily cultivable (Amann *et al.,* 1995). This limits the range of a search for new biocatalysts for the bioprocess industry, so the use of complex communities and the effort to overcome the problem of noncultivability attract not only scientific attention but also biotechnological innovation. Methods have been developed and used to overcome the non-cultivability of environmental microorganisms for biotechnology, namely cloning and the expression of metagenomes in suitable expression hosts. Proteases are present in all living forms as they are involved in various metabolic processes. They are mainly involved in hydrolysis of the peptide bonds (Gupta *et al.,* 2002). Proteases find a wide range of applications in food, pharmaceutical, leather and textile, detergent, diagnostics industries and also in waste management. In order to discover new proteases from metagenomic libraries, we screened for proteolytic activity from a constructed metagenomic library by direct cloning of environmental DNA of large DNA inserts. A novel gene encoding proteolytic enzyme was picked up, sequenced, expressed in *E. coli* and characterized. Several microbial proteases from the culturable organisms have been characterized. However, very few proteases have been identified through culture independent metagenomic approach.

Key words: Metagenomics, Marine Soil, HMW DNA, Cloning, Screening, Protease.

Introduction

The screening of metagenomic libraries, which is a critical step for the successful isolation of new and improved biological activities, can be performed by sequence homology or activity based assays (Lorenz and Eck, 2005).Screenings for novel enzymes, including proteases, have mainly used the cultivation-dependent approach. Many valuable enzymes originated from cultivable microorganisms; however, the rate of screening for novel enzymes is significantly decreased when standard cultivation methods are used owing to a high rediscovery frequency (Strohl 2000). The structure of microbial communities in various environments is diverse and complex. Nonetheless, the cultivable microorganisms constituting these resources correspond to only a small fraction of the microbial diversity less than 1% of the microorganisms in various environments are readily cultivable (Amann *et al.,* 1995). This limits the range of a search for new biocatalysts for the bioprocess industry, so the use of complex communities and the effort to overcome the problem of noncultivability attract not only scientific attention but also biotechnological innovation. Methods have been developed and used to overcome the non-cultivability of environmental microorganisms for biotechnology, namely cloning and the expression of metagenomes in suitable expression hosts.

Functional profiling of metagenomic libraries, in which all clones in a library that express a certain function are identified and then sequenced, may provide insight into the genomic context of the genes encoding the function and the phylogeny of the organisms that contain these genes (Handelsman, J. 2004, Riesenfeld *et al.,*2004, Schloss and Handelsman 2003). This approach has recovered novel biocatalysts and bioactive compounds, and has expanded our knowledge of uncultured microorganisms (Daniel, 2004, Handelsma 2004, and Riesenfeld *et al.,* 2004). However, the outstanding challenges in functional metagenomics include screening the massive libraries needed to represent all of the genomes in an environmental sample and overcoming barriers to heterologous gene expression in the host species harboring the library. These challenges are significant barriers in identifying small molecules such as antibiotics and signal compounds (Handelsman, 2004, Handelsman, 2005, Lorenz *et al.,* 2002, Streit *et al.,* 2004, Uchiyama *et al.,* 2005).

Proteases are present in all living forms as they are involved in various metabolic processes. They are mainly involved in hydrolysis of the peptide bonds (Gupta *et al.,* 2002). Proteases find a wide range of applications in food, pharmaceutical, leather and textile, detergent, diagnostics industries and also in waste management (Rao *et al.,* 1998). Thus, they contribute to almost 40% of enzyme sales in the industrial market. Though proteases are found in plants and animals, microbial proteases account for two-third of share in the commercially available proteases (Kumar and Takagi 1999).

