# Assessment of Antifungal Activity of Chitinase Produced by *Bacillus licheniformis* EG5 Isolated from Egyptian Soil

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**Abstract:** The chitinolytic activity of the isolate *B. licheniformis* EG5 obtained from agricultural Egyptian soil was investigated. This isolate degraded chitin with the development of distinct zone of clearance on colloidal chitin agar. The isolate was identified by classical bacteriological examination, metabolic fingerprinting using Biolog Micro Plates and phylogenetic analysis of 16S ribosomal RNA gene nucleotide sequence. Evaluation of antifungal activity of the produced enzyme was done and revealed the potential antifungal activity especially against *F. graminearum* (NRRL 5883) & *F.* sp. (NRRL 37262) among strains of *Fusarium* tested. Chitinase enzyme from the isolate under study was produced under the determined optimum conditions. These conditions were static incubation for three days at initial pH 8.00 & incubation temperature 40ºC with chitin concentration 0.7 % (w/v). Under these conditions, the enzyme activity in culture supernatant was 6.18 $UmL^{-1}$. Purification of the produced enzyme was performed and the results revealed that the enzyme activity recorded, 11.35 $UmL^{-1}$, had increased by about factor of two. Chitinolytic activity of the partially purified enzyme was examined again in contrast to controls maintained with heat inactivated enzyme which did not record any chitinolytic zones. Final assessment of antifungal activity of the partially purified chitinase produced by this isolate was confirmed against controls inoculated and maintained with heat inactivated enzyme which also did not show any activity against *Fusarium* spp. tested. Molecular weight determination revealed the presence of one distinct band of about 63 KDa.
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## 1. Introduction

*Bacillus* spp., the most often isolated bacteria from natural environments, has been used widely in agricultural applications. Biopesticides, whereby a natural organism or its' metabolites are used as the controlling agent, are the material basis and an important means of pesticidal control (Marc and Philippe, 2007). For example, the fungicides Serenade and Sonata that are made of *B. subtilis* QST713 and QST2808 have been registered and applied in America (Cao *et al.*, 2010). Yin *et al.* (2011) indicated that *B. amyloliquefaciens* PEBA20 has the potential to serve as a biological control agent for the poplar canker disease caused by *Botryosphaeria dothidea* and for diseases caused by other phytopathogens. Li *et al.* (2012) indicated that *Bacillus subtilis* ZZ120 showed strong growth inhibition activity *in-vitro* against the replant disease phytopathogens *Fusarium graminearum*, *Alternaria alternata*, *Rhizoctonia solani*, *Cryphonectria parasitica* and *Glomerella glycines*. The antifungal compounds were isolated from n-butanol extract as a mixture of iturins.

*Bacillus licheniformis* is a Gram-positive, spore-forming soil bacterium that is used in the biotechnology industry to manufacture enzymes, antibiotics, and biochemical and consumer products. This species is closely related to the well studied model organism *Bacillus subtilis*, and produces an assortment of extracellular enzymes that may contribute to nutrient cycling in nature. Chitin is the second most abundant polysaccharide in nature, next to cellulose. It is composed of (1-4)-β-linked N-acetyl-D-glucosamine (NAG) subunits and is an important component of both carbon and nitrogen cycles. This polysaccharide compound can be found in fungi, insect exoskeletons, and marine invertebrates (Huang *et al.*, 2005). The initial step in microbial chitin degradation is usually the chitinase-mediated hydrolysis of the polymer into monomers and oligomers. Since, chitin is a major cell wall constituent of fungi, therefore, chitinases, the hydrolytic enzymes that specifically degrade chitin, are gaining much attention worldwide (Wang *et al.*, 2006).

