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Identification and Characterization of a Protease Encoding Metagenome Clone from Dairy Effluent

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Metagenomic DNA was isolated from dairy effluent collected from a milk processing unit. The *Hpa* I digests of metagenome were cloned in pEZ BAC vector. The transformed DH5 colonies were selected by blue white screening on chloramphenicol (25µg/ml) containing plates. Functional screening of the library on skim milk agar showed a clone having protease activity. This clone was confirmed to have a metagenome insert of size approx. 9kb. The crude cell extract of this clone at 24th h of growth showed protease activity of 166.99U/ml (111.32U/mg protein). The protease was found to be alkaline and had maximum activity at 40^{0}C and pH 9.0.

Keywords: Metagenome, DNA, Transformed

Introduction

Proteases, the protein degrading enzymes, play a pivotal role among the industrially valuable enzymes. Extra cellular alkaline proteases from microbial sources find multiple applications in industries such as detergent laundry, food processing, leather processing, pharmaceutical, waste disposal and heavy metal recovery[1]. Proteases hold second position in the enzyme industry and are expected to grow with a CAGR of 14.6% from 2014 to 2020 http://www.bccresearch.com/marketresearch/biotechnology/microbial-products-technologies-market-bio086c.html. Though many enzymes have been identified, the requirement of industries for maximum efficiency still exists and novel enzymes suitable to withstand the conditions prevailing in each industry are always in good demand. In the present study metagenomic approach is adopted to isolate protease encoding gene/genes from dairy effluent by functional screening. Dairy effluent is the untreated waste water from milk processing unit which contains higher amounts of milk proteins that can act as a source for protease producing microbes.

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Materials and methods

Preparation of genomic library

Dairy effluent collected from a milk processing unit was centrifuged at 4000 RPM to remove suspended particles and metagenome was isolated following the method proposed by Wilson[2]. The integrity and quantity of the metagenomes were analyzed using gel electrophoresis and spectrophotometer. The integrity and quantity of the metagenomes were analyzed using gel electrophoresis and spectrophotometer. The metagenome was partially digested using restriction enzyme HpaI (Haemophilus parainfluenzae I) at 37°C at different time interval. The fragments produced were ligated to the linearized polynucleotide kinase treated pEZ BAC vector, at the HpaI site using clone smart ligase of Lucigen (USA). After ligation, transformation was performed in competent E. coli (DH5α) cells using heat shock method. Transformed colonies with inserts were selected by blue white screening on chloramphenicol (25µg/ml) containing plates.

Functional screening of the library for protease activity

The transformed colonies were plated on skimmed milk agar for identification of protease positive clones by observing clear zones.

Estimation of enzyme activity

The selected clone was grown in a specified medium containing (% w/v) glucose 0.1, peptone1.0,

yeast extract 0.02, $MgSO_4$ 0.01, $CaCl_2$ 0.01, K_2HPO_4 0.05, pH 7.0 for different intervals of time (12, 24, 36 and 48 hour). The cultures were centrifuged at 1000 rpm for 5 min and the crude enzyme extract was collected. The crude enzyme (0.05ml) was diluted to 0.5 ml and was incubated at 70^0C for 15 min in 0.5 ml substrate solution (1% casein in 100mM glycine NaOH). The reaction was stopped by adding 0.5ml of 20% trichloroacetic acid. Then, the mixture was incubated at room temperature for 15 min. The precipitate was removed by centrifuging at 10,000 x g for 15 min. The absorbance of tyrosine in the supernatant, released by the hydrolysis of casein, was measured at 280nm. Quantity of tyrosine released was determined from the standard graph of tyrosine. A control was kept without enzyme. One unit of protease is defined as the quantity of enzyme that releases 1µg of tyrosine per ml per minute at 70^0C. The stability of the crude enzyme was assessed by estimating the remaining activity of crude enzyme at different temperature and pH[3]. Effect of pH on stability of the enzyme was studied by pre incubating crude extract in buffers at pH over a range of 7.0 to 13.0 for 1 hour at 37^0C. The buffer systems used were phosphate buffer saline for pH 6-7, Tris- HCL buffer for pH 8-9 and glycine- NaOH for pH 9-13. The effect of temperature on the enzyme was determined by pre-incubating the crude enzyme at 37, 40, 45, 50, 55, and 60^0C for one hour.