In order to discover new proteases from metagenomic libraries, we screened for proteolytic activity from a constructed metagenomic library by direct cloning of environmental DNA of large DNA inserts. A novel gene encoding proteolytic enzyme was picked up, sequenced, expressed in *E. coli* and characterized. Several microbial proteases from the culturable organisms have been characterized. However, very few proteases have been identified through culture independent metagenomic approach (Schloss and Handelsman 2003)

Despite the success rate, very few attempts were made on the identification of proteases from metagenomic libraries. Functional screens using skimmed-milk agar to obtain protease activity is a common approach. In the present study was to determine the efficacy of this screen to obtain protease activity from metagenomic libraries.

Materials and methods

The soil samples were collected from the study area at coastal regions of Samiyarpettai, Caudalore District, Tamil Nadu, (South India), during the month of April 2010. For the Isolation of eDNA from soil sample, procedure has been modified from nature protocols (Sean F Brady, 2007) and also Purification of eDNA procedure was followed by Sean F Brady (2007) with modification of protocol of Chromous Gel Extraction Kits (chromous biotech, Bangalore). Construction of metagenomic library by isolated DNA from soil sample, procedure has been modified from nature protocols (Sean F Brady 2007). The optimal size of high molecular weight DNA size was (30-45 kb) for ligation into the pWEB-TNC vector. The blunt end an aliquot of the highest quality of DNA sample was using the end repair enzyme mixture from epicenter biotechnologies.

Screening of metagenomic library for proteolytic activity was followed by Lee *et al.,* 2007. Screening of the metagenomic library for protease activity was performed on Luria-Bertani (LB) agar supplemented with 1% (w/v) skimmed milk, and chloramphenicol (12.5μg ml^{-1}). SMA (skimmed milk agar) was prepared using a 10% (w/v) stock solution of commercially available nonfat milk powder and LB agar made separately. LB was autoclaved at 121_C for 15 min, while 10% (w/v) milk powder solution was autoclaved at 115_C for 10 min. Exactly 10% (w/v) milk solution was mixed with LB agar to a final concentration of 1% (w/v) while still hot. Plates were incubated for 3 days at 37_C after which time any clone with a clear halo was picked and stored for subsequent analysis. Photolytic clones were selected based on the formation of halo zone of clearance around the colony.

Proteolytic Enzyme assay

Total protease activity was measured using a casein substrate by a modification of the Anson Method (Keay and Wildi 1970). A 1 ml of the culture supernatant was mixed with 1 ml 0.05 M phosphate buffer-0.1 M NaOH (pH 7.0 adjusted with phosphoric acid) containing 2% casein, and incubated for 10 min at 37 °C. The reaction was stopped by adding 2 ml 0.4 M Trichloroacetic acid. After 30 min stand at room temperature, the precipitate was removed by centrifugation and the optical density of the assays was measured at 660 nm. A standard curve was generated using solutions of 0–60 μg/mL tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg/mL tyrosine under the experimental conditions used.

Results and discussion

A total of about 12000 clones with average `size 40kbp size insert were obtained from the metagenomics library of marine soil sediments. Among the 12000 clones only ten clones were picked up for expression of protease activity. In the present investigation for the activity of screening, clones of the metagenomic libarary from the marine soil sediments was transferred to the host strain *E.Coli* that has non functional Protease enzyme, which allows selection for grow on protease. After bacterial transformation cells were plated in defined media supplemented with protease and incubated it 37^{o} C for three to ten days. The screen for genes conferring protease activity was based on the library containing *E.Coli* clones to form halos (fig 1&2) when grown on indicator agar medium containing skimmed milk agar medium. Halo formations caused by hydrolysis of the milk protein. Screening of recombinant clone for protease activity revealed two clones carrying recombinant plasmid designated as *PMSP I* that exhibited a zone of clearance on LB skim milk agar plate after 3 days of incubation at 37°C, Skimmed milk agar was showed maximum proteolytic activity in plate A (20-18 mm)(Fig.1), when compare to plate B (15-12 mm) by *PMSP I* clones from marine soil sediments. The proteolytic activities of clones were assayed using skim milk agar and casein agar, and exhibited as diameter of clear zone in mm. Skim milk agar was the best than casein agar for Qualitative test of protease. Casein agar was

showed minimum proteolytic activity in plate C (10-8 mm) and plate D (10-8) (Fig.1) by *PMSP I* clones from marine soil sediments.

