ANTIFUNGAL ACTIVITY AND PROTOPLAST FORMATION BY CHITINASE PRODUCED FROM *Bacillus licheniformis* NK-7

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Abstract. Extracellular chitinase was produced by a chitinolytic bacterium, *Bacillus licheniformis* NK-7, under submerged fermentation. Maximum chitinase production (398.36 U/mL) was obtained when fermentation was carried out at 30°C for 72 h using 1500 µL of 24 h old bacterial culture in a 50 mL minimal salt medium (pH-7) amended with chitin (0.5% w/v; pH-6) under 150 rpm agitation rate. Supplementation of additional carbon, nitrogen and metal ion sources such as lactose, yeast extract and manganese (Mn^{2+}) respectively increased the chitinase production. Further, *B. licheniformis* NK-7 chitinase demonstrated antifungal activity against *Fusarium* sp., *Curvularia* sp. and *Aspergillus niger*. Protoplasts were also isolated from *Aspergillus niger* by the action of chitinase produced from *B. licheniformis* NK-7.

Key words: Chitin; chitinase; submerged; enzyme; fermentation; antifungal; protoplasts.

INTRODUCTION

Chitin, the most abundant naturally occurring polysaccharide in marine environment, consisted of N-Acetyl-D-glucosamine units joined by β-1,4-glycosidic linkages. Chitin is found as structural components of fungi, crustaceans, insects and arthropods. Aquatic biosphere (shrimp, crab, lobster and squid) alone is responsible for about 10^{12}-10^{14} tons of chitin production annually [9, 19]. Large amount of chitin gets accumulated in nature due to its insolubility and rigid structure. It is degraded by chemicals which results in production of non-specific products. In contrast, biological methods are safe. Biological degradation can be brought about by enzymes extracted from different microorganisms. Bacteria and fungi use chitin as a carbon source by producing chitinolytic enzymes. Chitinase is a complex enzyme which belongs to glycosyl hydrolases of families 18 & 19 based on amino acids homology in the catalytic domain and degrades chitin polymer by breaking the glycosidic bonds to produce various oligomers and monomers [4]. The oligomeric products of enzymatic hydrolysis of chitin have been of interest in the past few decades due to their broad range of medical, agricultural and industrial applications. Degradation of chitin helps in maintaining the global circulation of carbon and nitrogen and also can be used by microorganisms as the only source of energy. Chitinase is present in wide range of organisms [6].

Chitinase is a complex of enzymes divided into two categories (a) Endochitinase and (b) Exochitinase. Endochitinase cleave randomly at internal sites in polymer of chitin generating low molecular mass multimers of glucosamine. Exo-chitinases which are further divided into two categories: (1) Chitobiosidases-catalyze progressive release of diacetylchitobiose $(GlcNAc)_2$ from terminal non-reducing end and (2) N-acetylglucosaminidases that cleave oligomeric products obtained by endochitinase and chitobiosidases into monomers of N-acetyl glucosamine (GlcNAc) [27]. Due to multiple applications of chitinases in biocontrol, waste management, medicine and biotechnology they have become enzymes of interest for study. Chitinase can be used in control of pathogenic fungi, treatment of chitinous waste, preparation of pharmaceutically important chitooligosaccharides and N-acetyl D-glucosamine, isolation of protoplasts from fungi and yeast etc. The aim of the present study was to isolate, identify, characterize the most potential chitinase producing bacterial isolates with unique properties from different sources and optimize their fermentation conditions for maximum chitinase production and its applications.

MATERIAL AND METHODS

Microbial origins

A chitinase producing bacterium, *Bacillus licheniformis* NK-7, isolated in the laboratory from soil samples taken from Botany Garden of Kurukshetra University, Kurukshetra was used for the production of chitinase enzyme under submerged fermentation and also tested for its potential applications. *Aspergillus niger* MTCC 1344 procured from MTCC, Chandigarh was used for protoplast formation. The cultures of *Fusarium* sp., *Curvularia* sp. and *Alternaria* sp. were procured from Botany department of Kurukshetra University and used for antifungal activity.