Results and Discussion

Metagenomics provide information on nonculturable microbes in the environment having industrial importance. Isolation of good quality metagenome is a key factor which determines the success of metagenomics. Dairy effluents contain high amounts of milk proteins which act as a source for protease producing microorganisms. Dairy effluent used in this study was colloidal in nature with lot of suspended particles. Since filtration was not easy, the protocol reported for DNA isolation from water samples was not found suitable for this sample. So in the present study, method proposed by Wilson[2] for bacterial genomic DNA isolation was used to isolate the metagenome from dairy effluent. The purified metagenome isolated had a concentration of 2.8µg/µl with a A_{260}/A_{280} value 1.81. The size of the DNA was larger than 50 Kb. Metagenomic library construction is a powerful tool to isolate novel enzymes from the environment having complex microbial flora[4]. In the present work pEZ BAC vector was used to construct library from the isolated DNA for identifying protease encoding genes. BAC vector was preferred since large inserts can provide complete genes or operons encoding specific functional pathways and also the number of clones could be less, which could make the screening process easier[5]. Metagenomic DNA was digested with the restriction enzyme HpaI to create fragments of desirable length with sticky ends suitable for inserting in the HpaI cloning site of the BAC vector used. To get suitable fragments, digestion was carried out for different time intervals. The fragments obtained after 1h digestion yielded fragments of size more than 40kb and those fragments were cloned and transformed into *E. coli* (DH5α) by heat shock method. Blue white screening on chloramphenicol (25µg/ml) yielded 80 transformed colonies. The transformed colonies were screened for protease activity and in the present study, only one clone showed clear zones indicating the presence of protease activity (Figure 1). Functional screening of metagenomic library is used for identifying genomic fragments with a desired function[6]. It enables screening based on activity of the gene and this approach has been able to provide novel enzymes from uncultured microorganisms[7]. Casein or skimmed milk agar plate assays allow qualitative determination of protease activity[8,9]. There are several reports on the successful use of this screening strategy for the identification of proteases from diverse samples[10]. The clone showing protease activity was confirmed for the presence of metagonome insert by isolating the plasmid and digesting with HpaI. The gel picture