Chitinases are found in a broad range of organisms (fungi, bacteria, parasites, plants, insects and yeast) and play different roles in their origin. In microorganisms, chitinase has been found as biocontrol agents for different types of fungal diseases of plants (Huang *et al.*, 2005). This enzyme is used in many fields such as pest control, pollution abatement and commercial biology (Felse and Panda, 2000). Chitinases (EC 3.2.1.14) are produced by

several bacteria. These chitinases are used in various applications such as biological control of fungal pathogens (Chang *et al*., 2007). Microorganisms produce chitinase primarily for assimilation of chitin as carbon and/or nitrogen source. Chitinases have been isolated from variety of bacteria including *Bacillus* spp. and some of them are reported to produce multiple forms of chitinases with different molecular masses. Previous reports have shown that species of *Bacillus* including *B. licheniformis* are known to produce chitinolytic enzymes (Waldeck *et al*., 2006 & Chang *et al*., 2007).

So, the aim of the current study was to isolate and investigate a chitinolytic bacterium and determine its potential as a biological control agent active against fungi. Mainly, chitinolytic action was concerned due to the great potential of chitinases as biological control agents. Also, production optimization for such enzymes was concerned.

## 2. Material and Methods

### 2.1 Preparation of colloidal chitin

Colloidal chitin was prepared from the chitin flakes (Crab shell chitin, Sigma Chemicals Company, USA) by the method of Mathivanan *et al*. (1998). The chitin flakes were ground to powder and added slowly to 10 N HCl (10 % percentage) and kept overnight at 4°C with vigorous stirring. The suspension was added to cold 50% ethanol with rapid stirring and kept overnight at 25°C. The precipitate was collected by centrifugation at 10,000 rpm for 20 min and washed with sterile distilled water until the colloidal chitin became neutral (pH 7.0). It was freeze-dried to powder and stored at 4°C until further use.

### 2.2 Soil sampling, isolation, and culture conditions

The soil samples were collected aseptically from the upper most 0-5 cm soil layer of different agricultural fields in Giza governorate, Egypt. About 1.0 g of soil sample was transferred to 99.0 ml sterilized normal saline in 250 ml conical flask and agitated (100 rpm) at 30ºC for 15 minutes on water bath shaker (Eyela, Japan). The soil suspension was then diluted in serial up to $10^{-7}$ dilutions. One ml of each dilution was poured into Petri plates containing modified colloidal chitin agar (CCA) medium described by Hsu & Lockwood (1975). The medium composed of (g/L): Chitin (5 g of the dry preparation, or the equivalent volume of colloidal chitin suspension to give about 5 g of chitin per liter); Yeast extract, 0.5; $NaNO_3$, 2.0; $K_2HPO_4$ (anhydrous basis), 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.01 and tap water, 1000 ml (pH 7.0). The inoculated plates were then incubated at 30ºC and checked regularly for five days for the presence of zones of clearance around the developed bacterial colonies. The best bacterial isolate capable of degrading chitin with the largest distinct zone of clearance on CCA was selected. Pure culture of this isolate (referred to as EG5) was maintained in Luria-Bertani broth (LBB), amended with 20% glycerol & 0.5% chitin, and stored at -80ºC. Also, agar slant cultures of the same isolation medium and another set on LB slants for this bacterial isolate was stored at 4ºC for regular testing and subculturing. Also, colloidal chitin of the same composition above was used for the next experiments used for cultivation and enzyme production and assay of activity.

### 2.3 Detection of chitinase activity *in-vitro* for the isolate EG5

This test was performed with the culture supernatant of the selected isolate using agar well diffusion method. The isolate was grown in 0.5 % colloidal chitin. One ml inoculum with 0.5 OD was used to inoculate 50 ml of medium and incubated at 30ºC. After three days of incubation, the culture was harvested, centrifuged at 10,000 rpm for 15 min at 4ºC and the supernatant was collected. Colloidal chitin (0.5%) agar plates were prepared and wells were made using 1 cm sterile cork borer. Culture supernatant was placed at 100 µl in each well and incubated at 30ºC. After 12 h, the development of clear zone around the well was observed and recorded in triplicates. Also, the same culture supernatants were used to detect the potential antimicrobial activities as described briefly below.

