

## Characterization of an Indigenous *Serratia marcescens* Strain TRL Isolated From Fish Market Soil and Cloning of its *chiA* gene

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**Abstract:** Chitinases are of mammoth biotechnological importance. Firstly, these enzymes may be used to change chitin-containing biomass into functional (depolymerised) components. Secondly, these may be subjected for the control of fungal and insect pathogens of plants. Thus, in this study, an attempt was made to isolate and characterize potential chitinolytic bacteria from fish market soil samples. A total of 10 chitin hydrolyzing strains were isolated whose color ranged from white to red with distinct colony shapes. Morphological, biochemical and physiological characteristics of a potential chitinolytic isolate revealed it is *Serratia marcescens*. A comparative investigation of 16S rRNA gene sequence of TRL strain confirmed its identity as *Serratia marcescens*. From this strain Chitinase A gene (*chiA*) was PCR amplified, ligated in pTZ57R/T vector and transformed in *Escherichia coli* DH5 $\alpha$ . Cloned product was sequenced and submitted into GenBank under accession number KM044038. In future expression studies of this gene will be studied so that it can be exploited in the field of agriculture, medicine, waste management and industrial applications.

**Key words:** *Serratia marcescens* • Chitinase Genes • Fish Market • Cloning of ChiA Genes

### INTRODUCTION

Chitin, a 1, 4- $\beta$ -linked polymer of N-acetyl- $\beta$ -D-glucosamine (GlcNAc) is the most abundant polymer of a natural organic compound after cellulose [1]. It is widely dispersed in the structural components of many organisms that include exoskeleton of insects and crustaceans and it also occurs in the cell walls of a variety of fungi [2, 3]. Chitinases play an imperative role in the breakdown of chitin and potentially in the utilization of chitin as a renewable resource. Production of these enzymes is prevalent in a variety of organisms such as bacteria, plants, fish [4-6]. These enzymes have received a great biotechnological attention for being exploited in the field of medicine, agriculture, waste management, industrial applications, food quality enhancers [7-9].

*Serratia marcescens*, a Gram-negative bacterium, produces an array of extracellular enzymes including chitinases. In the presence of chitin this gram-negative soil bacterium produces at least three chitinases: ChiA, ChiB, ChiC, a chitinase and a chitin-binding protein (CBP21) [10, 11]. All these three chitinases belong to family 18 of the glycoside hydrolases [12]. In the present

study we have isolated and characterized a potential chitinolytic *Serratia marcescens* TRL from soil sample collected from a fish market. The bacterium was found to produce a chitinase enzyme. PCR with Chitinase A gene (*chiA*) primers have shown the presence of *chiA* gene. This PCR amplified gene was cloned and sequenced. In future expression studies of this gene and its application in different aspects of biotechnology will be studied.

### MATERIALS AND METHODS

#### Sample Collection and Isolation of Chitinolytic Bacteria:

A total of 30 soil samples were collected from fish market of Tamil Nadu Erode district for the isolation of chitin degrading bacteria. The samples were collected in a sterile ziplock bag and brought to the laboratory. One gram of sample was weighed and transferred to a vial containing 10 ml of sterile water and kept on a rotary shaker at 100 rpm for 30 min, to dislodge bacterial cells from the sample particles. Denser sample aggregates were then allowed to settle down for 30 min. The supernatant was diluted 10-fold and 0.1 ml ( $10^{-5}$ ,  $10^{-6}$ ,  $10^{-7}$  dilutions) was spread on

the nutrient agar medium supplemented with 0.1% (w/v) chitin (pH 7.5). These plates were incubated at 37 °C for 24 hrs. Colonies with zone of clearance were selected, subcultured, purified by quadrant streaking stored on nutrient agar slants at 4 °C. The bacterial isolate TRL which exhibited a large zone of hydrolysis on chitin nutrient agar medium was taken for further study.

**Characterization of Isolated Bacteria:** Morphological, biochemical and physiological characteristics of the potential chitinolytic isolate was studied according to Bergey's Manual of Systematic Bacteriology [13]. Biochemical tests such as fermentation of carbohydrates, utilization of citrate, decarboxylation of lysine, deamination of phenylalanine, arginine dihydrolase; decomposition of urea; nitrate reduction; production of indole and acetyl methyl carbinol was performed. The ability of the bacterial isolate to grow at temperatures of 5°C, 30°C, 50°C and 60°C and its growth in 2%, 4% and 8% of NaCl was also studied.

