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ORIGINAL ARTICLE

Cloning, characterization and expression of the chitinase gene of *Enterobacter* sp. NRG4

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Abstract A chitinase producing bacterium *Enterobacter* sp. NRG4, previously isolated in our laboratory, has been reported to have a wide range of applications such as antifungal activity, generation of fungal protoplasts and production of chitobiose and N-acetyl D-glucosamine from swollen chitin. In this paper, the gene coding for *Enterobacter* chitinase has been cloned and expressed in *Escherichia coli* BL21(DE3). The structural portion of the chitinase gene comprised of 1686 bp. The deduced amino acid sequence of chitinase has high degree of homology (99.0%) with chitinase from *Serratia marcescens*. The recombinant chitinase was purified to near homogeneity using His-Tag affinity chromatography. The purified recombinant chitinase had a specific activity of 2041.6 U mg^{-1}. It exhibited similar properties pH and temperature optima of 5.5 and 45°C respectively as that of native chitinase. Using swollen chitin as a substrate, the $K_{m,}$ k_{cat} and catalytic efficiency (k_{cat}/K_m) values of recombinant chitinase were found to be 1.27 mg ml^{-1}, 0.69 s^{-1} and 0.54 $s^{-1}M^{-1}$ respectively. Like native chitinase, the recombinant chitinase produced medicinally important N-acetyl D-glucosamine and chitobiose from swollen chitin and also inhibited the growth of many fungi.

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Introduction

Chitinase (Chi; EC 3.2.1.14) is an enzyme that cleaves β-1, 4 linkages in polymers of N-acetyl D-glucosamine. It has potential applications in the production of pharmaceutically important chitooligosaccharides and N-acetyl D-glucosamine (Patil *et al*. 2000), treatment of chitinous waste and preparation of single-cell protein (Vyas and Deshpande 1991), isolation of protoplasts from fungi (Mizuno *et al*. 1997) and yeast (Deshpande *et al*.1987), control of plant pathogenic fungi (Dahiya *et al*. 2005a) and insects (Barboza-Corona *et al*. 2003). Looking at the wide importance of chitinases, *chi* genes from many microbial spp., viz., *Serratia*, *Bacillus*, *Aeromonas*, *Vibrio, Pseudomonas, Alteromonas*, *Enterobacter*, *Clostridium*, *Streptomyces* and *Trichoderma* have been cloned and characterized (Morimoto *et al*. 1997; Felse and Panda 1999; Orikoshi *et al*. 2003)

Recently, our laboratory has isolated a Chi producing bacterium, subsequently identified as *Enterobacter* sp. NRG4 (Dahiya *et al*. 2005b). The Chi enzyme has been purified as well as characterized (Dahiya *et al*. 2005b). Purified Chi demonstrated antifungal activity (Dahiya *et al*. 2005c) and generated pharmaceutically important N-acetyl D glucosamine and chitobiose from swollen chitin (Dahiya *et al*. 2005b). In the present study, the *chi* gene of *Enterobacter* sp. NRG4 has been cloned in pET-21d(+) vector and expressed in *E. coli* BL21(DE3). The recombinant Chi was purified and characterized. The applications of the recombinant Chi in the production of N-acetyl D-glucosamine and

chitobiose from chitin and its antifungal activity were also studied.

Materials and methods

Bacterial strains and plasmids

Enterobacter sp. NRG4, a Chi producer, was isolated from soil in our laboratory (Dahiya *et al*. 2005b). The bacterial strains, *E. coli* DH5α and *E. coli* BL21(DE3) were used as cloning and expression hosts respectively. pUC18 plasmid was used as the vector for cloning the *chi* gene and pET-21d(+) was used as the expression vector.