### 2.4 Detection of chitinase antimicrobial activity for the isolate EG5

For assessment of potential antifungal activity of chitinase under study, using agar well diffusion test according to Taechowisan *et al*. (2003), a wide range of fungal strains (listed below), especially *Fusarium* spp. were used, as well as, some common bacterial strains. The bacteria and filamentous fungi were cultured on nutrient agar (NA) medium and potato dextrose agar (PDA) medium, respectively. The agar plates were incubated at 35°C for 24 h (bacteria) and at 25°C for 3 days (fungi). The yeast, *C. albicans* were grown in yeast peptone dextrose (YPD) agar at 30ºC. Inhibition of microbial growth was assessed on the basis of presence or absence of an inhibition zone around the agar wells created in the previous used media using 1 cm sterile cork borer. Each well was contained about 100 µl of the culture supernatant mentioned above of the isolate EG5. All plates were kept at 4ºC for at least three hours before incubation at the specified temperature. The inhibition zone in each case was measured as the distance from edge of the created

agar well till the boundary of inhibition zone (An average of three replicates). The following microbial cultures were used as test strains:

**2.4.1 Bacteria**: *Bacillus subtilis* (NCTC 10400), *Staphylococcus aureus* (NCTC 7447), *Escherichia coli* (NCTC 10416), & *Pseudomonas aeruginosa* (ATCC 10145).

**2.4.2 Fungi**: *Candida albicans* (IMRU3669), *Aspergillus niger* (LIV131), *Aspergillus flavus* (NRRL 6541), *Fusarium graminearum* (NRRL 5883), *Fusarium pseudograminearum* (NRRL 28062), *Fusarium cerealis* (NRRL 25491), *Fusarium acuminatum* (NRRL 29154), *Fusarium mesoamericanum* (NRRL 25797), *Fusarium acaciae-mearnsii* (NRRL 26752), *Fusarium asiaticum* (NRRL 6101), *Fusarium tucumaniae* (NRRL 34546), *Fusarium virguliforme* (NRRL 34552), and *Fusarium* sp. (NRRL 37262).

**2.5 Identification of the isolate EG5**

For the purpose of identification, the isolate EG5 was subjected for metabolic fingerprinting (using Biolog plate 3rd generation) in addition to some essential identification tests according to Logan and De Vos (2009). In addition, the isolate was identified by phylogenetic analysis of 16S ribosomal RNA gene nucleotide sequence using *16S rDNA* as the template for PCR amplification of the 16S rRNA gene.

**2.5.1 Biolog Identification**

Before inoculation into Biolog MicroPlate the isolate was grown at 30ºC on LB agar. When sufficient plate growth was noted, the isolate was suspended in 0.85% saline and inoculated into Biolog MicroPlate (150 µl per well) according to the manufacturer's instructions and incubated for 24 hours at 30ºC. Biolog plates were read using semi-automated Biolog Microstation System and Biolog software. Biolog microbial identification system using the powerful new GENIII redox chemistry is applicable to an extraordinary range of both gram negative and gram positive bacteria. This work was supported by "Research Services O" (Nasr City, Cairo, Egypt).

**2.5.2 Molecular identification of *16S rDNA* gene for the isolate EG5**

**2.5.2.1 DNA extraction, PCR amplification and purification**

The isolate EG5 was inoculated into 5 mL aliquots of LBB and incubated at 30ºC on a rotary shaker at 180 r/min for 16-18 h. Total genomic DNA for PCR amplification of *16S rDNA* sequence was extracted from the EG5 isolate according to the used kit's instruction manual (QIAamp DNA mini kit cat number, 51304). The *16S rDNA* region was amplified (approx 1500 bp) by polymerase chain reaction (PCR) using the forward primer P3 5'-AGAGTTTGATCMTGGCTCAG-3' and the reverse one P5 5'-TACGGYTACCTTGTTACGACTT-3' according to Moreno *et al.* (2002) and Yin *et al.* (2011). The PCR mixtures were prepared in 50 µl volumes containing 0.5 µM of primer, 200 mM of deoxyribonucleotide triphosphate, 5 µl of the 10X PCR buffer (100 mM Tris–HCl, 15 mM $MgCl_2$, 500 mM KCl; pH 8.3), 1 U of Taq DNA polymerase (Tianwei), and 1 µl of the extracted DNA. DNA amplification was performed in TProfessional Basic Thermocycler PCR system with an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 60 s, annealing at 58°C for 60 s, elongation at 72°C for 90 s, and a final extension at 72°C for 10 min. The amplicon was identified by horizontal electrophoresis on 1% agarose gel against the used DNA size marker (UMR-100) and finally was purified using PCR purification kit (Tianwei). This work was supported by "Research Services O" (Nasr City, Cairo, Egypt).