**Molecular Characterization of the Chitin Degrading Bacterial Strain:** Genomic DNA was extracted from bacterial isolate TRL using HiPurA™ Bacterial and Yeast Genomic DNA Purification Spin Kit (Himedia). To determine the sequence of the 16S rRNA gene, a DNA fragment of approximately 1.5-kb was amplified by PCR from the genomic DNA of the sample using universal eubacteria-specific primers: 27F (5-AGAGTTTGATCMTGGCTC AG-3) and 1492R (5-GGYTACCTTGTTACGACTT-3), synthesized at Xcelris Labs Ltd. For polymerase chain reactions (PCR), 0.1 µg of total DNA from isolate was mixed with a solution containing each primer at a concentration of 1 µM, 0.25 mM dNTPs and 2.5 units of Taq polymerase (Genei Bangalore) in PCR buffer with 2.0 mM MgCl<sub>2</sub>. PCR amplification was performed in an Eppendorf thermal cycler using the program: a 5 min denaturation step at 94 °C, 30 amplification cycles of 1 min at 94 °C, 1 min at 56 °C and 1 min at 72 °C, with a final extension step of 10 min at 72 °C. The amplified PCR product was purified using GeneJET™ PCR purification kit (Fermentas life science) and sequenced by automated sequencer (ABI 3730xl Genetic) at Xcelris Labs Ltd. The forward and reverse sequences were edited using Bioedit program [14]. The sequence obtained from strain TRL was compared to 16S rRNA gene sequences available in the databases of the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/>) by BLASTN homology search [15]. Phylogenetic analysis

included the 16S rRNA gene sequences of the local isolate TRL and the reference strains *Serratia marcescens* obtained from Gen Bank. *Enterobacter cloacae* VS3A (KJ162238) was used as an outgroup [16]. The evolutionary history was inferred using the Neighbor-Joining method [17]. Cluster support was assessed through 1,000 bootstrap replicates [18]. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distance was computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site [19]. The analysis involved seventeen nucleotide sequences. All positions containing gaps and missing data was eliminated. There were a total of 1366 nucleotides positions in the final dataset. A Phylogenetic and Evolutionary analysis was conducted in MEGA5 [20]. This sequence was submitted in GenBank and accession number was obtained.

**Enzyme Production:** The chitinase was produced using the enzyme production medium consisting of 0.8% nutrient broth, 1% malt extract, 1% peptone, 0.5% chitin and 0.1% NaCl [21]. For the study of chitinase production 100ml of medium was poured into 500ml of Erlenmeyer flask capacity and was sterilized at 121 °C for 15 minutes. After cooling 0.5ml of stationary phase culture of strain TRL was inoculated and incubated on shaker at under shaking condition for about 8 days and culture was allowed to stand overnight. The pellet was centrifuged at 10,000g for 20 min. Then the precipitate was dissolved in a small amount of 20 mM citrate phosphate buffer (pH 7.8) and extensively dialyzed against the same buffer. The dialysate was used for chitinolytic activity [22].

**Determination of Protein Content and Assay of Chitinolytic Activity:** The protein concentration was determined by taking bovine serum albumin as standard [23]. Chitinase activity was measured with colloidal chitin as a substrate. The reaction mixture containing 0.5 ml of 1% (w/v) colloidal chitin in 0.1 M citrate phosphate buffer (pH 5.0) and 0.5 ml of the crude enzyme was incubated at 30 °C for 1 h at 120 rpm. The reaction was stopped with the addition of 3 ml of DNS followed by heating at 100 °C for 5 min. Following centrifugation at 300rpm, the concentration of the reducing sugar in the supernatant was determined using the modified DNS method. The absorption of the appropriately diluted test sample was measured at 540 nm using a UV spectrophotometer along with substrate and enzyme

blanks. One enzyme unit was defined as the amount of enzyme that produces 1 mol of reducing sugar per hour under reaction conditions [24, 25].

#### Screening for the Presence of Chitinase Genes:

Total cellular DNA isolated from indigenous Chitinolytic strain TRL was used for identification of the chitinase A gene. Primers (5'-GGAATCACATATGCGCAATAATTTAA-3'; 5'-GCAACCGATTATTGAACGCCGG-3') designed for the detection of *chiA* gene was used as described by the authors [26]. The PCR amplification was carried out in an Eppendorf thermal cycler in a 25µL reaction volume containing 100 ng DNA, 0.5 mM of primers, 100 mM deoxynucleoside triphosphate, 1XTaq DNA polymerase buffer and 3U Taq DNA polymerase (Genei Bangalore). After confirmation of the size of the amplicon, the amplified PCR product was purified using GeneJET™ PCR purification kit (Fermentas life science).

