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# Cloning and Expression of Variant *chi*A from Native *Serratia marcescens* AUDS227

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***Serratia marcescens*** **a gram-negative and one of the most effective chitinolytic bacterium used as a model to study the degradation of chitin. The present study was carried out to identify the variant *chi*A in comparison with the reference *Sm*141*chi*A and to study the expression of variant *chi*A in *E. coli* and to identify the bioefficacy of variant *chi*A against the fungal pathogens. Sixty native isolates of *S. marcescens* were screened to check their chitinolytic activity in comparison with the reference strain *Sm*141. In order to find out the variability amongst *chi*A, PCR-RFLP analysis of native *chi*A positive isolates was done along with the reference *Sm*141*chiA*. The variant *Sm*AUDS227*chiA* identified through PCR-RFLP was cloned in pTZ57R/T and expressed in *E. coli* BL21 (DE3) by subcloning into prokaryotic expression vector pET32C$^{+}$ with a molecular mass of ~79.86 kDa. The extracted protein from the cloned *Sm*AUDS227*chi*A was found effective to inhibit the fungal pathogens viz. *R. solani and S. rolfsii.***

**Key words:** *Serratia marcescens*, chitin, chitinases, PCR-RFLP, antifungal activity.

*Serratia marcescens* is a gram-negative and soil dwelling facultative anaerobe and well known for its effective mechanism for degradation of chitin[1, 2]. Chitin is a homopolymer of N-acety l-D-glucosamine (GlcNAc) residues linked by β-1, 4 bonds and widely distributed in nature as a constituent of insect's exoskeleton, shells of crustaceans and fungal cell walls[3]. Chitinases (EC 3.2.1.14) are the enzymes which hydrolyze the β-1, 4 linkages in the insoluble chitin microfibril that are extensively distributed among plants, most fungi, yeasts and some prokaryotes and are required for morphogenesis of cell wall and exoskeletons[4]. Chitinases are classified as endochitinases- that cleave chitin chain polymer randomly at internal sites and exochitinases that catalyze the progressive release of diacetylchitobiose starting at the non-reducing ends of chitin chains[5]. Chitinases have a wide-range of applications such as preparation of pharmaceutically important chitooligosaccharides and N-acetyl D-glucosamine, single-cell protein, isolation of protoplasts from fungi and yeast, control of pathogenic fungi, treatment of chitinous waste[6].

Studies by number of groups clearly showed that *S. marcescens* produces at least three chitinases (ChiA, ChiB and ChiC), a chitobiase and a putative chitin-binding protein (CBP21). It represents the chitinolytic machineries of the bacterium[7]. The genes encoding chitinases from different strains of *S. marcescens* have been cloned and expressed in *E. coli* for characterization and increased enzyme production as well as in other bacteria such as *Bacillus thuringiensis* to increase their pesticidal activity.[8]

Due to its wide range of biotechnological applications especially in agriculture, chitinases have received increased interest for biocontrol of phytopathogenic fungi and harmful insects. The

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present study describes the cloning and expression of variant *chi*A from native *S. marcescens* AUDS227 and functional validation of variant *chi*A against the fungal pathogens.

## MATERIALS AND METHODS

During this study, sixty native isolates of *S. marcescens* isolated from the soils of Western Ghats of India in the Dandeli region obtained from the culture collection of the Department of Biotechnology, UAS Dharwad, Karnataka were used and *S. marcescens* 141 was used as the reference strain.

**Screening the native *S. marcescens* isolates for chitinolytic activity**

Colloidal chitin was prepared by the method of Robert and Selitrennikoff [9]. Individual isolates grown in Luria broth (LB) were inoculated on chitin plates with *Sm*141 as positive control. The chitinolytic activity of the native isolates of *S. marcescens* was scored by their ability to produce a halo representing the clearance of colloidal chitin after incubation at 28± 2°C for 48 h.