**2.5.2.2 *16S rDNA* gene Sequencing, data analysis and phylogeny**

Sequencing was performed using an Applied Biosystems 310 sequencer (ABI 310 DNA sequencer, Big Dye Terminator cycle sequencing ready kit applied biosystems) and the same primers used for PCR. The sequence was compared with similar sequences retrieved from DNA databases by using the NCBI n-BLAST search program in the National Center for Biotechnology Information (NCBI) and aligned with ClustalW (Ver.1.74) program (Thompson *et al.*, 1994). The nucleotide distances were estimated considering alignment gaps by using Jukes and Cantor (1969) method for correction of superimposed substitutions using the Molecular Evolutionary Genetics Analysis (MEGA) software (Ver. 4.0) (Tamura *et al.*, 2007). Neighbour Joining (NJ) implemented through MEGA 4.0 software and bootstrap analysis (1000 replicates) was performed to assess the reliability of the constructed phylogenetic tree.

**2.6 Chitinase activity assay and protein content determination**

The activity of enzyme produced as a result of the cultivation as described above in colloidal chitin was assayed in the following manner. Extracellular chitinase activity was determined by incubating 1 ml of crude enzyme (culture supernatant) with 1 ml of 0.5% colloidal chitin in a

0.05M phosphate buffer, pH 7.0 at 30ºC for 1 h. After centrifugation of reaction mixture, the amount of N-acetyl-D-glucosamine released in the supernatant was determined by the method of Reissig *et al.* (1955). The reaction was terminated by adding 0.1 ml of 0.08 M potassium tetraborate, pH 9.0 to 0.5 ml of reaction mixture and then boiled in water bath for 3 min. Then 3 ml of diluted *p*-dimethylaminobenzaldehyde (Sigma, USA) reagent was added and again incubated at 30ºC for 15 min. The released product in the reaction mixture was read at 585nm in UV/VIS spectrophotometer against the blank prepared with distilled water without the enzyme presence. Chitinase activity was determined using N-acetylglucosamine (Sigma Chemicals Company, USA) as the standard. One unit (U) of chitinase activity was expressed as the amount of enzyme which released 1 µmole of N-acetyl-D-glucosamine or its equivalent per min from colloidal chitin in 1 ml of reaction mixture under standard assay conditions (Mathivanan *et al*., 1998). Also, the total protein concentration of the supernatant sample was determined and expressed as mg per ml using the procedure of Lowry *et al*. (1951) with bovine serum albumin as a standard. In each case, the recorded values were averages of three replicates.

**2.7 Enzyme production optimization**

Effect of different incubation factors on the production of chitinase enzyme by the isolate under study was conducted. The variables tested were the incubation period, aerobic incubation under static and shaking conditions, incubation temperature, the pH of the culture medium, and different concentrations of the used substrate under study. The above cultivation medium (colloidal chitin) was used and at the end of incubation, both enzyme activity and the protein concentrations, for each culture under the tested variable were determined. In each case, for testing a different variable the initial state used for isolation was kept constant. The incubation under static and shaking conditions (100 & 200 rpm) at 30ºC for five days was tested first. Then, the incubation period was conducted for 1, 2, 3, 5 and 7 days. The effect of different pH values (5, 6, 7, 8 & 9), and temperature range (10, 20, 30, 40 & 50ºC) on chitinase production was conducted. Also, the substrate concentrations tested of the used substrate (Colloidal Chitin) were 0.1, 0.3, 0.5, 0.7 & 0.9 % (w/v).

