SUPPLEMENTARY MATERIAL

MOLECULAR CHARACTERIZATION OF BACILLUS CHITINASE FOR BIOCONVERSION OF CHITIN WASTE

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EXPERIMENTAL

Purification and extraction of chitin from Shrimp shells

Shrimp shells were collected from seafood processing area Fisheries Market, Karachi. The waste was washed thrice with distilled water and dried. Shrimp shells were weighed and soaked in 2% NaOH with constant stirring for 30 min. The deproteinated shells were filtered through Wattman 42 filterpaper and rinsed thoroughly with sterile distilled water. The shells were then treated with 5% HCl with constant stirring for 30 min, again filtered and rinsed with the distilled water. The deproteinated and demineralized shells were placed in an oven at 60°C overnight for drying (Pinelli-Saavedra et al. 1998). The dried shrimp shells were grinded and stored in a sterile air tightened jar at room temperature.

Qualitative analysis of extracted chitin

The chitin sample was oven dried overnight at 60°C and its 1mg was mixed in 100mg of KBr to prepare 0.25 mm thick disc. FTIR spectra of the sample (chitin powder) in KBr disc was obtained by using M2000, MIdac Corporation spectrometer with a resolution of 8 cm^{-1}.

Screening of chitinolytic **Bacillus** *sp.*

A total of seventeen bacterial isolates were obtained from School of Biological Sciences, University of the Punjab, Lahore including twelve strains of *Bacillus thuringiensis* i.e. SBS Bt1-6 (Saleem and Shakoori 2013) and CMBL Bt1-6, three strains of *Bacillus firmus* i.e. CMBL F1-3

and two strains of *Bacillus brevis* i.e. CMBL B1-2. Bacterial strains were cultured on nutrient agar, incubated overnight at 30°C and stored at 4°C till further use.

Preparation of colloidal chitin and selective medium

Five gram of chitin powder was suspended in 60ml of conc. HCl and stirred constantly for 1 hour. Solution was filtered through glass wool and 200ml of 50% ethanol was added to the filtrate. It was mixed thoroughly and then centrifuged in tabletop centrifuge (Hermle Z 300K) at 4°C at 6000 rpm (rcf 3904) for 15 min. Pellet was washed with sterile distilled water and centrifuged at 4^0C at 6000 rpm for 15 min. About 2L sterile distilled water was used for the repeated washings until pH of the colloidal chitin became neutral. Then colloidal chitin was then weighed and stored in dark at 4°C (Roberts and Selitrennikoff 1988). The bacterial strains were screened on selective medium containing 1% colloidal chitin for their chitinolytic potential (Mejía-Saulés et al. 2005).

Optimization of temperature and pH for chitinolytic activity

The *Bacillus* strains showing chitinolytic activity were cultured on the selective medium containing 1% colloidal chitin and incubated at 25°C, 30°C, 37°C and 45°C, for 96 h, while for optimization of pH they were cultured on the selective medium at initial pH of 5.5, 7.0, 8.5 and 10.0. Zones of chitin hydrolysis were observed daily up to 96 h. The chitinolytic *Bt* were selected based upon their high chitinolytic activity, as assessed through the chitin hydrolysis zones.

Genomic DNA Isolation and Quantification

Total DNA was isolated from the selected *Bacillus* strain as described by Saleem and Shakoori (2013). Bacterial pellet was resuspended in 200 µl of lysis buffer, placed in water bath at 45°C for 40 min and then at 94°C for 10min. Following phenol chloroform extraction, the DNA was precipitated from the aqueous layer by addition of ice cold ethanol and then centrifuged at 12000rpm for 5 min. The DNA pellet was washed with 70% ethanol, dried and dissolved in TE buffer. DNA quantification was done by visualizing on 1% agarose gel as well as by spectrophotometric calculations. The quantity of DNA was calculated by applying the following formula

Amount of DNA (mg/ml) = 50 x OD_{260} x dilution factor.

PCR amplification of* chitinase *gene

The following primer pair was designed to amplify full length Chitinase gene from *Bacillus* species by analysing *Bacillus* Chitinase gene sequences available in DNA databases (NCBI).

CHI-F 5′ CGAGAAATGCATATGAGGTCTC 3′

CHI-R 3′ AACTGCAGCGAAAGCCTTTCCCTAACAGGTGAC 5′

PCR reaction mixture (50 µl) contained 1X Taq buffer, 2mM $MgCl_2$, 0.2mM dNTPs, 1µM each primer, 350 ng template DNA, 2.5 units of Taq DNA. The reaction cycle consisted of pre-PCR denaturation at 94°C for 3 min followed 35 cycles, each of 45s at 94°C (denaturation), 40s at 58°C (annealing), 1 min at 72°C (extension) and final extension was done for 5 min at 72°C. The results of PCR were checked on 1% agarose gel.

DNA extraction (Gene Clean)

PCR products were purified from agarose gel in 1X TAE buffer, using DNA Extraction Kit (Vivantis Cat # GF-GP-100) according to manufacturer's instructions. The extracted DNA was eluted and stored at -20 ºC till further use.

Cloning of* chitinase *gene and restriction analysis:

PCR products were cloned in InsT/Aclone^{TM}PCR Product Cloning Kit (cat # K1214) from Fermentas Life Sciences. Vector and the PCR product were used in a ratio of 1:3 in the ligation reaction (Sambrook et al. 1989). The plasmid DNA was subjected to single and double digestion with *Eco*R1 (Fermentas) and *Hin*dIII (Fermentas). Restricted fragments were analysed on 1% agarose gel.

DNA Sequencing

The recombinant plasmid containing the gene of interest was purified from 10 ml culture using Thermo Scientific GeneJet Miniprep Kit #K0502) following the manufacturer's instructions. The quality and quantity of the purified DNA was determined by agarose gel electrophoresis. The sample was sent to Macrogen (S. Korea) for sequencing. The finalized sequence was submitted to EMBL database.

Sequence analysis

Sequence of *chitinase* gene was analysed using available tools for gene and protein analysis. The alignment of the sequences was done using CLUSTAL W (Thompson et al. 1994). Homologues were selected from result of BLASTn search of GenBank. Sequence analysis was done using web available programs at NCBI and DDBJ. The sequences were compared to check the variations and restriction sites, using NEBcutterV.2. Molecular weight and isoelectric point calculations were performed by using Swissprot online software tools (Hitachi Software, Yokohama, Japan). Peptide mass, %age composition of amino acids and stability of protein was found using Protparam. Three dimensional structure of deduced amino acid sequence was generated using ITESSER.

References

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