**Molecular characterization of *chi*A**

The genomic DNA was isolated from the native chitinolytic *S. marcescens* isolates by the protocol of Sambrook and Russell[10] and used as a template for screening of *chi*A. The *chi*A gene specific primers *chi*A-F (5'gcccatggaaggaatcagttatgcgcaaat3') and *chi*A R (5'gcggatcccaacgcactgcaaccgattat3') were designed from reported *S. marcescens* nucleotide sequences available in the NCBI database. PCR amplification of *chi*A gene was carried out in a Eppendorff Master cycler in 20 µl reaction volume containing 100 ng template DNA, 25mM $MgCl_2$, 5 pm each primer, 1 mM dNTPs, (10x) Taq assay buffer, 3U/ µl Taq DNA Polymerase (Bangalore Genie). The PCR cycling conditions were followed as: initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, followed by a final extension for a period of 10 min at 72 °C. The amplified PCR product was checked on 1 % agarose in 1 X TAE buffer.

***In silico* analysis of reference *Sm*141*chi*A**

The reference *Sm*141*chi*A (DQ990373.1) gene sequence was retrieved from the NCBI database http://www.ncbi.nlm.nih.gov. The restriction sites present in the reference were analyzed by using BTI Gene Tool software v.1.0. The nucleotide sequence of *Sm*141*chi*A (~1692bp) revealed the presence of unique restriction sites viz. *Xma*I at 948$^{th}$, *Sma*I at 950$^{th}$, *Sal*I at 526$^{th}$ and *Pst*I at 905$^{th}$ position. Based on this analysis, the restriction enzymes *Pst*I, *Xma*I, *Sma*I and *Sal*I (Bangalore Genie Pvt. Ltd. Bangalore) were selected for restriction fragment length polymorphism of native c*hi*A.

**RFLP of *chi*A positive isolates**

Based on the chitinolytic activity the *Sm*141*chi*A, *Sm*AUDS227*chi*A, *Sm*AUDS274*chi*A and *Sm*AUDS365*chi*A (~1692bp) of 25 ng/µl were restricted with *Pst*I, *Sal*I, *Sma*I and *Xma*I enzymes at 10 U/µl separately. The restriction reaction was incubated at 37°C for 1.30 h and the restricted products were separated on 1.2 per cent low melting agarose with λ *Hind*III */Eco*RI double digest as DNA molecular weight marker and 100 bp ladder (Bangalore Genie Pvt. Ltd. Bangalore).

**Cloning of *Sm*AUDS227*chi*A**

Based on the chitinolytic activity and difference in RFLP profile of the *Sm*AUDS227*chi*A with the reference *Sm*141*chi*A, the *Sm*AUDS227*chi*A (~1692 bp) was cloned into pTZ57R/T (~2886 bp) cloning vector (MBI, Fermentas, USA) and transformed to *E. coli* DH5á. The clones containing recombinant molecules were selected based on blue-white assay, confirmed through PCR amplification by using *chi*A specific primers and by restriction analysis using *Eco*RI and *Hind*III. The *Sm*AUDS227*chi*A was subcloned into prokaryotic expression vector pET32C$^+$ (Novagen, Germany) followed by standard recombination technique downstream to T7 promoter. The ligated mixture was transformed into competent *E. coli* BL21 (DE3) (Novagen, Germany) and confirmation was also done by restriction analysis.

**Induction and expression of *Sm*AUDS227*chi*A**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in gels containing 12% acrylamide and 0.1% SDS using a discontinuous Tris–glycine buffer system[11]. The crude protein concentration was determined by the Lowry's method[12] and subjected to SDS-PAGE analysis. The molecular mass of the crude protein was determined by calculating the relative mobility of standard protein molecular

weight markers 10 to 250 kDa (Page ruler™ plus prestained protein ladder '"SM1811 (Thermo Scientific, USA).