Chitinase production and optimization of cultural parameters under submerged fermentation (SmF)

The cultural conditions such as temperature (in Celsius) viz. 20, 25, 30, 37, 40, 45 and 50; pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0), incubation period in hours (h) viz. 24, 48, 72, 96, 120 and 144), inoculum size in microliters (µL) viz. 100, 500, 1000, 1500, 2000, 2500 and 3000; agitation rate in rpm viz. 0, 90, 120, 150, 180, 210, 240, 270 and 300), concentration of chitin (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0%), additional sources of carbon (glucose, lactose, N-acetyl-D-glucosamine (GlcNAc), starch, xylose, fructose, mannitol and sucrose; 0.5%), nitrogen (peptone, yeast extract, urea, ammonium nitrate &

chloride, sodium nitrate and potassium nitrate; 0.5%) and metal ions (Mn^{+2}, Fe^{+2}, Mg^{+2}, Cu^{+2} & Zn^{+2}; 0.1%) were optimized for enhancing the production of chitinase under submerged fermentation (SmF) [31]. One variable at a time approach was followed in which one parameter is tested in the experiment while keeping other parameters constant. The production medium {50 mL minimal salt medium chitin-0.5% (w/v), Na_2HPO_4-6.0, KH_2PO_4-3.0, NH_4Cl-1.0, NaCl-0.5, Yeast Extract-0.05 (g/L); pH-7} was inoculated with 1.0 mL of 24 h old *Bacillus licheniformis* NK-7 ($2x10^8$ CFU/mL) culture and incubated for 3 days at 37°C under shaking condition (120 rpm). Following incubation, medium was centrifuged (10,000 rpm; 4˚C) for 10 minutes. Supernatant (crude chitinase enzyme) obtained was used to measure chitinase activity by DNS assay. A standard curve for N-acetyl-D-glucosamine (GlcNAc) vs absorbance (at 540nm) was also prepared for determining the enzyme activity. All the experiments were conducted in triplicates and the average values were recorded.

Chitinase assay by DNS method

Chitinase was measured using colloidal chitin as a substrate by Dinitrosalicylic acid (DNS) method [21]. The crude enzyme extract (500µl) was added to tubes consisting a mixture of 500µl of 0.5% colloidal chitin dissolved in phosphate buffer (0.2 M; pH-7). Tubes were incubated at 37°C for 60 minutes. Following incubation, 3.0 ml DNSA reagent was added to each tube. Control was run at the same time containing all reagents except enzyme. The reactions were subjected to boiling for 10 minutes in a water bath and then cooled down. The amount of reduced sugar released was determined by recording the absorbance at 540 nm in a UV-Vis spectrophotometer. By definition, one unit of chitinase activity is the amount of enzyme which yields 1 μmol of reducing sugar as N-acetyl-D-glucosamine (GlcNAc) equivalent per minute.

Statistical analysis

One-way ANOVA with post-hoc Tukey HSD statistical program was used for data analysis.

Application of chitinase

The crude chitinase was used for its application as a potential antifungal agent against some phytopathogenic fungi and for isolation & formation of fungal protoplasts.

Antifungal activity

To a sterile petri plate containing chitinase, molten potato dextrose agar (PDA) was poured, mixed gently and allowed to solidify. The control plate was prepared using distilled water instead of the enzyme. A small fungal disc was cut from 5 days old fungal plate with the help of a well-borer and placed in the center of each solidified PDA plate with a sterilized needle. The plates were incubated at 27ºC and fungal growth was examined for 5 days. The method of Prapagdee [25] was used to determine the effect of the bacterial chitinase on radial growth of each fungi. The fungal growth inhibition was expressed as the percentage of inhibition of radial growth of fungi relative to control by the equation given below.

$$\text{Inhibition of radial growth (\%)} = \frac{\text{Diameter (contr. pl.) - Diameter (test plate)}}{\text{Diameter (control plate)}} \times 100$$

(Note: Test plate contains chitinase enzyme extract while control plate contains distilled water instead of enzyme extract)

Isolation and formation of fungal protoplast by chitinase

Protoplasts from *Aspergillus niger* were prepared by the method given by Patil [24]. *Aspergillus niger* was grown in Czepek Dox broth. Mycelium was harvested, washed and suspended in 20 mL stabilization buffer (0.2 M phosphate buffer, pH 6.8; 0.8 M sorbitol). Then, 1.0 mL chitinase enzyme from *B. licheniformis* NK-7 was added to the mixture and incubated at 30°C in a shaker incubator at 100 rpm. A control sample without the chitinase enzyme was run simultaneously. The suspension was observed under microscope after 1 hour. Then, after 2 hours incubation, mycelial debris was removed by centrifugation at 2000 rpm for 10 min. The protoplasts were washed in 45 mL cold STC buffer (1.33 M sorbitol, 10 mM Tris-HCl and 50 mM $CaCl_2$, pH-7.5) and observed under microscope.