**2.8 Enzyme production and purification**

Chitinase enzyme from the isolate under study was then produced under the determined optimum conditions. Purification of the produced enzyme was then performed according to the methods suggested by Nawani and Kapadnis (2001) & Narayana and Vijayalakshmi (2009). The culture filtrate (500 mL) of 72-h old culture broth was subjected to precipitation with ammonium sulphate up to 80% saturation and kept at 4ºC for 24 h. The precipitate thus obtained was collected by centrifugation at 10,000 g for 20 min. The pellet was dissolved in a 0.05M phosphate buffer, pH 7.0 and extensively dialyzed against the same buffer. The protein concentrate was loaded on Sephadex G-100 (Sigma, USA) column (2x40 cm) pre-equilibrated with a 0.05M phosphate buffer, pH 7.0 and eluted with the same buffer. Fractions thus collected were tested for chitinolytic activity *in-vitro*, as described above. Chitinolytic active fractions were recovered, concentrated and then refrigerated at 4°C until further analysis. This partially purified enzyme was then subjected to enzyme activity and protein concentration determinations as described above. In addition, assay of chitinolytic activity, inhibition of fungal growth, and molecular weight determination were all followed up as described briefly below.

**2.9 Assay of chitinolytic activity of the purified chitinase from the isolate EG5**

Chitinolytic activity of the partially purified enzyme was examined by agar well diffusion method as described before. Chitinase enzyme (100 µl) was loaded onto the wells of CC agar plates. Control was maintained with (100 µl) of heat inactivated enzyme (5-min boiled). Chitinolytic zones around the wells were observed after 12-24 h of incubation at 30ºC.

**2.10 Inhibition of fungal growth by the purified chitinase from the isolate EG5**

For final assessment of antifungal activity of chitinase of the isolate EG5, agar well diffusion test according to Taechowisan *et al*. (2003), was used again against only *Fusarium* spp. (listed above). They were cultured on potato dextrose agar (PDA) medium. Chitinase enzyme (100 µl) was placed in wells of the PDA plates. Controls were also maintained with (100 µl) of heat inactivated enzyme (5-min boiled). All plates were kept at 4ºC for three hours before incubation at 25°C for 3 days to observe inhibition of fungal growth.

**2.11 Molecular weight determination of the purified chitinase from the isolate EG5**

The molecular weight of chitinase enzyme obtained from the isolate under study was determined by using sodium dodecyl sulphate – poly acrylamide gel electrophoresis (SDS-PAGE) technique according to Laemmli (1970). This was carried out using 10% acrylamide gel in Tris-HCl buffer (pH 8.0) containing 0.1% SDS. After electrophoresis, the gel was stained with 0.025% Coomassie brilliant blue R-

250. The gel was then destained and the bands were then compared with standard protein marker (Bio-Rad, USA) and photographed.

## 3. Results

### 3.1 The isolate EG5

From the collected soil samples, twenty five different bacterial isolates were obtained based on their chitinolytic ability on colloidal chitin agar (CCA). Only one isolate was selected to carry out the current investigation due to its promising activity. This isolate was designated as the most chitinolytic active isolate among all the obtained isolates and also sought preliminary to belong to *Bacillus subtilis* group species. This isolate degraded chitin with the development of distinct zone of clearance on CCA reached to about 25 mm in diameter.

### 3.2. *In vitro* chitinase & antimicrobial activity of the isolate EG5

Chitinase activity was tested along with antimicrobial activity of this enzyme using the same culture supernatant (100 µl) of this isolate grown in 0.5 % colloidal chitin after three days of incubation. Agar well diffusion method was used in each case, and, the development of clearance and inhibition zones around the wells were observed and recorded. The enzyme degraded chitin with the development of distinct zone of clearance on CCA reached to about 18 mm in diameter. Also, inhibition of microbial growth was assessed on the basis of presence or absence of an inhibition zone around the agar wells. Table (1) shows the results of this test in order of decreasing activity against the tested microbial strains. The recorded data obviously reveal the potential antifungal activity especially against strains of *Fusarium* tested. Also, while the data represent means of three replicates, any diameters less than 3mm were neglected. This is was obvious through the absence of any appropriate activities against bacterial strains. The largest inhibition zone, reached to 16 mm in diameter, was recorded against *F. graminearum* (NRRL 5883) & *F.* sp. (NRRL 37262).