**Antifungal activity of *Sm*AUDS227*chi*A**

Antifungal activity of the *Sm*AUDS227*chi*A was determined using the agar-disc diffusion method. The phytopathogen *Rhizoctonia solani* and *Scelrotium rolfsii* were obtained from the culture collection of the Department of Biotechnology, UAS Dharwad, Karnataka. The 5 mm agar discs of fungal pathogens were placed on the center of the Potato Dextrose Agar (PDA) plates and incubated at 28±2°C for 48 h separately. After 48 h of incubation, the induced protein of 100 µg/µl was extracted from pSBK201B construct carrying in *E. coli* BL21 (DE3) and spotted separately on 5 mm of Whatman paper No. 3 against the fungal pathogens.

**Sequencing of *Sm*AUDS227*chi*A clones**

For sequencing, the clones (pTZ57R/T plus *Sm*AUDS227*chi*A) were selected on the basis of efficacy of expression in pET32C$^{+}$. The corresponding pTZ57R/T plus *chi*A clones were sequenced using M13 F- 5'gtaaaacgacggccagt 3' and R- 5' caggaaacagctatgac 3' primers and further primer walking by designing internal primers at Ocimum Biosolutions, Hyderabad. After sequencing, the complete nucleotide sequences of *Sm*AUDS227*chi*A and the reference *Sm*141*chi*A were assembled in BIO EDIT software v. 7.1.3 and subjected to *in silico* analysis using BTI Gene Tool software v. 1.0 for ORF finding, restriction site and *in silico* translation. BLAST analysis was done with the DNA sequences obtained by available online nucleic acids data base at http://www.ncbi.nlm.nih.gov. The pair-wise alignment of amino acid sequences *Sm*AUDS227*chi*A and *Sm*141*chi*A (reference) was done using BTI Gene tool software v. 1.0.

**Nucleotide sequence accession number**

The nucleotide sequence of the *Sm*AUDS227*chi*A was deposited in the NCBI Genbank under the accession No. HQ699804.

## RESULTS AND DISCUSSION

Chitin is a β-1, 4-linked polymer of *N*-acetyl glucosamine (GlcNAc), is an important structural component of insects, fungi and nematodes[13]. Hydrolysis of chitin to disaccharides

**Table 1.** Chitinolytic activity of native isolates of *Serratia marcescens* from Dandeli region of Western Ghats of India

| S. No. | Isolate | Zone of hydrolysis (mm) |
|---|---|---|
| 1 | AUDS190 | 2 |
| 2 | AUDS191 | 2 |
| 3 | AUDS192 | 2 |
| 4 | AUDS220 | 4 |
| 5 | AUDS221 | 2 |
| 6 | AUDS224 | 2 |
| 7 | AUDS226 | 4 |
| 8 | AUDS227 | 5 |
| 9 | AUDS228 | 4 |
| 10 | AUDS229 | 3 |
| 11 | AUDS230 | 2 |
| 12 | AUDS231 | 2 |
| 13 | AUDS232 | 3 |
| 14 | AUDS241 | 3 |
| 15 | AUDS242 | 5 |
| 16 | AUDS243 | 5 |
| 17 | AUDS256 | 4 |
| 18 | AUDS257 | 2 |
| 19 | AUDS258 | 2 |
| 20 | AUDS259 | 4 |
| 21 | AUDS260 | 2 |
| 22 | AUDS261 | 3 |
| 23 | AUDS263 | 5 |
| 24 | AUDS264 | 5 |
| 25 | AUDS265 | 5 |
| 26 | AUDS266 | 4 |
| 27 | AUDS267 | 2 |
| 28 | AUDS268 | 2 |
| 29 | AUDS272 | 2 |
| 30 | AUDS273 | 3 |
| 31 | AUDS274 | 5 |
| 32 | AUDS275 | 2 |
| 33 | AUDS276 | 2 |
| 34 | AUDS278 | 2 |
| 35 | AUDS285 | 4 |
| 36 | AUDS286 | 3 |
| 37 | AUDS287 | 3 |
| 38 | AUDS288 | 5 |
| 39 | AUDS294 | 2 |
| 40 | AUDS307 | 2 |
| 41 | AUDS308 | 2 |
| 42 | AUDS309 | 2 |
| 43 | AUDS311 | 2 |
| 44 | AUDS312 | 2 |