Cloning of *chi* gene of *Enterobacter* sp. NRG4 in *E. coli* DH5α

Genomic DNA of *Enterobacter* sp. NRG4 was isolated by the standard CTAB-NaCl protocol of Ausubel et al (1992). DNA was purified using phenol-chloroform treatments and finally dissolved in TE (100µl). Genomic library of *Enterobacter* sp. NRG4 was made by ligating *Sau*IIIA partials (1.0-6.0 kb) of genomic DNA with *Bam*HI digested pUC18 using T4 DNA ligase. The ligation mixture was electroporated into *E. coli* DH5α and plated on LB agar + amp (100 µg ml^{-1}) + X-gal (40 µg ml^{-1}) + IPTG (0.5 mM) plates and incubated at 37°C for 24 h. All white colonies growing on these plates were patched on two sets of LB agar + amp_{100} + swollen chitin + IPTG plates and incubated at 37°C for 24 h. One set of the plates was saved and the other set was exposed to chloroform vapors for 15 min and further incubated overnight at 37°C. A zone of clearance around the bacterial colonies was indicative of the production of chitinase by the transformants.

DNA sequencing

The *chi* gene containing DNA fragment was subjected to forward and reverse sequencing using M13 sequencing primers by the fluorescent di-deoxyterminator method using an ABI 3100 capillary sequencer (ACTG, Inc., USA). Sequencing was done in the DNA Sequencing Facility at Department of Biochemistry, University of Delhi, South Campus, New Delhi, India. The nucleotide sequence of the *chi* gene from *Enterobacter* sp. NRG4 was submitted to Gene Bank where it appears with accession number DQ013365.

PCR amplification of *chi* gene

Using PCR technology, *chi* gene sequence cloned in a recombinant plasmid was amplified using modified primers carrying restriction sites for *Nco*I and *Xho*I. The primer sequences were as follows:

*Nco*I

Forward primer: 5′- TATACCATGGGCAAATTTAATAAACCGCTG- 3′

*Xho*I

Reverse primer: 5′- AAACTCGAG TTGAACGCCGGCGCTATTG-3′

Thermocycler (BioRad Model: MyCycler Thermocycler) was programmed for a hot start of 94°C for 1 min followed by 30 cycles at 94°C (1 min), 56°C (1 min 30 s) and 72°C (2 min) using *Taq* DNA polymerase (Fermentas). Amplified DNA fragments were purified from agarose gel using Amersham Gel Extraction Kit.

Sub-cloning of *chi* gene in expression vector, pET-21d (+)

The amplified gene products were digested with *Nco*I and *Xho*I, purified and ligated with the help of T4 DNA ligase into *Nco*I and *Xho*I cut expression vector pET-21d (+). The ligation reaction was carried out at 14°C for 16 h in a cooling water bath. An aliquot of ligation mixture was electroporated into competent cells of *E. coli* BL21(DE3). These cells were plated on LB agar plates supplemented with IPTG (0.5 mM), X-gal (40 µg ml^{-1}) and ampicillin (100 µg ml^{-1}) and incubated at 37°C for 24 h. The transformants growing on the plates were checked for Chi activity by plate assay as described earlier. All the transformants displayed Chi activity on solid medium. One transformant, labeled as *E. coli* BL21(DE3) pMRT-1 was used for further studies. The recombinant plasmid, pMRT-1 purified from this clone had the expected size of 7. 13 kb (pET vector = 5.44 kb + *chi* gene =1.69 kb).

Production and purification of recombinant Chi

Overnight grown culture of *E. coli* BL21(DE3) carrying *chi* positive recombinant plasmid was inoculated (final inoculum, 1%) in liquid broth (LB + amp_{100}) and incubated (37°C, 200 rpm) in an orbital shaker (NSW, India) till the cell density OD_{600} = 0.7 was achieved. The culture was supplemented with IPTG (final concentration, 0.1 mM) and further incubated for 3 h at 30°C. The cells were harvested by centrifugation (12,000 rpm, 10 min., 4°C) and resuspended in 20% (w/v) lysis buffer (50 mM phosphate buffer, 300 mM NaCl, 10 mM imidazole) containing lysozyme (final concentration, 0.5 mg ml^{-1}) and a protease inhibitor PMSF (final concentration, 1 mM). The cell suspension was kept in ice for 30 min and lysed by sonication (20 cycles, 30 s pulse followed by 30 s interval) using ultrasonicator (Sonics, USA). The cell lysate was centrifuged at (13,000 rpm, 4°C, 20 min) to remove cellular debris. The recom-

binant Chi was purified from the cell free supernatant by affinity chromatography procedure (Ni-NTA agarose, Qiagen, USA) using phosphate buffer (50 mM, pH 8.0) supplemented with imidazole (final concentration, 200 mM).