### 3.3 Identification of the isolate EG5

Classical morphological & physiological bacteriological tests revealed that the isolate was characterized by positive Gram's stain reaction and normal rod shaped, without curved, filaments or coccoid cells. Central ellipsoidal endospores were produced and facultative anaerobic potential was recorded, but, no strict anaerobic conditions were required for growth. Motility, catalase, acid from glucose (without gas production), nitrate reduction & denitrification, and Voges-Proskauer test were all positive. Based on these tests, the isolate was strongly recommended to belong to *Bacillus subtilis* group especially *B. licheniformis* due to the ability of anaerobic growth.

#### 3.3.1 Biolog Identification

Also, the isolate EG5 was subjected for metabolic fingerprinting using GENIII Biolog plates read using Biolog software. Metabolic fingerprint obtained from Biolog identification plate for the isolate EG5 revealed 99% similarity percentage to *B. licheniformis*. As shown in table (2), GENIII dissects and analyzes the ability of the cell to metabolize all major classes of biochemicals, in addition to determining other important physiological properties such as pH, salt & lactic acid tolerance, reducing power, and chemical sensitivity.

#### 3.3.2 Molecular identification of *16S rDNA* gene for the isolate EG5

PCR amplification of the *16S rDNA* gene produced the expected amplicon size of approximately 1500 bp (Figure 1). The partial nucleotide sequence of isolate EG5 *16S rDNA* gene (370 nucleotides) was compared with similar sequences retrieved from DNA databases by using the NCBI n-BLAST search program in the National Center for Biotechnology Information (NCBI) and was multiple-aligned at the same partial sequences of 8 reported *Bacillus* sequences in GenBank using ClustalW program with minor manual adjustments, resulting in 370 positions including the gaps (Figure 2). A phylogenetic tree was generated using the Neighbour-Joining method and bootstrap analysis of 1000 repetitions (Figure 3). It was revealed that the bacterium belonged to the genus *Bacillus* and was closely clustered together with *Bacillus licheniformis.* The amplified *16S rDNA* gene sequence of isolate EG5 was most closely related to that of *B. licheniformis* B16 (GenBank accession number, JX112647) and showed 99.7% identity with the sequence from *B. licheniformis* B16. On the other hand, it was showed 99.2% identity with *B. subtilis* SCS-3 (GenBank accession number, EU257431). The sequence has been deposited in GenBank with accession number JX462644. The partial nucleotide sequence of *B. licheniformis* isolate EG5 *16S rDNA* gene (GenBank accession number, JX462644) revealed highest content for Guanine (G) 118 (31.9%) followed by Adenine (A) 94 (25.4%), then Cytosine (C) 89 (24.1%) and Thymine (T) 69 (18.6%). Data also showed that, C+G content was 207 (56%) and A+T content was 163 (44%). Moreover, the ratio between G+C to A+T was 1.27. On the other hand, bases composition data for *B. licheniformis* isolate EG5 *16S rDNA* gene (GenBank accession number, JX462644) and 8 *Bacillus*

sequences in GenBank was tabulated to determine G+C and A+T ratio (Table 3).

On the basis of the results of the classical bacteriological tests, the Biolog and the analysis of *16S rDNA* gene, it concluded that the isolate was a strain of *B. licheniformis* and was named *B. licheniformis* EG5.

