Identification, Cloning and Sequence Analysis of Chitinase Gene in *Bacillus halodurans* Isolated from Salted Fish

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Abstract: Chitin, a linear N-acetylglucosamine is a structural component of fungal cell wall and exoskeletons of invertebrates. Chitinases catalyse the conversion of chitin and it is produced by a wide range of bacteria. The gene for chitinase biosynthesis *Chi* was PCR amplified from genomic DNA of *Bacillus halodurans*. The amplified genes were cloned and nucleotide sequences were determined. The sequencing results showed that *Chi* gene contain 1800 bp long ORF encoding 599 amino acids respectively (GenBank accession No. GU481106). Computational sequence analysis of nucleotides and amino acids revealed that the *Chi* sequences of *Bacillus halodurans* were conserved in many eubacteria.

Key words: PCR, cloning, gene transformation, *in silico*, bioremediation

INTRODUCTION

Chitin, a linear β-(1, 4)-linked N-acetylglucosamine (Glc-NAc) polysaccharide (Gooday, 1999), is a main structural component of the fungal cell wall and the exoskeletons of invertebrates, such as insects and crustaceans (Wang and Hwang, 2000). It is one of the most abundant naturally occurring polysaccharides and has attracted tremendous attention in the fields of agriculture, pharmacology and biotechnology (Antranikian et al., 2005; Muzzarelli et al., 2005). Chitin can be hydrolysed by an enzyme chitinases. Chitinases catalyse the conversion of chitin and it is produced by a wide range of organisms, including bacteria (Kitamura and Kamei, 2003), plants (Masuda et al., 2001) and fungi (Ike et al., 2006). Chitin is a structural material in many marine invertebrates, such as cuttlefish, crab, lobster, fungi and algae (Li and Roseman, 2004). Marine bacteria possess the effective capability in chitin depolymerization (ZoBell and Rittenberg, 1938). Among eubacteria, marine bacteria are the excellent source for chitinases (Austin, 1988). Chitinase enzyme has been well characterized in several marine bacteria (Bassler et al., 1991; Tsujibo et al., 1993; Hirono et al., 1998; Aunpad and Panbangred, 2003; Howard et al., 2003). Chitinases can be used to treat fungal infections (Liu et al., 2002). Plants produce chitinases as defence agent against fungal pathogens (Taia et al., 2005). Chitin also has multiple applications mainly in the bioremediation of environmental pollutants like Pb<sup>2+</sup>, Zn<sup>2+</sup> and Bi<sup>3+</sup> (Rae and Gibb, 2003). Chitinase enzyme possesses added value in food and pharmaceutical industries, anti-bacterial agents, elicitors, lysozyme inducers and immunoenhancers (Kato et al., 2003; Muzzarelli et al., 2005). Due to the massive biological applications, the characterization and synthesis of chitinase is highly anticipated in biotechnology industry. *Bacillus halodurans* is an alkalophilic bacterium (Takami and Horikoshi, 1999) that can grow well at pH 7-10.5 in saline environments. Even though *B. halodurans* is well characterized physiologically, biochemically and genetically (Horikoshi, 1999; Takami and Horikoshi, 1999) the functional characterization of chitinase biosynthesis genes from *B. halodurans* has not been reported till date and ours is the first report on this subject. The aim of the present study is the identification, characterization and *in silico* analysis of the chitinase biosynthesis gene (*Chi*) in alkalophilic *Bacillus halodurans* isolated from fish.

MATERIALS AND METHODS

Bacterial strains and culture media: *Bacillus halodurans* was isolated from salted anchovies procured from local fish markets in Cochin, Kerala, India. Microbial identification and biochemical characterization of *B. halodurans* was carried out using conventional
method as per (Nielsen et al., 1995) as well as 16S rDNA study. The 16S rDNA sequence of the isolate showed 100% homology with that of B. halodurans C-125. Other bacterial strains included in this study includes Escherichia coli JM109. Bacillus halodurans was grown aerobically on alkaline bacillus medium at 37°C. Chitinolytic activity of Bacillus halodurans was observed as the clear zone of chitin in alkaline bacillus agar containing 1% chitin from shrimp exoskeleton. Genomic DNA was prepared as described by Ausubel et al. (1994).

**Polymerase chain reaction:** The chitinase gene, Chi was amplified by using gene specific primers. The PCR reaction was performed with the final volume of 50 μL that contained, 0.5 μM each of forward and reverse primers, 1.0 μL of crude genomic DNA, 200 μM of dNTPs, 1X Taq buffer, 2.5 mM MgCl2, 1U Taq DNA polymerase (MBI Fermentas, Hanover, Maryland, USA) and autoclaved MilliQ water. The PCR was performed using a Master cycler (PC-818, Astec Co., Ltd., Japan) with the following conditions: initial denaturation at 94°C for 3 min, followed by 30 repeated cycles of 94°C for 30 sec, 50°C for 1 min and 72°C for 2 min and final extension at 72°C for 5 min. The PCR amplified product was analyzed on 1.5% agarose gel along with DNA ladder (MBI Fermentas) and documented using a gel documentation system (Vilber Lourmat, France).

**Cloning of PCR product:** The PCR amplicon of Chi was purified by the use of Perfectprep Gel Cleanup Kit (Eppendorf, Germany) and cloned into the cloning vector, pTZ57R/T (MBI Fermentas). The cloned inserts were transformed into E. coli JM109 and plated on Luria Bertani (LB) agar containing ampicillin (100 μg mL−1), IPTG (50 μM) and X-gal (80 μg mL−1). The plates were incubated at 37°C and the transformants were selected and inoculated in 5 mL LB broth with the corresponding antibiotic. The recombinant plasmids were isolated from the overnight culture by alkaline lysis method (Sambrook and Russell, 2001).