#### Cloning and Sequencing of Chitinase Gene:

InsTAclone™ PCR Cloning Kit (Fermentas Life Science) was used for cloning of purified PCR products. The PCR amplified *chiA* product was ligated in pTZ57R/T vector as per instructions in user manual. The recombinant vector pTZ57R/T-*chiA* was transformed in *Escherichia coli* DH5α. Positive clone was identified by blue white screening. The nucleotide sequence of gene *chiA* in pTZ57R/T-*chiA* construct was confirmed by PCR and restriction digestion. Complete sequencing of *chiA* gene was performed using an automated DNA sequencer (ABI 3730xl Genetic) at Xcelris Labs Ltd. The forward and reverse sequence was edited using Bioedit program and blast was performed using BLASTN [14]. This sequence was submitted in Gen Bank and accession numbers was obtained.

## RESULTS

**Bacterial Soil Isolate:** A total of 10 chitin hydrolyzing strains were isolated from the fish market soil samples. The color of the colonies ranged from white to red. Each colony had a distinct shape. A strain which produced a clear zone with larger diameter was selected for further studies. This potential chitinolytic strain TRL was found gram negative, rod shaped bacterium, flagellated, motile, non endospore former. The colony morphology was found red pigmented convex,

transparent in nature. The biochemical tests showed indole negative, methyl red negative, vogues proskauer positive and citrate positive and hydrogen sulphide gas production negative (Table 1). Blast analysis of partial sequence of 16S rRNA gene from strain TRL showed 99% similarity with the *Serratia marcescens*. Following the physiological and biochemical characteristics and comparison of its 16S rRNA gene sequence, the selected strain was identified as *Serratia marcescens*. The partial 16S rRNA gene sequence of the selected bacterial strain obtained in this study was deposited in the GenBank nucleotide sequence database under the accession number KM044037.1 (Figure 1).

**Enzyme Production and Activity:** Our results showed that the partially purified enzyme hydrolyzed colloidal chitin which indicates the presence of chitinases in purified enzyme preparation. During hydrolysis the enzyme released reducing sugars from the colloidal chitin.

#### Effects of Incubation Time on Chitinase Production:

*Serratia marcescens* produced the highest chitinase after 2 days of incubation at 30°C on a rotary shaker (200 rpm). Enzyme levels remained constant during the third day of incubation. However, chitinase production started to decline, thereafter, this being perhaps due to the lack of nutrients in the medium.

#### Cloning and Sequencing of Chitinase Genes:

InsTAclone™ PCR Cloning Kit (Fermentas Life Science) was used for cloning of purified PCR products of chitinase (A) gene. The PCR amplified *chiA* sequences was ligated in pTZ57R/T vector as per instructions in user manual. The recombinant vector pTZ57R/T-*chiA* was transformed in *Escherichia coli* DH5α. Positive clones were identified by blue white screening. The recombinant plasmids were isolated and double digestion with *Bam*H1 and *Eco*R1 released the expected products. The nucleotide sequence of *chiA*, gene from the recombinant construct pTZ57R/T-*chiA* of positive clone was sequenced using an automated DNA sequencer (ABI 3730xl Genetic) at Xcelris Labs Ltd. The edited forward and reverse sequence of *chiA* gene when blasted showed 99% identity with *chiA* gene (Accession no. Z362941) of *Serratia marcescens* strain and sequence was submitted in Gen Bank under accession numbers KM044038.

Table 1: Morphological, biochemical and physiological characteristics of the potential chitinolytic isolate

S.No.	Property	Strain TRL	S.No	Property	Strain TRL
1	Colony Morphology	Red colored, Moist round	10	Lysine decarboxylase	-
2	Gram stain	-	11	H2S production	-
3	Acid from Glucose	+	12	Decomposition of urea	-
4	Acid from Sucrose	+	13	Catalase production	+
5	Acid from maltose	+	14	Oxidase	-
6	Acid from lactose	-	15	Indole production	-
7	Acid from Mannital	+	16	Methyl red	-
8	Citrate utilization	+	17	Voges-Proskauer	+
9	Phenylalanine deaminase	-	18	Mortality	+
Identified as				Serratia marcescens	