Table 1. *In-vitro* chitinase antimicrobial activity of the isolate EG5

| Test Strain | *Diameter of inhibition zone (mm) |
|---|---|
| *Fusarium graminearum* (NRRL 5883) | 16.00 |
| *Fusarium* sp. (NRRL 37262) | 16.00 |
| *Fusarium pseudograminearum* (NRRL 28062) | 15.00 |
| *Fusarium acaciae-mearnsii* (NRRL 26752) | 15.00 |
| *Fusarium cerealis* (NRRL 25491) | 14.00 |
| *Fusarium acuminatum* (NRRL 29154) | 13.00 |
| *Fusarium asiaticum* (NRRL 6101) | 13.00 |
| *Fusarium virguliforme* (NRRL 34552) | 12.00 |
| *Fusarium mesoamericanum* (NRRL 25797) | 11.00 |
| *Fusarium tucumaniae* (NRRL 34546) | 11.00 |
| *Aspergillus flavus* (NRRL 6541) | 9.00 |
| *Aspergillus niger* (LIV131) | 7.00 |
| *Candida albicans* (IMRU3669) | 5.00 |
| *Bacillus subtilis* (NCTC 10400) | 1.00 |
| *Staphylococcus aureus* (NCTC 7447) | 1.00 |
| *Escherichia coli* (NCTC 10416) | 0.00 |
| *Pseudomonas aeruginosa* (ATCC 10145) | 0.00 |

*****Zone diameters not include the diameter of the agar well (10 mm)

Table 2. Metabolic fingerprint obtained from Biolog identification plate for the isolate EG5

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Negative Control | Dextrin | D-Maltose | D-Trehalose | D-Cellobiose | Gentiobiose | Sucrose | D-Turanose | Stachyose | Positive Control | pH 6 | pH 5 |
| | - | + | + | + | + | + | + | + | - | + | + | + |
| B | D-Raffinose | α-D-Lactose | D-Melibiose | β-Methyl-D-Glucoside | D-Salicin | N-Acetyl-D-Glucosamine | N-Acetyl-β-D-Mannosamine | N-Acetyl-D-Galactosamine | N-Acetyl Neuraminic Acid | 1% NaCl | 4% NaCl | 8% NaCl |
| | - | - | - | + | + | + | - | - | - | + | + | + |
| C | α-D-Glucose | D-Mannose | D-Fructose | D-Galactose | 3-Methyl Glucose | D-Fucose | L-Fucose | L-Rhamnose | Inosine | 1% Sodium Lactate | Fusidic Acid | D-Serine |
| | + | + | + | - | - | - | - | + | - | + | - | - |
| D | D-Sorbitol | D-Mannitol | D-Arabitol | myo-Inositol | Glycerol | D-Glucose- 6-PO4 | D-Fructose- 6-PO4 | D-Aspartic Acid | D-Serine | Troleandomycin | Rifamycin SV | Minocycline |
| | + | + | - | ? | + | - | - | - | - | - | - | - |
| E | Gelatin | Glycyl-L-Proline | L-Alanine | L-Arginine | L-Aspartic Acid | L-Glutamic Acid | L-Histidine | L-Pyroglutamic Acid | L-Serine | Lincomycin | Guanidine HCl | Niaproof 4 |
| | + | - | - | - | - | - | - | - | - | - | + | - |
| F | Pectin | D-Galacturonic Acid | L-Galactonic Acid Lactone | D-Gluconic Acid | D-Glucuronic Acid | Glucuronamide | Mucic Acid | Quinic Acid | D-Saccharic Acid | Vancomycin | Tetrazolium Violet | Tetrazolium Blue |
| | + | + | - | + | + | + | + | - | - | - | - | - |
| G | p-Hydroxy-Phenylacetic Acid | Methyl Pyruvate | D-Lactic Acid Methyl Ester | L-Lactic Acid | Citric Acid | α-Keto-Glutaric Acid | D-Malic Acid | L-Malic Acid | Bromo-Succinic Acid | Nalidixic Acid | Lithium Chloride | Potassium Tellurit |
| | - | ? | - | + | + | - | - | + | - | - | + | + |
| H | Tween 40 | γ-Amino-Butryric Acid | α-Hydroxy-Butyric Acid | β-Hydroxy-D,L-Butyric Acid | α-Keto-Butyric Acid | Acetoacetic Acid | Propionic Acid | Acetic Acid | Formic Acid | Aztreonam | Sodium Butyrate | Sodium Bromate |
| | ? | ? | - | - | - | - | - | - | - | + | + | - |

(+): Positive reaction (-): Negative reaction (?): Positive or Negative reaction (not read)