The potential of this protease activity from independent cloning events are not the results duplicate cloning. A clone with skimmed milk agar protease activity was selected and its 40kbp further include for complete sequencing. From the present investigation the marine soil sediments support high biodiversity and vast ecosystem services and microbial populations widespread more in marine environment than terrestrial earth (Snelgrove 1999). Although several novel enzymes for biocatalyst have been screened from metagenomic libraries, there are very few metagenomic studies targeting proteases from a marine environment. To the best of our knowledge, this is the first study on expression and characterization of a protease enzyme from marine soil sediments. The eukaryotic DNA concentration was lower in the metagenomic DNA prepared using the indirect methods than the direct method (Gabor *et al.,* 2003). So we have been used the direct method for extraction of DNA to construct metagenomics library. Kayalvizhi and Gunasekaran (2008) have been earlier reported that goat skin contains diverse species of bacteria including several unculturable bacteria in addition to the culturable proteolytic bacteria that are predominant and are involved in the degradation of the skin (Kayalvizhi and Gunasekaran 2008).

Identification of protease gene from metagenomic library was previously unsuccessful (Jones *et al.,* 2007; Rondon *et al.,* 2000). However, few other functional metalloproteases were identified through metagenomic approach (Lee *et al.,* 2007; Waschkowitz *et al.,* 2009; Gupta *et al.,* 2002). The unsuccessful attempts in identification of protease genes from metagenomic library could be attributed to the problems associated with the expression of cloned gene in the heterologous host (Handelsman 2004) and low frequency of target sequence in the metagenomic library (Henne *et al.,* 1999).

To identify enzymatic functions of individual clones, chemical dyes and insoluble or chromophore-containing derivatives of enzyme substrates can be incorporated into the growth medium (Daniel 2005; Ferrer *et al.,* 2009; Handelsman 2004). Lee *et al.,* (2007) and Waschkowitz *et al.,* (2009) have been simple activity-based approach are the detection of recombinant *E. coli* clones exhibiting protease activity on indicator agar containing skimmed milk as protease substrate.

Conclusion

Thus the above-accumulated evidence provides a window into a world of microbial diversity that is astonishing in its magnitude and is open to tap the vast genetic potential of microorganisms to obtain protease enzyme for process of biotechnological value. Metagenome approach thus provides a means to view both the structural and functional genomics of microbial diversity and pave a pathway to search out and discover novel genes for obtaining newer and useful industrial proteolytic enzymes with better properties.

References

[1] R.I., Amann W. Ludwig, and K.H. Schleifer**,** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev, 59:143-169.

[2] C. Simon and R. Daniel, 2009. Achievements and new knowledge unraveled by metagenomic approaches. Appl Microbiol Biotechnol, 85:265–276.

[3] R.Daniel, 2004. The soil metagenome: a rich resource for the discovery of novel natural products. Curr. Opin. Biotechnol. 15:199–204.

[4] R. Daniel, 2005. The metagenomics of soil. Nat. Rev. Microbiol. **3:**470–478.

[5] M. Ferrer, A. Beloqui, K.N. Timmis, P.N. Golyshin, 2009. Metagenomics for mining new genetic resources of microbial communities. J Mol Microbiol Biotechnol 16:109–123.

[6] E.M. Gabor, E.J. de Vries, D.B. Janssen, 2003. Efficient recovery of environmental DNA for expression cloning by indirect extraction methods. FEMS Microbiol Ecol 44:153-163.

[7] R.Gupta, Q.K. Beg, P.Lorenz 2002. Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol 59:15-32.

[8] J. Handelsman2004. Soils: the metagenomics approach, p. 109–120. In A. T. Bull (ed.), Microbial diversity and bioprospecting. ASM Press, Washington, D.C.

