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ZYMOGRAPHIC IDENTIFICATION AND BIOCHEMICAL CHARACTERIZATION OF CHITINASE AGAINST PHYTOFUNGAL PATHOGENS

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ABSTRACT

An endospore forming Gram positive bacterium (MBCU4) was isolated from a vermicompost amended soil, and confirmed as *Bacillus subtilis* through the 16S rRNA sequence analysis. An extracellular chitinase was detected from this strain of *B. subtilis* under specific environmental condition. An attempt was made to purify the enzyme by ammonium sulfate precipitation followed by DEAE sepharose CL-6B column chromatography. The purified enzyme was demonstrated as a single band, having the molecular weight 31kDa on SDS PAGE analysis and its activity in the gel was determined by clear zone on zymogram. Further characterization of the isolated enzymes has showed that this enzyme is most active at pH 6.0 and at the optimized temperature of 50 ^{0}C. The purified chitinase exhibited high degree of antifungal activity particularly by degrading their cell wall components of plant pathogens *Macrophomina phaseolina* (69.0%) and *Rhizoctonia solani* (52.0%). It infers that the chitinase produced by *B. subtilis* could play an important role for biopesticidal activity.

Keywords: Antifungal, *Bacillus subtilis*, Biocontrol, Chitinase, Zymography

INTRODUCTION

The endospore forming genus *Bacillus* is one of the most widely researched and commercialized biocontrol agents **(Paulitz and Belanger, 2001).** Their biocontrol mechanisms include production of antibiotics and extracellular hydrolytic enzymes such as chitinase, laminarinase, lipase, and protease. These hydrolase enzymes contribute to degradation of fungal cell wall **(Korsten *et al*., 1993; Paulitz and Belanger, 2001; Helisto *et al*., 2001)** thus suitable for control of phyopathogenic fungi. Among those hydrolases chitinases considered as key hydrolytic enzymes in the lysis of cell walls of fungi, and play important factors in biological control **(Guthrie *et al.*, 2005).** Chitinases (E.C. 3.2.1.14.) are enzymes that are capable of hydrolyzing chitin to its oligomers and/or monomers. Chitinase from various origins has different enzymological properties and usually constitutes a complex chitinolytic enzyme. These enzymes play an important role in the nutrition and parasitism of bacteria and fungi. Identification and characterization of chitinase are prerequisites for understanding their role in the pathogenesis of infectious diseases as well as to improve their application in biotechnology. Zymography in protein chemistry provides reliable identification of enzymes like chitinase, based on the molecular mass of their active forms after gel electrophoresis **(Grudkowska *et al*., 2013)**. It is based on visualization of areas where the specific substrate is digested by the enzyme of interest. It can be used to perceive the total enzymatic activity or to see activities of enzyme isoforms following the protein electrophoretical separation on polyacrylamide gels **(Vandooren *et al.*, 2013)**. The present study attempts to identify and characterize chitinase from newly isolated *B. subtilis* MBCU4 using zymography technique to enhance resolution and for molecular size determination, and evaluate the antifungal activities of the isolated chitinase against common fungal pathogens.

MATERIAL AND METHODS

Identification of bacterial strains

The bacterial strains used in this study were isolated from vermicompost amended site (fertile land of Dantiwada Agricultural University, Dantiwada district, Gujarat, India) as explained earlier **(Pandya *et al.*, 2014).** They were screened on colloidal chitin agar plates (contains a semi minimal medium supplemented with 1% colloidal chitin) as explained by **Sampson and Gooday (1998)**. After incubating the plates at 30^{0}C for five days, they were flushed with 1% Congo red solution for 10 min followed by washing with 1M NaCl. The zones of degradation of chitin became visible as translucent halos around the colonies. The colonies from the higher zone of chitinase activity were selected and maintained on minimal medium for retesting. Among the retested colonies, the most potent MBCU4 was selected for further study. The bacterial strain MBCU4 was preliminary identified as *Bacillus subtilis* on the basis of its morphological, physiological and biochemical parameters which were then confirmed by 16S rRNA sequences. The sequence was submitted to NCBI and an accession number KC907389 was obtained.

Purification of Chitinase

Strain MBCU4 was cultured in 1000 ml of semi minimal medium containing 1% colloidal chitin at 37 ^{0}C for 48 hrs. The supernatant was harvested by centrifugation at 4 ^{0}C for 20 min at 12,000g and used for further purification using chromatography techniques. Column chromatography technique was used according to the modified method of **Wang *et al*., (2012).** Ammonium sulfate was added (608 g/l) to the culture supernatant (940 ml) and stored at 4 ^{0}C overnight. After centrifugation at 4 ^{0}C for 20 min at 12,000g, the precipitate was dissolved in a 0.5ml of 50 mM sodium phosphate buffer (pH 7) and dialyzed against the same buffer. The resultant dialysate was loaded onto a DEAE-Sepharose CL-6B column (5 cm × 30 cm) equilibrated with 50 mM sodium phosphate buffer (pH 7). The fractions containing the chitinase were individually collected and concentrated using ammonium sulfate. The resultant precipitates were collected by centrifugation and dissolved in 0.5ml of 50 mM sodium phosphate buffer (pH 7).