Regeneration of fungal protoplast

The method of Patil [24] was followed for the regeneration of fungal protoplasts on solid medium. The plates containing minimal medium (0.95 M sucrose and 1.2% agar, pH 6.0), inoculated with protoplasts, were incubated at 27°C temperature for 24-48 hours and observed for mycelium development and formation of fungal spores.

RESULTS

Chitinase production and optimization of cultural parameters under submerged fermentation (SmF)

Temperature is the most significant parameter needed for the efficient production of the enzyme. The chitinase production was studied using the different temperature ranging from 20-50˚C. Among various temperatures, 30°C was found as the best for production of chitinase with 122.64 U/mL, followed by 37°C with 100.1 U/ mL and 40°C with 77.25 U/ mL whereas further increase in temperature lead to progressive decrease in chitinase production (Fig. 1a). Among different pH used, pH 6.0 was found to be the best for chitinase production with 154.81 U/ mL. At pH 5.0 and pH 9.0, approx. 45%, however at pH 7.0 and pH 8.0, approx. 80% and 54% chitinase was produced respectively (Fig. 1b). Maximum chitinase production was given after an incubation of 72 h with enzyme activity of 170.63 U/ mL (Fig. 1c). The production of chitinase enzyme was decreased after 96 h of incubation (98.19 U/ mL) and lowest activity was recorded at 144 h (23.52 U/ mL). Gradual increase in chitinase activity was recorded with inoculum size of 100 µL up to 1500 µL (Fig. 1d). Maximum chitinase yield was observed at inoculum size 1500 µL with 199.86 U/ mL while lowest yield was recorded at 3000

µL with 32.46 U/ mL. Out of different shaking conditions (0-300 rpm), highest chitinase production was observed with agitation rate of 150 rpm (242.67 U/ mL) while at much higher speed (Fig. 1e), it resulted in the decrease of the enzyme production. Moreover, production of chitinase was recorded lower at stationary condition (77.76 U/ mL) & at 300 rpm (59.46 U/ mL).

At 0.5% of chitin concentration, maximum production of chitinase with 251.21 U/ mL was observed (Fig. 2a). Among various carbon sources glucose, lactose, xylose, fructose and sucrose enhanced the production of chitinase while N-acetyl glucosamine (GlcNAc), starch and mannitol decreased the chitinase production as compared to control (Fig. 2b). Maximum enzyme production was observed with lactose (317.78