| | | |
|---|---|---|
| 45 | AUDS365 | 5 |
| 46 | AUDS366 | 3 |
| 47 | AUDS367 | 2 |
| 48 | AUDS368 | 3 |
| 49 | AUDS370 | 3 |
| 50 | AUDS409 | 2 |
| 51 | AUDS515 | 2 |
| 52 | AUDS518 | 2 |
| 53 | AUDS531 | 4 |
| 54 | AUDS532 | 2 |
| 55 | AUDS533 | 2 |
| 56 | AUDS534 | 4 |
| 57 | AUDS643 | 2 |
| 58 | AUDS644 | 4 |
| 59 | AUDS645 | 2 |
| 60 | AUDS646 | 2 |

and larger oligomeric saccharides usually takes place extracellularly by the action of chitinases and it has been shown to play an important role in biological control of soil plant pathogens. In the present study, the *S. marcescens* isolates AUDS227, AUDS232, AUDS242, AUDS243, AUDS263, AUDS264, AUDS265, AUDS274, AUDS288 and AUDS365 were found very effective in releasing chitinase enzyme when different strains of *S. marcescens* were grown on colloidal chitin plate with a range of zone of hydrolysis of 5 mm (Table 1).

On the basis of colloidal chitin degradation assay, the highly potent isolates of *S. marcescens* were selected further for PCR-RFLP analysis to identify the presence of variant of *chi*A using an approach similar to that of Kuo and Chak[14].

**Table 2.** PCR-RFLP profile of *chi*A positive isolates with *Xma*I, *Sma*I, *Sal*I and *Pst*I,

| S. No. | Enzymes | Restriction fragment sizes in (bp) | | | |
|---|---|---|---|---|---|
| | | *Sm*141*chi*A (Reference) | *Sm*AUDS227*chi*A | *Sm*AUDS274*chi*A | *Sm*AUDS365*chi*A |
| 1 | *Xma*I | 948, 744 | 948, 744 | 948, 744 | 948, 744 |
| 2 | *Sma*I | 950, 742 | 950, 742 | 950, 742 | 950, 742 |
| 3 | *Sal*I | 1100, 526 | 1100, 526 | 1100, 526 | 1100, 526 |
| 4 | *Pst*I | 905, 787 | 787, 361, 283, 261 | 787, 361, 283, 261 | 787, 361, 283, 261 |

The PCR-RFLP typing system is a facile method to detect the organization and differential expressions both known and novel cry genes. The 14 distinct *cry*-types have been identified through PCR-RFLP from 20 *B. thuringiensis* strains[14]. The RFLP pattern of *Sm*AUDS227*chi*A, *Sm*AUDS274*chi*A and *Sm*AUDS365*chi*A generated by *Sal*I revealed the 526 bp and 1166 bp fragments, by *Xma*I 948 bp and 744 bp fragments and by *Sma*I 950 bp and 742 bp fragments which gave the similar banding pattern compared to the reference *Sm*141*chi*A. Through *Pst*I, the *Sm*AUDS227*chi*A, *Sm*AUDS274*chi*A and *Sm*AUDS365*chi*A revealed 787 bp, 361 bp, 283 bp and 261 bp fragments, which were found to be different compared to *Sm*141*chi*A. The reference *Sm*141*chi*A gave 905 bp and 787 bp fragments (Fig. 1). The restriction site and the DNA fragments generated after restriction of *Sm*141*chi*A, *Sm*AUDS227*chi*A, *Sm*AUDS274*chi*A and *Sm*AUDS365*chi*A by *Xma*I, *Sma*I, *Sal*I and *Pst*I separately are presented in (Table 2).

Based on the chitinolytic activity and difference in RFLP profile the variant *Sm*AUDS227*chi*A (~1692 bp) identified through PCR-RFLP technique was cloned into pTZ57R/T cloning vector and transformed to *E. coli* DH5á. The transformants were confirmed by PCR and restriction analysis (Fig 2 A) and labeled as pSBK101A, pSBK101B. In the similar study, *S. marcescens chi*A was PCR cloned, sequenced and heterologously expressed in an anti-coleopteran *B. thuringiensis* (strain 3023-SCHl). The specific *chi*A activity of recombinant *B. thuringiensis* was found higher compared to the source organism[8].