Characterization and Zymogram studies

The protein content was determined using the Bradford method with a Bio-Rad dye reagent concentrate and bovine serum albumin as the standard. After column chromatography, the protein concentration was estimated by measuring the absorbance at 280 nm **(Wang *et al.*, 2006).** Chitinase activity was measured with colloidal chitin as a substrate. Enzyme solution (0.5 ml) was added to 1 ml of substrate solution and 1.5% (w/v) suspension of colloidal chitin in phosphate buffer (50mM, pH 7.0) and the mixture was incubated at 37^{0}C for 15 min. After centrifugation, the amount of reducing sugar produced in the supernatant was

determined using N-acetylglucosamine as a standard **(Imoto and Yagishita, 1971).** One unit (U) of chitinase was defined as the amount of enzyme that produced reducing sugars corresponding to 1 µmol of N-acetyl glucose-amine equivalents from colloidal chitin per minute under the assay conditions. The optimum pH for the chitinase activity was measured under standard assay conditions, using the following buffers (all at 50 mM): citrate phosphate (pH 3.0-5.0), sodium acetate (pH 5.0-6.0), sodium phosphate (pH 6.0-8.0), Tris–HCl (pH 8.0-9.0), and carbonate-bicarbonate buffer (pH 9.0-11.0). The optimal temperature for the chitinase activities was determined by performing the condition assay **(Prasanna *et al.*, 2013)** at temperatures ranging from 10 to 100 ^{0}C. The molecular mass of purified chitinase was estimated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10 % gel according to Laemmli (1970) method. The proteins were visualized by staining with Coomassie brilliant blue R-250. For zymogram analysis of chitinase, the protein samples were mixed with loading buffer without reducing agent and heating. After electrophoresis, the gel was incubated in 100mM sodium acetate buffer, pH 5 at 40 ^{0}C for 18h. The activated chitinase on the gel was visualized by staining with 0.1 % Congo red followed by de staining with 1N NaCl **(Yamabhai *et al.*, 2008).**

Antifungal properties of Chitinase

Both crude and purified chitinase were tested for inhibitory activity against the fungal strains such as *Macrophomina phaseolina, Rhizoctonia solani, Fusarium oxysporum, Sclerotium rofsii* and *Sclerotinia sclerotiorum.* Spore suspensions of these fungi were prepared from 7 day old culture with sterile water, and 3 ml suspension (1x10^8 spores/ml) was seeded in 20ml of Potato Dextrose Agar (PDA). After 48h, about 200 µl of cell-free culture filtrate of each fungal strain was added in three 8mm wells of PDA. The treated plates were incubated at 28 ^{0}C. The zone of inhibition on PDA was measured at different time intervals up to 72hrs. The antifungal activity of chitinase was estimated using a growth inhibition assay described by **Wang *et al.*, (1999).**

RESULTS AND DISCUSSION

Identification of chitinolytic bacterium

Many biocontrol agents from *Bacillus* and *Pseudomonas* have ability to produce chitinase and digest chitin as a source of carbon and energy **(Xiao *et al.*, 2009; Wang *et al.*, 1997**). Application of vermicompost as a biofertilizer can provide available nutrients to improve the productivity and control of soil borne fungal pathogens by the specific bacterial flora like *Bacillus, Strptomyces, Aeromonas, Lysobacter* and *Salinispora* spp. that showed important role in the environmental degradation of chitin **(Xiao *et al.*, 2005; Yasir *et al.*, 2009).** In the present study, our isolate MBCU4 showed a strong chitinolytic ability on agar medium containing 1% (w/v) colloidal chitin, showing clear zone of inhibition around the colonies after 3 days of incubation at 30 ^{0}C. The isolated strain was observed as a Gram positive rod shaped bacterium. On the basis of the nucleotide sequences of 16S rRNA gene, the bacterium was identified as *Bacillus subtilis* (Fig. 1).

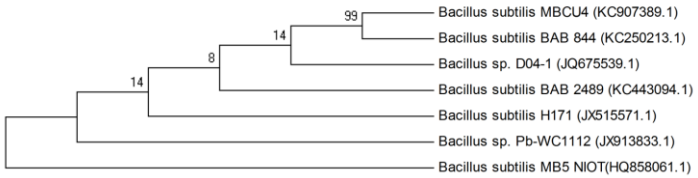

Figure 1 Phylogenetic analysis based on 16S rRNA gene sequences of newly isolated *B. subtilis* MBCU4 compared with other available *B. subtilis* strains from the European Molecular Biology Laboratory data library constructed after multiple alignments data by Clustal X. Distances and clustering with the neighbor-joining method was performed by using the software packages Mega version 4.0.