[9] J.Handelsman, 2005. Sorting out metagenomes. Nat. Biotechnol. 23:38–39.

[10] A Henne R. Daniel, R.A. Schmitz, G. Gottschalk, 1999. Construction of environmental DNA libraries in Escherichia coli and screening for the presence of genes conferring utilization of 4-hydroxybutyrate. Appl Environ Microbiol 65:3901–3907.

[11] B.V. Jones, F .Sun, and J.R. Marchesi 2007. Using skimmed milk agar to functionally screen a gut metagenomic library for proteases may lead to false positives. Lett Appl Microbiol 45:418–420.

[12] N. Kayalvizhi and P.Gunasekaran 2008. Production and characterization of a lowmolecular- weight bacteriocin from Bacillus licheniformis MKU3. Lett Appl Microbiol 47:600–607.

[13] L. Keay, and B.S. Wildi, 1970. Proteases of the genus Bacillus II. Alkaline proteases. Biotechnol. Bioeng., 12, 213–249.

[14] C.G. Kumar, H,Takagi 1999. Microbial alkaline proteases: from a bioindustrial viewpoint. Biotechnol Adv 17:561-594.

[15] D.G. Lee, J.H.Jeon, M.K. Jang, N.Y. Kim, J.H.Lee, S.J. Kim G.D. Kim, S.H. Lee, 2007. Screening and characterization of a novel fibrinolytic metalloprotease from a metagenomic library. Biotechnol Lett 29:465–472.

[16] P. Lorenz, K. Liebeton, F. Niehaus, and J. Eck. 2002. Screening for novel enzymes for biocatalytic processes: accessing the metagenome as a resource of novel functional sequence space. Curr. Opin. Biotechnol. 13:572–577.

[17] P.J. Lorenz and J. Eck, 2005. Metagenomics and industrial applications. Nat Rev Microbiol 3, 510–516.

[18] M.B. Rao A.M.Tanksale, M.S Ghatge, V.V. Deshpande 1998. Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev 62:597–635.

[19] C. Riesenfeld, S. P. D. Schloss, and J. Handelsman. 2004. Metagenomics: genomic analysis of microbial communities. Annu. Rev. Genet. 38:525–552.
[20] Schloss PD, Handelsman J (2003) Biotechnological prospects from metagenomics. Curr Opin Biotechnol 14:303–310.
[21] P.V.R. Snelgrove, 1999.Getting to the bottom of the marine biodiversity: sedimentary habitats: ocean bottoms are the most widespread habitat on earth and support high biodiversity and key ecosystem services. Biosciences. 129-138.
[22] W. R. Streit, R. Daniel, and K. E. Jaeger. 2004. Prospecting for biocatalysts and drugs in the genomes of noncultured microorganisms. Curr. Opin. Biotechnol. 15:285–290.
[23] W.R. Strohl 2000. The role of natural products in modern drug discovery. Drug Discov Today 5:39–41.
[24] T. Uchiyama, T. Abe, T. Ikemura, and K. Watanabe. 2005. Substrate-induced gene-expression screening of environmental metagenome libraries for isolation of catabolic genes. Nat. Biotechnol. 23:88–93.
[25] T. Waschkowitz Rockstroh S, Daniel R (2009) Isolation and characterization of metalloproteases with a novel domain structure by construction and screening of metagenomic libraries. Appl Environ Microbiol 75:2506–2516.

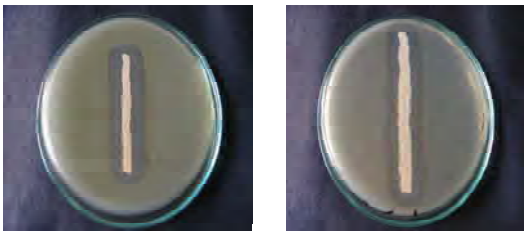

Fig 1:Qualitative assay for protease activity in the medium of skimmed milk agar plate (A&B)

Fig 2: Qualitative assay for protease activity in the medium of casein agar plate (C&D)