**Characterization of recombinant plasmid:** The recombinant plasmid was double digested with BamHI and XbaI restriction enzymes. The reaction mixture contained recombinant plasmid 2 μL, Enzyme buffer (10X) 2 μL, each restriction enzyme (10U μL−1) 0.5 μL and volume up to 20 μL with autoclaved MilliQ water. The reaction mixture was incubated overnight at 37°C in a water bath. The digested products were analyzed on 1.5% agarose gel. The clone with the correct insert as judged by size was sequenced on an ABI PRISM 377 genetic analyzer (Applied Biosystems, Perkin Elmer Co., Foster City, CA, USA).

**In silico sequence analysis:** The nucleotide sequences obtained were compared to the available database sequences by BLAST analysis using the NCBI (http://www.ncbi.nlm.nih.gov) database. The sequences were aligned and clustered using CLUSTAL-X version 1.81 (Thompson et al., 1997). The molecular masses and the theoretical pI values of the polypeptides were predicted using the ProtParam tool (http://www.expasy.org/tools/protparam.html).

**RESULTS**

The Chi gene was PCR amplified and is encoded by polyribonucleotides of 1800 bp (Fig. 1). The Chi encodes proteins of 599 amino acids with the pI value of 4.62. The molecular mass of the protein is 67059 Da, based on in silico estimates (Fig. 3). After PCR amplification, the products were purified from the agarose gel and cloned into pTZ57R/T cloning vector. The recombinant transformants with Chi gene was also confirmed by double digestion with restriction enzymes (Fig. 2). The nucleotide sequence of Chi gene was submitted to GenBank and have been given accession No. GU481106.

The search for homologous genes and deduced amino acid sequence were performed using BLAST. The nucleotide sequence of Chi gene matches significantly with the chitinase genes from other organisms. The Chi sequences from the B. halodurans isolate were compared with the reported nucleotide and amino acid sequences of other eubacteria viz., Bacillus clausii (GenBank accession No. AP006627), Bacillus pumilus (DQ859055), Bacillus subtilis (DQ661650), Bacilluslicheniformis (GQ899144), Bacillus amylogluefaciens (EF088513), Bacillus circulans (AF154827) and Bacillus thuringiensis (GQ183831) using Clustal W software. In silico nucleotide sequences analysis of Chi gene revealed a high degree of

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**Fig. 1:** Agarose gel electrophoresis of amplified product of Chi gene.
amino acid substitutions were observed in N-terminal, middle and C-terminal regions of all eubacterial chitinases.

Phylogenetic tree based on evolutionary distances was constructed for nucleotide and amino acid sequences of Chi with the MEGA software (Molecular Evolutionary Genetics Analysis, version 3.1), Kumar et al. (2004) using the neighbour-joining method. The tree at nucleotide and amino acid sequence of Chi (data not shown) reveals that, B. halodurans and B. clausii forms a single cluster with that of other eubacteria. Many bacterial species switched to different clusters for Chi gene at nucleotide and amino acid level indicating the divergence among the organisms and the degree of divergence in the sequences.

**DISCUSSION**

Chitinases (1, 4-beta-poly-N-acetylglucosaminidase) catalyse the conversion of chitin and is produced by a wide range of organisms, including bacteria, plants and fungi (Ike et al., 2006). Based on the enzyme activity, chitinase enzymes are classified into three types: endochitinases, exochitinases and N-acetylglycosaminidases (Lee et al., 2007). Endochitinases cleave β-1, 4-glycosidic bonds of chitin randomly, while exochitinases cleave the nonreducing end of the chitin chain to form diacetyl chitobiose. N-acetylglycosaminidases hydrolyse diacetyl chitobiose into N-acetylglycosamine (Taraka et al., 2001). Up to now, chitinases has been functionally characterized from various aerobic eubacteria: *Bacillus circulans* (Alam et al., 1995), *Serratia marcescens* (Burberg et al., 1996), *Aeromonas* sp. (Shiro et al., 1996), *Alphomonas* sp. (Tanjib et al., 1992) and *Janthinobacterium lividum* (Gleave et al., 1995). Bacterial chitinases contains Chitin Binding Domain (CBD) and Wronnicki type III-like domain. The chitin binding domain mainly involves in the degradation of insoluble chitin (Morimoto et al., 1997). Chitinase enzyme tenders massive applications in food and pharmaceutical industries (Muzzarelli et al., 2005).

Based on the sequence analysis, it was previously reported that the Chi gene of *Bacillus* sp. codes for chitinase (Lee et al., 2007). To date, only least information on the characterization of Chi gene from marine source has been reported (Li and Roseman, 2004). In this study we cloned and analyzed the Chi gene from *B. halodurans* cells isolated from salted fish, as a first step towards the molecular characterisation of chitinase. Evaluation of the deduced amino acid sequences of Chi gene with reported sequences in the database revealed a maximum similarity. However, the sequence analysis of Chi of present isolate showed several base substitutions with that of reported
sequences, resulting in the altered amino acid sequences of the translated proteins.

We conclude that this study represents the first instance in which Chi gene from *B. halodurans* isolated from salted fish has been cloned and characterized in detail. Moreover, the determination of protein structure modification due to the nucleotide substitutions will certainly provide the basis for performing site-directed mutagenesis to improve the production and configuration of the chitinase of biotechnological interest.

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**REFERENCES**