Purification of chitinase

The isolate MBCU4 released chitinase into the culture broth showed specific activity of chitinase as 0.25 unit/mg protein with a purification factor of 2.08 (Table 1). Since, Chitinolytic zymogram analysis has been proved as one of the best detection techniques for the detection of active chitinase by substrate gel and imprint techniques was employed in the present study **(Trudel and Asselin, 1990; Tronsmo and Harman, 1993).** The resolution of zymogram is determined by the Laemmli condition of electrophoresis **(Laemmli, 1970).** The deviations of chitinolytic zymography-estimated molecular masses and calculated molecular masses of chitinases were calculated as reported earlier **(Pechsrichuang *et al.*, 2013; Songsirittigul *et al.*, 2010**). Our chitinolytic zymography assay used in this study revealed a high resolution, excellent linearity, and range of calibration to determine molecular masses of chitinases. Our results showed presence of single zymographic band, which had molecular weight of 31kDa (Fig. 2). In a similar study, purified chitinase from sequential chromatography showed 31kDa of chitinase from *B.subtilis* **(Yan *et al.*, 2011; Chang *et al.*, 2010).**

Table 1 Purification of chitinase from *B. subtilis* (MBCU4)

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor	Yield (%)
Crude enzyme (supernatant)	1083	138	0.12	1.00	100.00
80% Ammonium sulfate precipitation	898	121	0.14	1.17	87.61
DEAE- Sepharose CL-6B chromatography	57.85	14.37	0.25	2.08	10.41

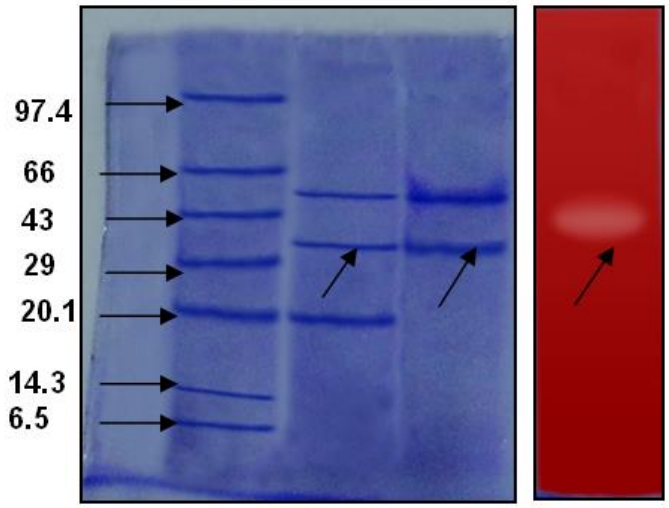

Figure 2 SDS PAGE and zymogram analysis of activated chitinase from stain *B. Subtilis* MBCU4. Lanes: M, Molecular marker; 1, crude enzyme; 2, the adsorbed chitinase fractions after DEAE sepharose CL-6B chromatography; 3, activated chitinase detection on zymogram analysis.

Effect of pH and temperature

The optimum pH for the enzyme activity was found to be 6.0 which suggested that the chitinase from our strain has near neural pH range (Fig. 3). Chitinase also works better at an acidic or near neutral pH as reported in other *Bacillus spp.*, such as *B. pumilus SG2* at pH 6.0 **(Ghasemi *et al.*, 2010)** and *B. cereus* YQ308 at pH 7.0 **(Chang *et al.*, 2003).** We observed 50 % of enzymatic activity was lost at extremely low and high pH. The optimal chitinase activity was observed at 40 ^{0}C to 60 ^{0}C while low activities were noticed in the ranges of 10 ^{0}C to 30 ^{0}C or 80 ^{0}C to 100 ^{0}C respectively. The optimal temperature was 50 ^{0}C, above which chitinase activity decreased (Fig. 4). These results showed similarity with optimum temperature for maximum chitinase production by *B. subtilis* as reported earlier **(Chang *et al.*, 2010; Yan *et al.*, 2011).**

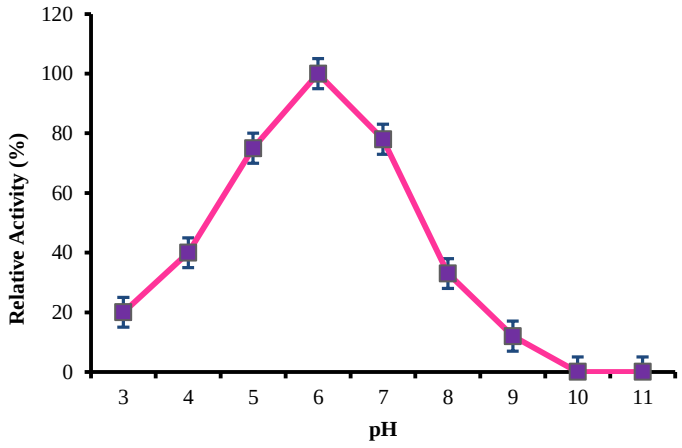

Figure 3 Effect of pH on relative activity of chitinase of newly isolated *B. subtilis* MBCU4