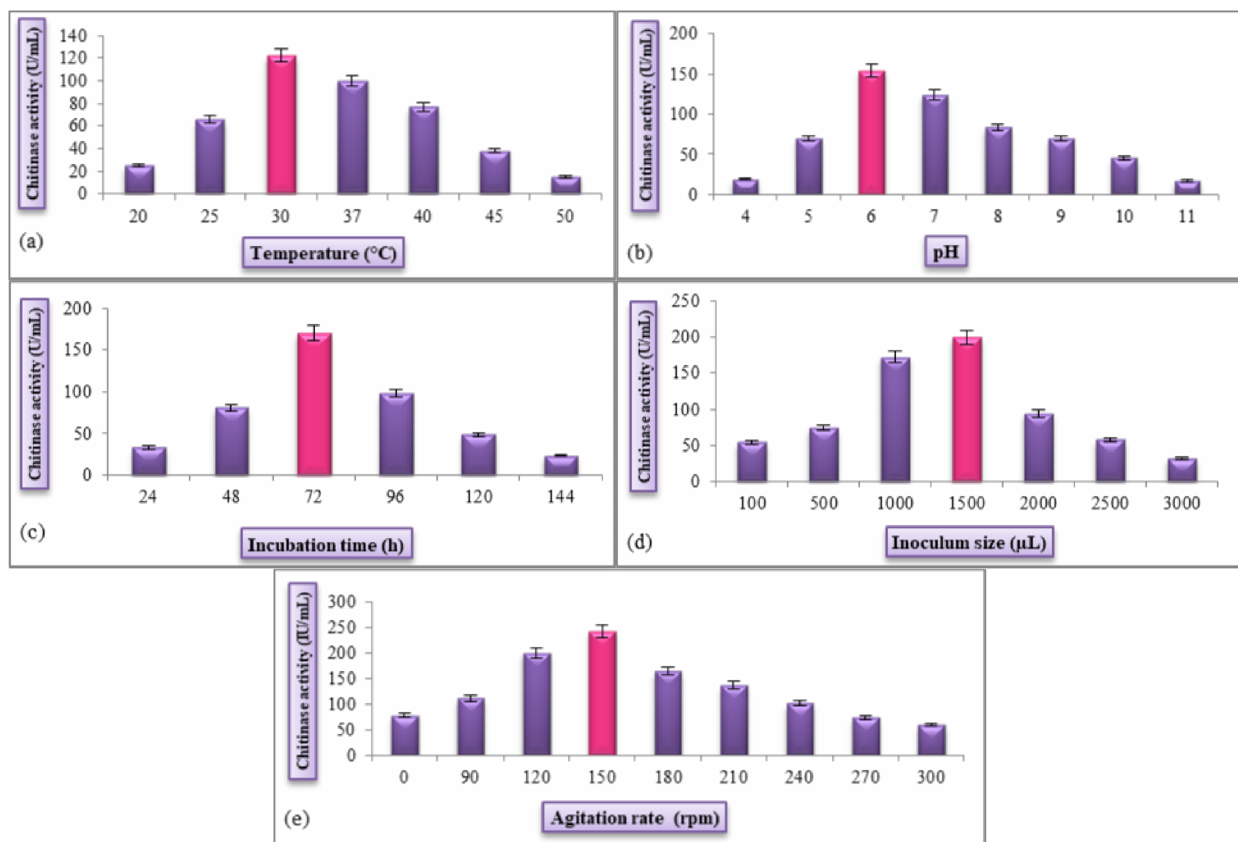

Figure 1. Effect of various parameters on chitinase production from *Bacillus licheniformis* NK-7 (a) Temperature (b) pH (c) Incubation time (d) Inoculum size (e) Agitation rate. (Note: The data represents mean ± S.D. (n=3) and P values less than 0.05 were considered significant with a 95% confidence limit.)

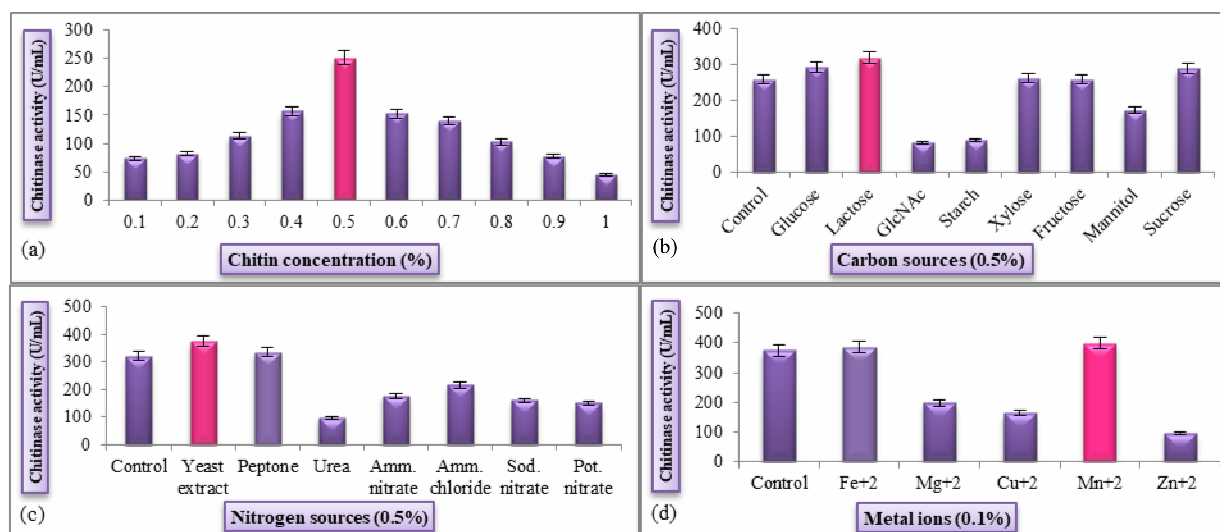

Figure 2. Effect of various parameters on chitinase production from *Bacillus licheniformis* NK-7 (a) Chitin concentration (b) Carbon sources (c) Nitrogen sources (d) Metal ions sources (Note: The data represents mean ± S.D. (n=3) and P values less than 0.05 were considered significant with a 95% confidence limit.)