Chi assay

Flake chitin (HiMedia Laboratories, India) was used to prepare various forms of chitin, i.e., swollen chitin, colloidal chitin and glycol chitin by the methods of Monreal and Reese (1969), Jeuniaux (1966) and Yamada and Imoto (1981) respectively. These forms of chitin were used as substrates for Chi assay. The reaction mixture contained 1.0 ml substrate {0.5% (w/v) chitin preparation in 50 mM citrate phosphate buffer, pH 5.5} and 0.5 ml of enzyme solution. The tubes were incubated at 45°C for 15 min. The reaction was stopped by placing the tubes in boiling water bath for 5 min. The reaction mixtures were centrifuged at 5000 rpm for 10 min. The amount of N-acetyl D-glucosamine released was determined by the method of Reissig et al (1955), using N-acetyl D-glucosamine as the standard. One enzyme unit was defined as the amount of enzyme that catalyzes the release of 1 µmol of N-acetyl D-glucosamine in 1 h at 45°C. The protein concentration was measured using the method of Lowry et al (1951). Kinetic parameters K_m and V_{max} were determined by the Lineweaver-Burk representation of the Michaelis – Menten model.

Physical and biological parameters of recombinant Chi

Chi activity was assessed at different pH values and temperatures under a standard assay condition with swollen chitin as the substrate as reported earlier (Dahiya *et al*. 2005b). The enzymatic breakdown products of swollen chitin were analyzed on HPLC (Dahiya *et al*. 2005b). The amount of products formed was calculated from the peak area in the HPLC chromatogram. The percentage yield of the products was calculated as

$$\left\{\frac{\text{Amount of product formed}}{\text{Amount of substrate used}}\right\}\times 100$$

Antifungal activity tests

The antifungal activity of recombinant Chi was assayed by disc diffusion method. Briefly, 80 µl of actively growing cultures ($O.D._{600nm}$ 0.7, diluted 10^5 times) of fungal strains were uniformly spread on YEPD (yeast extract peptone dextrose) agar plates and allowed to stand for 10 min to evaporate water from the surface of the plates. Sterile filter discs were gently placed on the plates and 40 µl of the purified Chi (60 U) was loaded on these discs. A single disc on each plate was loaded with inactive Chi to serve as a control. It was prepared by keeping 2.0 ml of purified Chi in a boiling water bath for 5 min. The plates were incubated at 30°C for 24 h and zone of inhibition around the discs was observed.

Results and discussion

Cloning and expression of *chi* gene

The genomic library of *Enterobacter* sp. NRG4 was constructed in pUC18 vector and expressed in *E. coli* DH5α. Five out of 2000 transformant colonies screened were surrounded by a clear transparent zone on LB agar + amp_{100} + swollen chitin + IPTG plates, which was indicative of the Chi activity of the clones. The recombinant plasmids were isolated from all *chi*$^+$ clones and their size varied from 4.4-6.7 kb. As the size of the zone of chitin clearance around the *chi*$^+$ transfromants was nearly the same i.e. 8 mm diameter, smallest *chi*$^+$ plasmid (pNR1, 4.4 kb) containing 1.81 kb *chi*$^+$ DNA insert was used for further studies. The nucleotide sequence of this insert showed that it comprised of 1812 bp. NCBI-BLAST search of this fragment revealed a single complete ORF (1,692 bp) showing homology with the *chi*A genes of *Serratia marcescens* (97%), *Enterobacter agglomerans* (87%) and *Aeromonas caviae* (81%). A 22-bp sequence preceded the start codon in which a putative Shine-Dalgarno sequence, AAGGAA, was located 12-bp upstream from the ATG start codon. No putative promoter region could be seen in the insert suggesting that the cloned *chi* gene was being expressed by the *lac* promoter of the vector. The recombinant Chi was expressed intracellularly in *E. coli* cells although a signal peptide identical to that of *Serratia marcescens* (Jones *et al*. 1986), *Enterobacter agglomerans* (Chernin *et al*. 1997) and *Aeromonas caviae* (Sitrit *et al*. 1995) chitinases was found to be present in the amino acid sequence.