As pTZ57R/T is more of a cloning vector, the *Sm*AUDS227*chi*A was further sub cloned into a prokaryotic expression vector pET32C$^+$ to study the expression of cloned *chi*A in *E. coli* BL21 (DE3). The obtained recombinants were analyzed by PCR and restriction analysis (Fig. 2 B) and labeled as pSBK201A, pSBK201B. Expression of the transformants was analyzed on 12% SDS-poly-

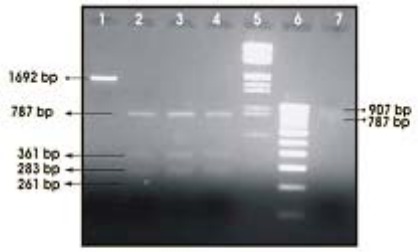


Fig. 1. PCR-RFLP pattern of *ChiA* poistive *S. marcescens* isolates

1. *Sm*141*chiA* 2. *Sm*AUDS227*chiA* with *Pst*I 3. *Sm*AUDS274*chiA* with *Pst*I 4. *Sm*AUDS365*chiA* with *Pst*I 5. Lambda DNA/ *Eco*RI +*Hind*III marker 6. 100 bp ladder.7. *Sm*141*chiA* with *Pst*I

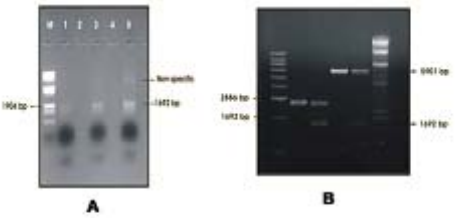


Fig. 2 PCR (A) and restriction confirmation (B) of pSBK101A and pSBK201A

(A) M Lambda DNA/EcoRI + *Hind*III marker 1. *Sm*141chiA (positive control), 2. Plain pTZ57R/T (2886bp), 3. pTZ57R/T with insert (1692bp), 4. Plain pET32C+ (5901bp), 5. pET32C+ with insert (1692bp)

(B) M1. 1 kb ladder M2. lambda DNA/*Hind*III digest 1. Plain pTZ57R/T (2886 bp) 2 pSBK101A (pTZ57RT+chiA) 3 Plain pET32C+ (5901 bp) 4 pSBK201A (pET32C+ + chiA)

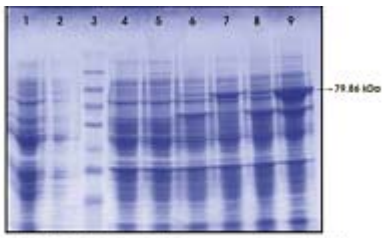


Fig. 3. SDS-PAGE analysis for expression of *chiA*

1. Uninduced *E. coli* BL21 (DE3), 2. Induced *E. coli* BL21 (DE3), 3. Page rulerTM plus prestained protein ladder # SM1811, 4. Uninduced pET32C+, 5. Induced pET32C+, 6. Uninduced pSBK201A, 7. Induced pSBK201A, 8. Uninduced pSBK201B, 9. Induced pSBK201B

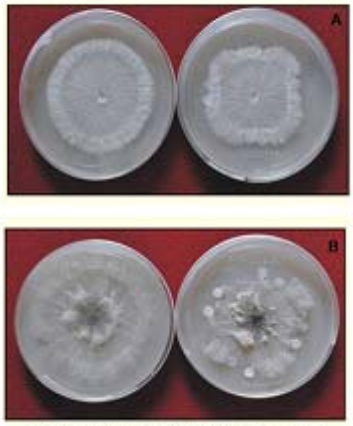


Fig. 4. Antifungal activity of chiA against A. *Sclerotium rolfsii*, B. *Rhizoctonia solani*

acrylamide gels. A unique ~79.86 kDa protein was observed in IPTG induced *E. coli* BL21 (DE3) pSBK201A, *E. coli* BL21 (DE3) pSBK201B cultures compared to control (Fig. 3). For further functional analysis of the clones, antifungal bioassay was employed.