U/mL). Sucrose, xylose and fructose enhanced the production of chitinase by 31%, 3% and 1% respectively. Organic nitrogen sources such as yeast extract (372.45 U/ mL) and peptone (334.18 U/ mL) enhanced the production of enzyme whereas inorganic nitrogen sources such as urea, ammonium chloride, ammonium nitrate, sodium nitrate and potassium nitrate decreased the chitinase production (Fig. 2c). Out of different metal ions used, Mn^{+2} and Fe^{+2} ions enhanced the production while Zn^{+2}, Cu^{+2} & Mg^{+2} decreased production of chitinase (Fig. 2d). The highest chitinase production was observed with Mn^{+2} (398.36 U/ mL). Production of chitinase was found to be suppressed by Mg^{+2} (197.51 U/ mL); Cu^{+2} (164.25 U/ mL) & Zn^{+2} (96.71 U/ mL) ions.

Applications of chitinase

The chitinase enzyme from *Bacillus licheniformis* NK-7 was tested for its application as an antifungal agent against phytopathogenic fungi and for the isolation & formation of fungal protoplasts from *Aspergillus niger*.

Antifungal activity of chitinase

The *B. licheniformis* NK-7 chitinase inhibited the growth of phytopathogens (Fig. 3) and it was measured in terms of percent inhibition of radial growth of fungi (Table 1) such as *Fusarium* sp., *Curvularia* sp. and *Aspergillus niger* when subjected to poison plate assay whereas negligible effect was observed on the growth of *Alternaria* sp.

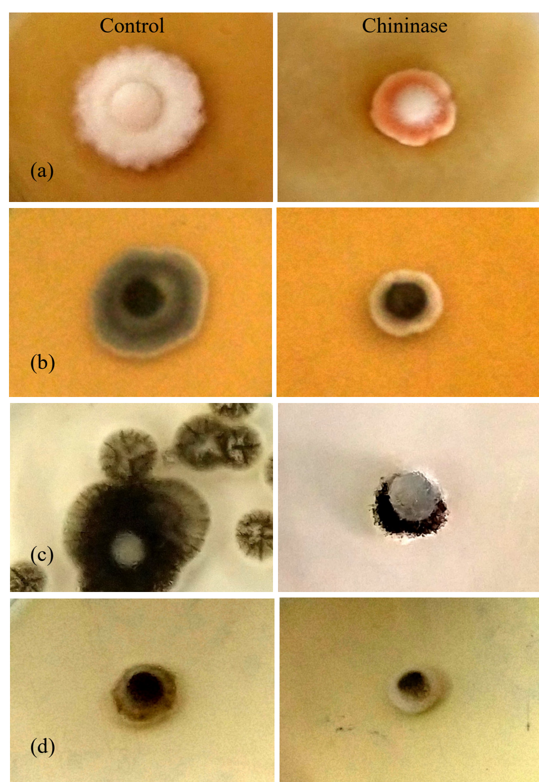

Figure 3. Antifungal activity of *B. licheniformis* NK-7 chitinase against various phytopathogenic species of fungi (a) *Fusarium* (b) *Curvularia* (c) *Aspergillus niger* (d) *Alternaria*

Table 1. Radial growth inhibition by *B. licheniformis* NK-7 chitinase

Sr. no.	Phytopathogenic fungi	Radial growth inhibition (%)
1.	*Fusarium* sp.	65.11
2.	*Curvularia* sp.	65.21
3.	*Aspergillus niger*	69.44
4.	*Alternaria* sp.	06.67

Isolation & formation of fungal protoplasts

Chitinase extracted from *Bacillus licheniformis* NK-7 was used to study the formation of protoplasts from mold *Aspergillus niger*. The fungal mycelium showed different alterations in its structure upon treatment with chitinase as compared to control such as formation of protoplasts within hyphae, digestion of mycelial fragments and release of protoplasts in the surrounding medium. Figure 4 (a-d) demonstrates the formation of protoplasts which was observed under light microscope (100x).