Using modified primers, the 1692 bp *chi* structural gene corresponding to ORF of Chi was amplified and ligated downstream T7 promoter of an expression vector pET-21d (+) and then electroporated into *E. coli* BL21(DE3). One *chi*$^+$ clone, labeled as *E. coli* pMRT-1, was used for purification of recombinant Chi by affinity chromatography as the recombinant Chi is a fused protein carrying poly-His tag at its carboxyl end. The purified recombinant Chi was homogenous as judged by the presence of single protein band on SDS-PAGE (Fig. 1, lane 5). The molecular weight of the recombinant Chi was found to be approximately 61 kDa (mature Chi 60 kDa + 6X His-tag 930 Da = 61 kDa approximately). The cell free supernatant obtained after

sonication of IPTG induced cells was used for purification of recombinant Chi by His-Tag affinity chromatography. The recombinant enzyme was purified 5.6 fold with enzyme recovery of 28.33% as summarized in Table 1.

Homology of the Enterobacter sp. NRG4 Chi

The deduced 563 amino acid sequence of the *chi* gene in the recombinant plasmid showed high similarity to family 18 chitinases. Using BLAST search, maximum homology was found to be with ChiA of *Serratia marcescens* (99%) followed by *Enterobacter agglomerans* (88.3%), *Aeromonas hydrophila* (75%), *Alteromonas* sp. O-7 (49.7%) and *Vibrio alginolyticus* (49.6%). A phylogenetic tree showing the evolutionary relationship of Chi from *Enterobacter* sp. NRG4 with chitinases from different organisms is shown in Fig. 2.

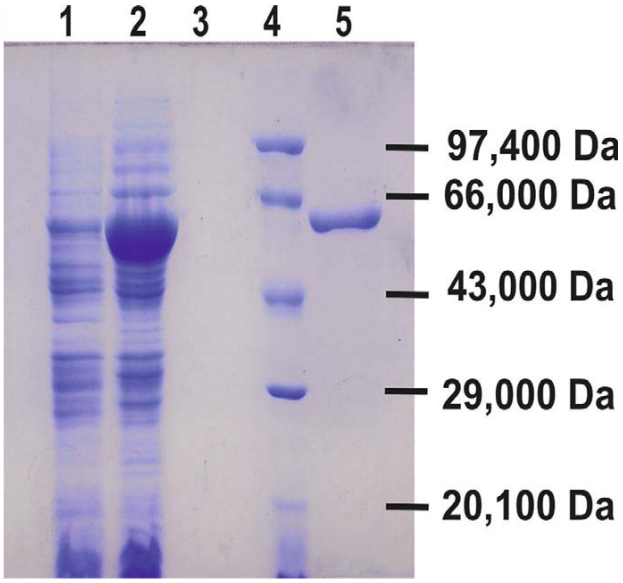

Fig. 1 SDS-PAGE analysis of expressed recombinant Chi. Lane1, Noninduced culture; Lane 2, Induced culture; Lane 4, Protein MW Marker; Lane 5, Purified recombinant Chi.