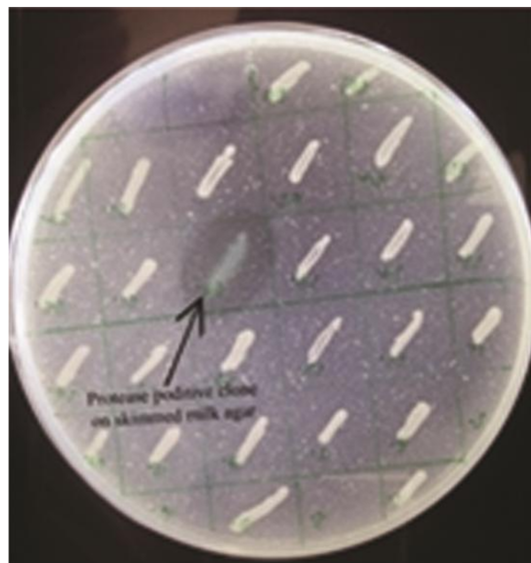

Fig. 1 — Metagenome clone showing protease activity

showed a band corresponding to the vector and another band of size approximately 9kb representing the metagenome insert (Figure 2), confirming the presence of metagenome insert. It is important to study the stability of enzymes under various environmental conditions to understand their suitability in industrial applications. The crude extract of the protease positive clone collected at 24th hour of growth showed maximum enzyme activity of 167.99 units/ml, which decreased gradually with time. The enzyme was found stable only in a narrow pH range and the maximum enzyme activity was observed at pH 9.0 (Figure. 3). The pH of the dairy effluent used in this study was 8.6. Temperature showed a significant effect on the stability of the enzyme. The enzyme was most stable at 40^0C (Figure 3), as reflected in the remaining activity (146.66units/ml). The enzyme activity was reduced drastically at temperatures above 45^0C. At 60^0C the enzyme retained only 35.71% of its original activity. Alkaline proteases are one among the most utilized enzymes in industries. Stability of the enzyme under harsh conditions is an important requirement for such purposes. Characterization of the enzyme can provide information on its possible utility. A few enzymes isolated from metagenomes have been assigned utility following this process. An alkaline protease isolated by Biver *et al*.[11] showed oxidant stability suggesting its possible applications in the detergent and bleaching industries. Two serine proteases isolated from surface sand of deserts were found to be relatively resistant to detergent, suitable for laundry industry[12]. A novel protease belonging to chymotrypsin-like S1 serine proteases was isolated by Niehaus *et al*.[13]. The thermostable serine protease isolated from aklaliphilic estuarine bacterium *Bacillus altitudinis* strain BR1 showed maximum activity at pH 9 and temperature 50°C. The bacterium was found to have 5 distinct isozymes of alkaline protease with molecular weight 17,22,43,64 and 88 KDa respectively[14]. LasB, a duel substrate specific protease from *Pseudomonas aeruginosa* MCCB 123 was having optimum pH and temperature, 9 and 60^0C respectively. the enzyme is having cytotoxicity against Hep2 cells with an IC50 value of 47.29 µg/ml[15]. Alkaline proteases find a suitable place in detergent industry. Enzymes which work in ambient temperature are considered as more economic for this purpose.

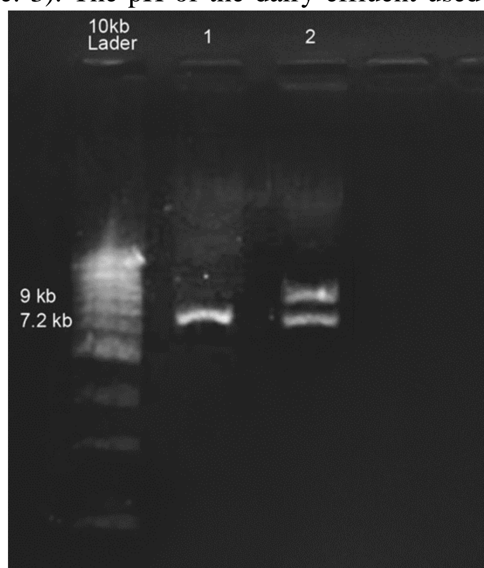

Fig. 2 — Metagenome insert in the clone showing protease activity (Lane1: Marker, Lane2: Plasmid DNA from the clone, Lane3: Plasmid DNA digested with Hpa1 showing 9kb insert)

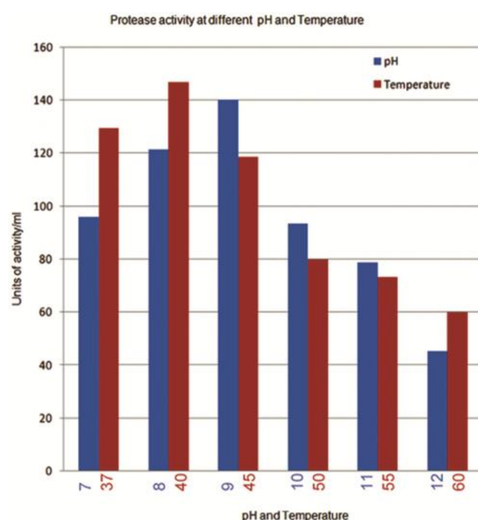

Fig. 3 — Effect of pH and temperature on the enzyme activity of the culture extract

Conclusion

Metagenome approach provides a means to identify genes encoding novel enzymes from environmental sources based on both structural and functional genomics. The protease encoding metagenome isolated in this study produces alkaline protease which shows maximum activity at 40^0C and a pH of 9, which suggests its suitability in detergent industry.

Acknowledgement

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