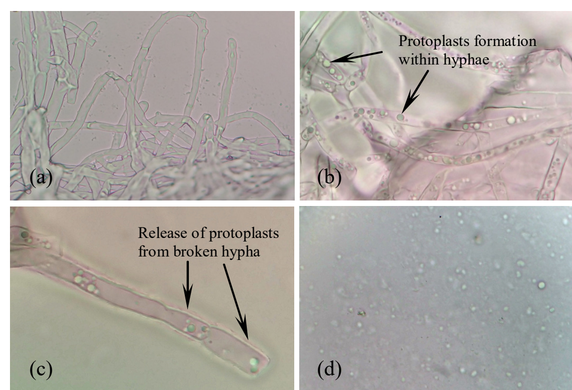

Figure 4. Isolation of protoplasts of *Aspergillus niger* by chitinase from *B. licheniformis* NK-7 and their observation under microscope (a) Control-vegetative mycelium (b) Protoplasts trapped within clusters of hyphae (c) Release of protoplasts from broken hypha (d) Released protoplasts of *Aspergillus niger.*

Regeneration of fungal protoplasts

A suspension of fungal protoplasts was spread-plated on minimal agar medium. The plates were incubated at 27°C for 24-48 hrs. Then, plates were observed for mycelium development and sporulation after incubation. Following incubation, the protoplasts of *Aspergillus niger* had readily regenerated into mycelial colonies and black colored spores were developed when cultured into minimal agar medium (Fig. 5).

Figure 5. Regeneration of the fungal protoplasts on a minimal agar medium

DISCUSSION

Optimization of different cultural parameters increased the production of chitinase enzyme. Temperature regulation is very necessary in modifying the speed of fermentation and maintaining cell viability. Maximum chitinase activity was observed at 30°C temperature. Similar results were reported by Shivalee [31] where 25-40°C range was best suited for maximum chitinase production under submerged fermentation. The results suggested that too high temperature may lead to undesirable intracellular changes, increase in number of dead cells and a greater concentration of unstable components whereas too low temperature may result in slower fermentation rate and may create undesirable products [18]. Optimum pH is a very important factor in the stability of enzymes. Extreme pH values usually result in complete loss of enzyme activity for most of the enzymes by affecting the shape and structure of the proteins and disrupting the bonds within, thus denaturing the enzyme [28]. Highest chitinase activity was observed after 72h of incubation by *B. licheniformis* NK-7. Karunya [17] also reported in their study that the chitinase production from *Bacillus subtilis* increased from day 1 up to day 4. Decrease in the chitinase production during incubation may be due to several factors such as depletion of nutrients, production of toxic wastes and oxygen availability in the production medium which limit the growth, reproduction and metabolism of bacteria. Inoculum size also significantly affects the activity of an enzyme. According to Sandhya [29] and Shivalee [31] the lower concentration of the inoculum may not be sufficient for maximum conversion of the substrate whereas in case of higher inoculum concentration there is decrease in the extracellular chitinase production. Agitation rate may greatly influence the enzyme production. Because at stationary or low agitation speed, bacterial cells would not get the proper dissolved oxygen as well as cell would get clumped due to improper mixing of the medium components while at higher speed cells may get ruptured and the enzyme production would not be at the desired frequency [5].