Enzyme characteristics of the recombinant Chi

The recombinant Chi showed similar enzymatic characteristics (physical as well as biological) as that of native Chi of *Enterobacter* sp. NRG4 (Dahiya et al 2005b). The enzyme was found to retain over 90% of activity over a wide range of pH ranging from 4.5–8.0 with optimum pH of 5.5. The optimum temperature was found to be 45°C. The enzyme was stable at 45°C for an hour and after 3 h at 45°C, about 80% of maximum activity retained. These values are quite similar to those observed with *Enterobacter* sp. NRG4 Chi. The K_m and V_{max} values of the purified recombinant Chi using different substrates at its optimum conditions were 1.27 mg ml^{-1} and 35.71 µmol $mg^{-1}min^{-1}$ for swollen chitin, 1.43 mg ml^{-1} and 31.25 µmol $mg^{-1}min^{-1}$ for colloidal chitin and 1.92 mg ml^{-1} and 13.8 µmol $mg^{-1}min^{-1}$ for glycol chitin, respectively. The corresponding values for native Chi were 1.42 mg ml^{-1} and 83.33 µmol $\mu g^{-1}h^{-1}$, 1.40 mg ml^{-1} and 74.07 µmol $\mu g^{-1}h^{-1}$ and 2.0 mg ml^{-1} and 33.33µmol $\mu g^{-1}h^{-1}$, respectively (Dahiya *et al*. 2005b). The slight differences in the kinetic parameters recorded for the native and the

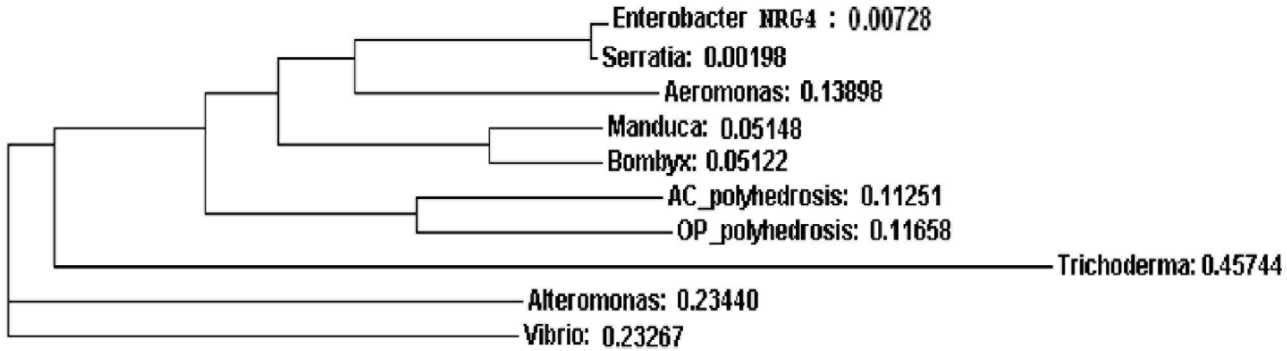

Fig. 2 Phylogenetic tree showing the relationship of the chitinases of *Enterobacter* sp. NRG4, *Serratia marcescans*, *Aeromonas caviae*, *Manduca sexta*, *Bombyx mori*, nucleopolyhedrovirus NPVAC, nucleopolyhedrovirus NPVOP, *Alteromonas* sp. strain O-7, *Vibrio alginolyticus* and *Trichoderma harzianum*. The tree distance value for each organism is given in brackets.

Table 1 Purification profile of the recombinant Chi

	Total Volume (ml)	Total Enzyme activity (U)	Total Protein content (mg)	Specify activity (U mg^{-1})	Purification (Fold)	Percentage recovery (%)
Crude Sample	10.0	3600.0	9.8	367.35	–	100.0
Affinity	10.0	1020.81	0.5	2041.6	5.6	28.33

1U was defined as the amount of enzyme that catalyzes the release of 1 µmol of N-acetyl D-glucosamine in 1h at 45°C.

recombinant Chi could be due to the presence of the His-tag fused to the recombinant enzyme as suggested by Lan *et al*. (2006). The turnover number (k_{cat}) and the catalytic efficiency (k_{cat}/K_m) values determined for the recombinant Chi using the three different substrates showed that the enzyme has more affinity for the swollen chitin than colloidal chitin and glycol chitin (Table 2).