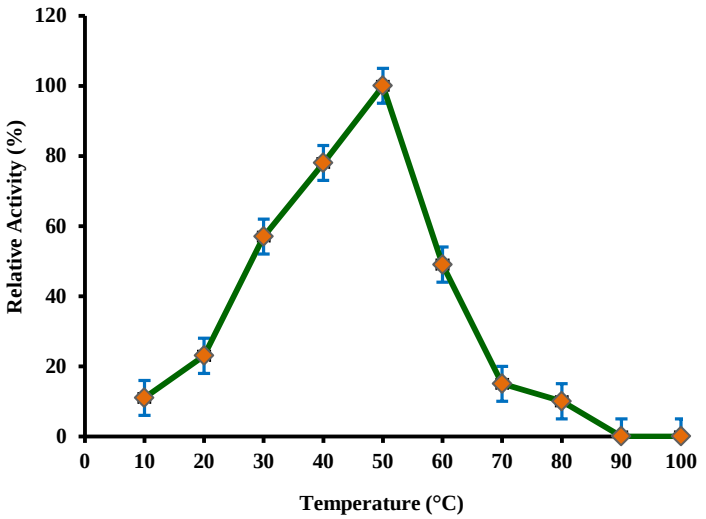

Figure 4 Effect of temperature on relative activity of chitinase of newly isolated *B. subtilis* MBCU4

Antifungal activity of crude and purified chitinase

An antifungal activity of purified chitinase was tested against five known fungal phytopathogens i.e. *Macrophomina phaseolina*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Sclerotium rofsii* and *Sclerotinia sclerotiorum*. The purified chitinase of newly isolated *B. subtilis* MBCU4 was capable of suppressing the growth of two pathogens *Macrophomina phaseolina* and *Rhizoctonia solani*. Antifungal activity of crude and purified chitinase was also observed against *M. phaseolina* and *R. solani* (Table 2). The growth inhibition of *M. phaseolina* was the strongest both by crude and purified enzymes (78 and 69 %, respectively). The growth of *R. solani* was also inhibited by crude and purified chitinase (73 and 52%). However, the growth of *Fusarium oxysporum*, *Sclerotium rofsii* and *Sclerotinia sclerotiorum* was only inhibited by crude chitinase. **Chang *et al.*, (2010)** reported that purified chitinase from *B. subtilis* NPU 001 inhibited hyphal extension of *F. oxysporum*. Partially purified chitinases (ChiS and ChiL) from *B. pumilus* were tested against fungal phytopathogens such as *Rhizoctonia solani*, *Verticillium sp*., *Nigrospora sp.*, *Stemphyllium botryosum* , *Bipolaris sp.*, *Phytophthora citricola* and *Phytophthora capsici* under *in vitro* conditions. Results revealed that purified antifungal chitinase showed growth inhibition for all the pathogens except *P. citrocola* and *P. capsici* **(Ghasemi *et al.*, 2010).** As reported earlier, *B. pumilus* SG2 secreted two chitinases which showed in vitro growth inhibition of the wheat pathogens *F. graminearum* and *B. sorokiniana* responsible for hyphal inhibitions of both the fungus in agar medium for a minimum 14 days **(Shali *et al.*, 2010).**

Table 2 Antifungal activity of chitinase from *B. subtilis* MBCU4 against various phytopathogens

Phytopathogens	Antifungal Activity	
	Crude chitinase	Purified chitinase
Macrophomina phaseolina	+++	+++
Rhizoctonia solani	+++	+++
Sclerotinia sclerotiorum	++	-
Fusarium oxysporum	+	-
Sclerotium rofsii	+	-

[a]Antifungal activity was classified as no inhibition (–; 0 %), moderate inhibition (+; 21–30 %), strong inhibition (++; 31–50 %), and very strong inhibition (+++; 50 %).

CONCLUSION

The newly isolated strain *B. subtilis* MBCU4 has better secretion capability for chitinase which could be used as a potential antifungal agent against plant fungal pathogens. The present study also revealed that zymography technique provides a sensitive, convenient, and inexpensive method for determination of activity of chitinase as employed in our newly isolated *B. subtilis* MBCU4. Further research to screen our purified chitinase from *B. subtilis* MBCU4 *in vivo* experiments against several other batteries of fungal pathogens will help to expand our studies.

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