As suggested by Ulhoa [32], increase in the concentration of substrate enhances the rate of enzymatic reaction to a definite point. When the enzyme entirely gets bound to the substrate, any further increase in substrate would affect the rate of reaction because the available enzymes would get saturated & work at their maximum rate. Maximum enzyme production was observed with lactose which could be due to the high level production of extracellular β-galactosidase enzyme in *B. licheniformis* [2] that converts lactose to glucose and galactose in an inducible expression. The results showed similarities with the studies reported by Gomaa [13], Jha [15] and Shivalee [31]. In nitrogen utilization, Narayana [22] and Vaidya [33] demonstrated the highest chitinase production in medium amended with yeast extract while Butti [7] and Agustiar [1] reported enhanced enzyme production with peptone. However, addition of urea into the fermentation medium decreased the chitinase activity [11, 16, 29]. These results support the findings of the researchers that the organic nitrogen sources can efficiently increase the enzyme yield as they are better growth and metabolic stimulators than inorganic nitrogen sources. Metal ions are crucial for catalytic action of some enzymes as they have the capability to donate/attract electrons. Mn^{+2} and Fe^{+2} exhibited higher activity which tend to have the stimulatory effects on the chitinase production than other metal ions. Similar results were reported in the findings of Shivalee [31] and Aliabadi [3] in their studies. Donderski [10] and Gomaa [13] reported in their research that the inhibition of chitinase enzyme by Mg^{+2}, Cu^{+2} and Zn^{+2} could be related to the residues of amino acids in the active site of chitinase which may interfere in the binding of substrate molecules.

Chitinase enzymes are able to degrade the chitinous cell wall of several fungi. Crude chitinase produced from *B. licheniformis* NK-7 was efficiently able to inhibit the growth of *Fusarium* sp., *Curvularia* sp. and *Aspergillus niger*. Various researchers have reported similar antifungal activity of chitinase against different species of fungi in their studies. Antifungal activity of chitinase from *Bacillus subtilis* was reported by Senol [30] against *Fusarium culmorum.* The lytic action of *Bacillus* sp. chitinase on fungus *Helminthosporium sativum* was demonstrated by Melent'ev [20] in their research. Moreover, Haggag [14] reported the antifungal activity of chitinase from *Streptomyces hygroscopicus* against several phytopathogens viz. *Rhizoctonia solani, Fusarium oxysporum, Alternaria alternata, Aspergillus niger, Aspergillus flavus, Sclerotinia sclerotiorum* and *Botrytis cinerea.* Fleuri [12] used chitinase from *Cellulosimicrobium cellulans* to study the mechanism of lysis in different fungi viz. *Streptomyces phaerochromogenes*, *Mucor miehei*, *Rhizopus oligosporus*, *Paecylomyces* sp., *Penicillium* sp. & *Trichoderma viride*. Thus, many phytopathogenic fungal diseases can be eliminated by potential chitinase producers to solve the problem of agricultural production worldwide.

Isolation of protoplast and its fusion is an essential tool in improvement of strains to promote genetic recombination techniques and development of hybrid strains from filamentous fungi. During isolation of protoplasts, chitinase produced from *B. licheniformis* NK-7 enzymatically hydrolyzed the cell wall due to which the hyphal tip of the fungal pathogen bulged out and the protoplasts were released gradually into the high osmotic strength lytic medium [24]. Thus, *B. licheniformis* NK-7 chitinase has the potential to be used for the isolation of protoplasts from fungi either directly or in combination with other commercial enzymes. Similarly, Yano [34] used culture filtrate of *Bacillus circulans* KA-304 to develop protoplasts from *Schizophyllum commune*. Dahiya [8] isolated protoplasts from various fungal species such as

Trichderma reesei, *Aspergillus niger*, *Agaricus bisporus* and *Pleurotus florida* by using various combinations of chitinase and cellulase from *Enterobacter* sp. NRG4.

Regeneration of protoplasts on minimal medium provides a relative measure of the consequences of enzyme treatment on the viability of cell. Hypertonic culture medium is required for the reversion of protoplasts to mycelial form where regeneration of cell wall takes place and this ability allows the organism to return back to its normal structure and morphology [24]. As reported by Patil [23], the regeneration frequency has been observed better with minimal medium amended with sucrose. Successful regeneration of the fungal protoplasts to normal mycelia is very important for its viability. The protoplasts which lack the capability to regenerate on a medium either lack nuclei or got damaged at a certain point during or after the enzyme treatment [26].

In the present study, chitinolytic bacterial isolate *Bacillus licheniformis* NK-7 was found to be a potential chitin degrader. Fermentation conditions had a strong influence on chitinase production which led to a four-fold increase in chitinase activity after optimization of cultural conditions. Crude chitinase enzyme was effective in the formation & release of protoplasts from *Aspergillus niger* without the addition of any other commercial enzyme which makes the chitinase enzyme industrially significant for biotechnological applications such as protoplast fusion, improvement of strains etc.

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Conflict of interest. There is no actual or potential conflict of interest in relation to this article.

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