Production of N-acetyl D-glucosamine and chitobiose

HPLC data revealed chitobiose $(GlcNAc)_2$ and N-acetyl D-glucosamine (GlcNac) products after treatment of swollen chitin with recombinant Chi (Fig. 3). The amount of chitobiose and N-acetyl D-glucosamine released were 32.52 mM (71.8%) and 2.44 mM (5.4%) respectively, from 10 mg ml^{-1} swollen chitin on treatment with 10 U ml^{-1} Chi for 5h. These results confirmed the similarity of recombinant Chi with native Chi of *Enterobacter* sp. NRG4.

Anti-fungal activity

Recombinant Chi displayed antifungal activity on plate by disc diffusion method against *Aspergillus niger, Fusarium* sp*., Mucor* sp. as reported earlier for native Chi of *Entero-*

Table 2 Kinetic parameters of purified recombinant Chi

Substrates	K_m (mg ml^{-1}) ± SE	V_{max} (μ mol $mg^{-1}min^{-1}$) ± SE	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}M^{-1}$)
Swollen chitin	1.27 ± 0.04	35.71 ± 1.79	0.69	0.54
Colloidal chitin	1.43 ± 0.03	31.25 ± 2.89	0.59	0.41
Glycol chitin	1.92 ± 0.21	13.8 ± 4.37	0.26	0.13

Note: Each value represents the mean of three independent experiments. The data show the value ± standard error of mean (SEM).