For functional validation of the cloned *Sm*AUDS227*chi*A, the ChiA protein from the *E. coli* BL21 (DE3) pSBK201B construct was extracted and subjected for bioassay against the fungal pathogens viz. *S. rolfsii* and *R. solani* and after 12 h of incubation at 28°C the crescent shaped zone of mycelial growth inhibiton of fungal pathogens was observed. The inhibition was found more in case of *R. solani* than *S. rolfsii*. This indicates the functional expression of variant *Sm*AUDS227*chi*A. The antifungal activity of chitinases cause rapid loss of fungal hypal tips and germinating spores. These enzymes are an effective tool for the complete degradation of mycelial or conidial walls of phytopathogenic fungi. In the similar study, the chitinase from novel isolate *S. marcescens* MO-1 has shown the antifungal activity against *Alternaria citri, Fusarium oxysporum, Trichoderma harzianum, Aspergillus niger, and Rhizopus oryzae*[15].

The *in silico* nucleotide sequence analysis of *Sm*AUDS227*chi*A (~1692 bp) revealed an ORF of 1691 bp encoding 563 amino-acid residues with the first 163 amino acids represent as *N*-terminal tag. The SDS-PAGE analysis revealed the size of ChiA is ~79.86 kDa and the average molecular weight of amino acid is ~0.11 kDa. Therefore, estimated size of recombinant ChiA with 563 and 163 amino-acid residues is ~79.86 kDa with signal peptide. In the similar study, the *S. marcescens chi*A gene has been cloned by PCR into a pRSET vector and expressed in *E. coli* with the molecular mass of the protein 58 kDa, as it was found similar with the theoretical calculation of the protein without signal peptide[16].

The *Sm*AUDS227*chi*A has shown the *Pst*I site at 361$^{th}$, 644$^{th}$ and 905$^{th}$ position whereas, reference *Sm*141*chi*A nucleotide sequence has unique site for *Pst*I enzyme at 905$^{th}$ position, which were found similar to the *in vitro* studies of *Sm*AUDS227*chi*A. The pair-wise alignment of amino acid sequences of *Sm*AUDS227*chi*A and reference *Sm*141*chi*A revealed the presence of variation in the catalytic site of *Sm*AUDS227*chi*A

at 96th and 240th position. The nucleotide sequences of clones pSBK101A, pSBK101B were analyzed using BLAST algorithm available at http://www.ncbi.nlm.nih.gov. *Sm*AUDS227*chi*A has shown 99 per-cent homology with S. *marcescens* Bn10 endochitinase (DQ165083), 98 per-cent homology with *chi*60 of *Serratia*. sp. TV09 (AY040610), 98 per-cent homology with *S. marcescens* BJL200 *chi*A (Z36294) and 97 per-cent homology with the reference *S. marcescens* strain 141 *chi*A (DQ990373.1). The amino acid sequence of cloned *chi*A showed 99 per-cent homology with reported *chi*A of *S. marcescens* (ABS70983.1), *Sanguibacter* sp. C4 (ABB91448.1), *S. marcescens chi*A (ABI79317.1) and *chi*A of *S. marcescens* (ACE78180.1).

## CONCLUSIONS

The present investigation oriented on the cloning and expression of variant *chi*A from native *S. marcescens* AUDS227 and functional validation of variant *chi*A against the fungal pathogens. Based on the results, the variant *Sm*AUDS227*chi*A identified through PCR-RFLP technique was expressed in *E.coli* BL21DE3 with a molecular mass of ~79.86 kDa and the extracted protein from the cloned *Sm*AUDS227*chi*A was found effective to inhibit the fungal pathogens viz. *R. solani and S. rolfsii.*

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