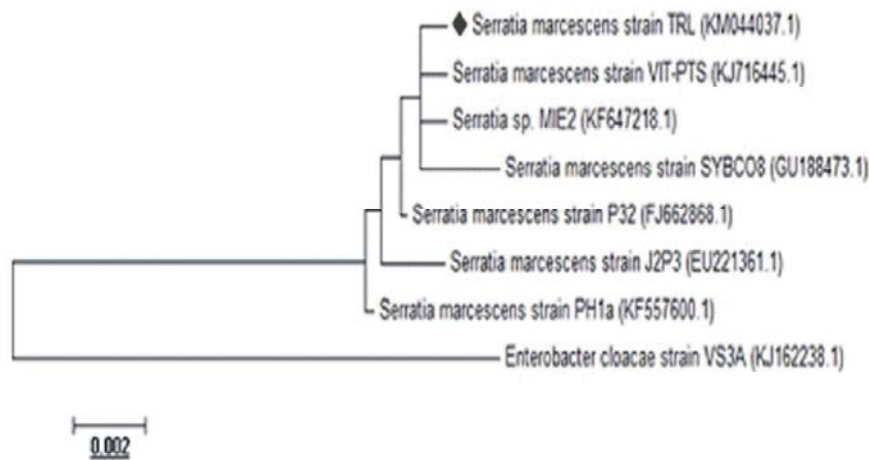


Fig. 1: The gene sequence of strain TRL was deposited in the National Center of Biotechnology Information, Bethesda, United States and the number was obtained under the gene bank account number KM044037.1. The phylogenetic tree was drawn by multiple sequence alignment with neighbour joining method. The sequences were submitted to tree topology online tree construction tool A.N.B institute, Russia and strain TRL showed 97% similarities with that of Serratia marcescens.

### DISCUSSION

Screening is one of the most efficient and successful ways of searching for new or suitable microbial enzymes [27]. A potential chitinase producing strain was reported from the wastewater of shrimp culture ponds in southern Iran [21]. In present study soil samples were collected from fish market keeping in mind bacteria present in chitin rich areas may over produce chitinolytic enzymes with more efficacy and activity due to selection and environmental stress. A total of 10 chitinolytic strains were isolated. Among these one isolate which has shown highest zone of clearance in a chitin based medium was identified as *S. marcescens* TRL based on its morphology, physiological tests and the 16S rRNA gene sequence. Now-a-days conventional characterization of bacteria which is based on phenotypic traits like biochemical

testing, cellular fatty acid analysis and numerical analysis, is considered to be more reliable along with 16S rRNA gene sequencing. Phylogenetic analysis including the sequence of the isolated bacterial strains 16S rRNA gene sequencing which provides, in many instances, useful information at the species level was also performed. *Serratia marcescens* produced the highest chitinase after 2 days of incubation at 30 °C. Enzyme levels remained constant during the third day of incubation. However, chitinase production started to decline, thereafter, this being perhaps due to the lack of nutrients in the medium [29]. In this report, the optimization of chitinase production by *Serratia marcescens* TRL was described. It was found to produce highest levels of chitinase during second day of growth. A similar chitinolytic activity has been observed earlier in a soil bacterium, *Serratia marcescens* B4A in two days at 30 °C and in

*Aeromonas schubertii* on chitin based media after 72 hours of incubation [30]. In the Antarctic bacterium, *Sanguibacter antarcticus* KOPRI 21702, maximal chitinase activity was reported to take place past 40 hours of incubation [31]. The major reasons for a decline in product formation subsequent to the optimum incubation time might be due to the abridged levels of nutrients in the culture medium and/or denaturation of chitinase by proteases [32]. The influence of the carbon sources on chitinase production demonstrated that monosaccharides inhibited chitinase biosynthesis. Inhibition of chitinase production in the presence of glucose and other simple sugars might be due to catabolite repression. These results are in concurrence with those of Mandana *et al.* [21]. The growth of microorganisms was found to soar in the presence of glucose and galactose, but chitinase production was at its lowest. In our study addition of glucose to the chitin-containing medium reduced the chitinase activity which was similar to the findings regarding chitinase production by *Streptomyces* sp. [33]. The influence of nitrogen sources on chitinase biosynthesis illustrated malt extract to be the most favorable nitrogen source for the production of chitinase. Evaluation of the results revealed that organic nitrogen sources increase chitinase activity considerably relative to inorganic ones. In corroboration of our findings, comparable observations have been reported in the case of *Serratia marcescens* B4A and *Streptomyces* sp. Da11 [34]. *Serratia marcescens* produces battery of chitinases (ChiA, ChiB and ChiC) which collectively facilitate the bacterium to proficiently degrade the insoluble chitin polymer. These enzymes vary in terms of their architecture and their competence towards chitinous substrates. Three Chitinases from *Serratia marcescens* 2170 had been cloned and expressed in *Escherichia coli*, their enzymatic properties and synergism on chitin degradation had been studied [10]. In present study Chitinase (A) was PCR amplified and cloned into *Escherichia coli* DH5 $\alpha$ . The recombinant plasmid was isolated and sequenced at Xcelris labs. Forward and reverse sequences were edited using bioedit software and the sequence was compared with already submitted sequences in NCBI using Blastn. This has shown 99% similarity with the already reported enzyme.

### CONCLUSION

An increase in knowledge of the chitinases and mechanisms involved in their degradation of insoluble abundant polymer chitin will play an important role in the

agriculture, medicine, nanotechnology etc. Thus Cloning and expression of chitinase gene from new species can be a big boost to the biotechnology. In future expression studies of this gene will be studied.

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