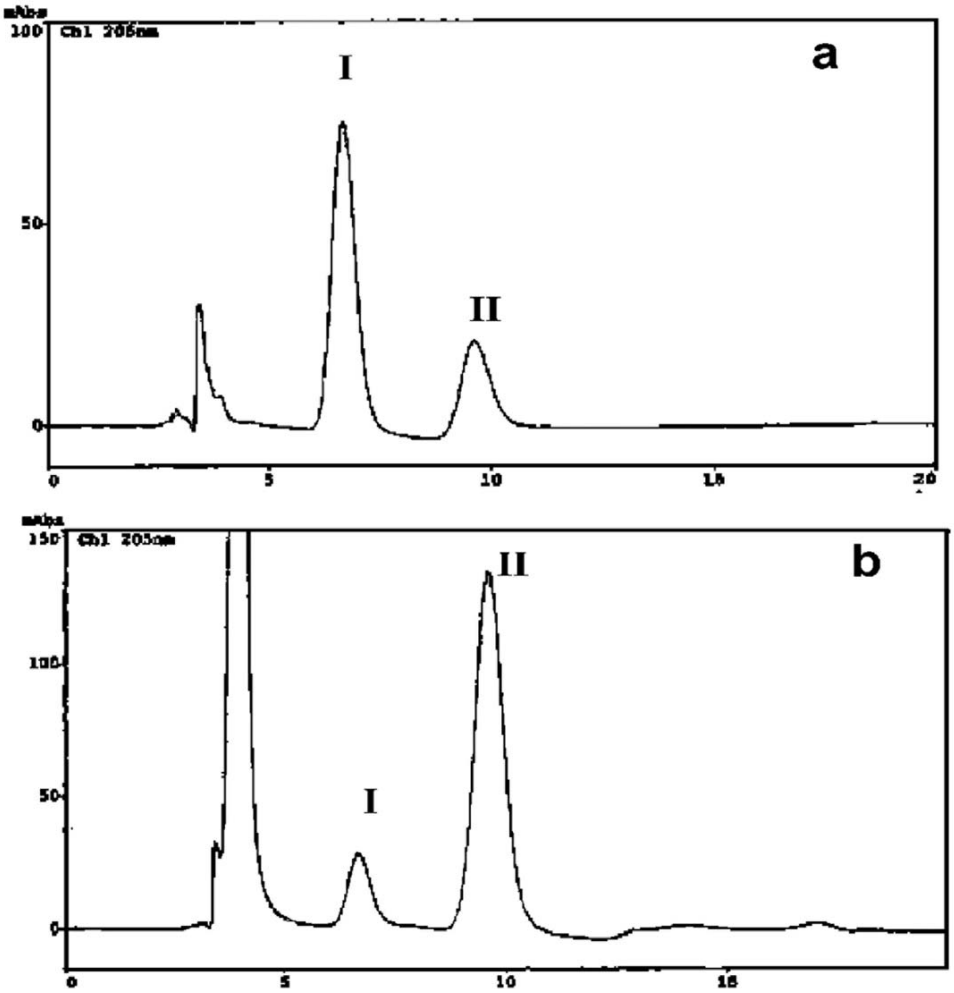

Fig. 3 HPLC analysis of the hydrolysis products of swollen chitin by purified recombinant Chi. (a) Standards: (I) N-acetyl D-glucosamine, (II) Chitobiose. (b) Hydrolyzed products of chitin after 5 h of enzyme-substrate reaction (10 mg ml^{-1} swollen chitin + 10 U ml^{-1} recombinant Chi).

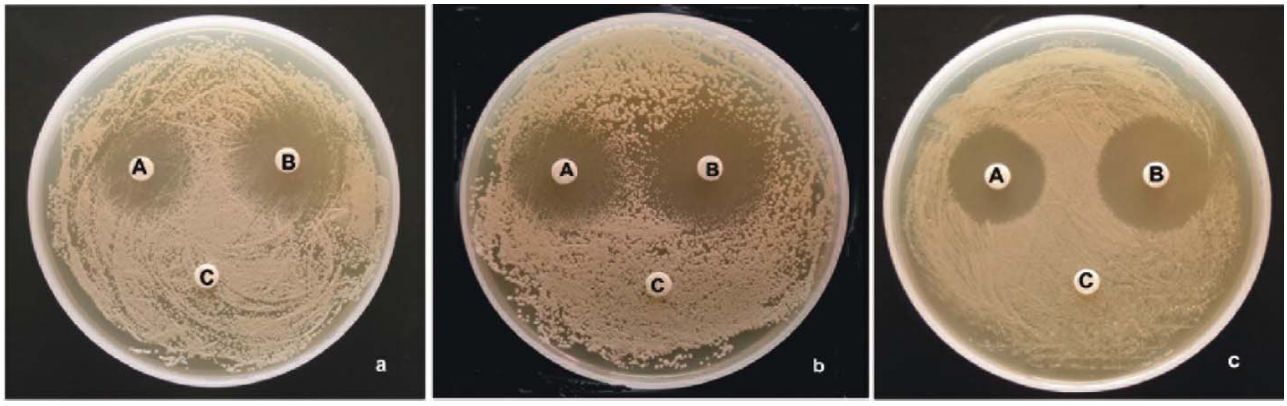

Fig. 4 Antifungal activities of purified recombinant Chi against (a) *Candida albicans*, (b) *Candida tropicalis*, (c) *Candida kruseii.* (A) Purified wild type Chi, (B) purified recombinant Chi, (C) negative control (5 min boiled preparation of the recombinant Chi).

bacter sp. NRG4. Both types of chitinases, native and recombinant also showed antifungal activity against *Candida albicans*, *Candida tropicalis* and *Candida kruseii* (Fig. 4), which has not been reported earlier.

We have successfully cloned and expressed the *chi* gene of *Enterobacter* sp. NRG4. The purified recombinant Chi has similar physical and biological properties as reported for native Chi of *Enterobacter* sp. NRG4. The over expression of recombinant Chi leads to production of soluble form of Chi (present in the cell free lysate of sonicated IPTG induced cells) as well as insoluble form i.e. inclusion bodies (data not shown). The results presented in this study were obtained with soluble form of Chi only. Our next goal is to optimize the conditions for maximal recovery of functionally active Chi from inclusion bodies. The purified recombinant Chi will be used for production of pharmaceutically important N-acetyl D-glucosamine and chitobiose.

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