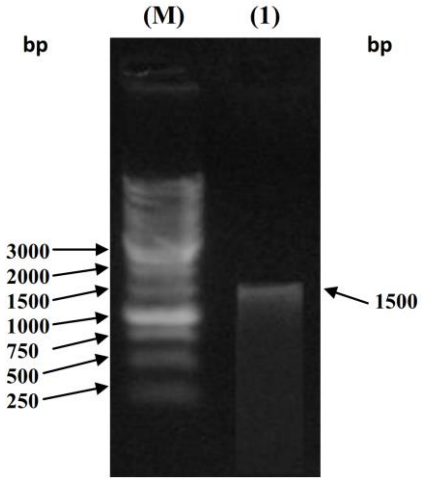


Figure 1. Agarose gel electrophoresis of amplified product of *16S rDNA* gene of the isolate EG5 (1) & the DNA size marker (M).

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EG5_JX462644                        ACGAACGCTG GCGGCGTGCC TAATACATGC AAGTCGAGCG GACAGAAGGG AGCTTGCTCC CTGATGTTAG CGGCGGACGG
Bacillus_licheniformis_JX112647     .......... .......... .......... .......... ......T... .......... .......... ..........
Bacillus_subtilis_EU257431          .......... .......... .......... .......... ......T... .......... .......C.. ..........
Bacillus_sonorensis_JQ712166        .......... .......... .......... .......... ...C..C... .......... ..T.G..C.. ..........
Bacillus_pumilus_AY211128           .......... .......... .......... .......... .......... .......... .G........ ..........
Bacillus_tequilensis_JQ619484       .......... .......... .......... .......... ......T... .......... .......... ..........
Bacillus_amyloliquefaciens_DQ389755 .......... .......... .......... .......... ......T... .......... .......... ..........
Bacillus_vallismortis_NR_024696     .......... .......... .......... .......... ......T... .......... .......... ..........
Bacillus_atrophaeus_AB680855        .......... .......... .......... .......... ......T... .......... .......... ..........

EG5_JX462644                        GTGAGTAACA CGTGGGTAAC CTGCCTGTAA GACTGGGATA ACTCCGGGAA ACCGGGGCTA ATACCGGATG CTTGATTGAA
Bacillus_licheniformis_JX112647     .......... .......... .......... .......... .......... .......... .......... ..........
Bacillus_subtilis_EU257431          .......... .......... .......... .......... .......... .......... .....A.... ..........
Bacillus_sonorensis_JQ712166        .......... .......... .......... .......... .......... .......... .......... ..........
Bacillus_pumilus_AY211128           .......... .......... .......... .......... .......... ..T....... .......... G...T.....
Bacillus_tequilensis_JQ619484       .......... .......... .......... .......... .......... .......... .......... G...T.....
Bacillus_amyloliquefaciens_DQ389755 .......... .......... .......... .......... .......... .......... .......... ....T.....
Bacillus_vallismortis_NR_024696     .......... .......... .......... .......... .......... .......... .......... ....T.....
Bacillus_atrophaeus_AB680855        .......... .......... .......... .......... .......... .......... .......... ....T.....

EG5_JX462644                        CCGCATGGTT CAATTATAAA AGGTGGCTTT TAGCTACCAC TTACAGATGG ACCCGCGGCG CATTAGCTAG TTGGTGAGGT
Bacillus_licheniformis_JX112647     .......... .......... .......... .......... .......... .......... .......... ..........
Bacillus_subtilis_EU257431          .......... .......... .......... .......... .......... .......... .......... ..........
Bacillus_sonorensis_JQ712166        .......... .......... .......... .......... .......... .......... .......... ..........
Bacillus_pumilus_AY211128           .......... ...AC..... .........C -G........ .......... .......... .......... ..........
Bacillus_tequilensis_JQ619484       .......... ...AC..... .........C -G........ .......... .......... .......... ..........
Bacillus_amyloliquefaciens_DQ389755 .......... ...AC..... .........C -G........ .......... .......... .......... ..........
Bacillus_vallismortis_NR_024696     .......... ...AC..... .........C -G........ .......... .......... .......... ..........
Bacillus_atrophaeus_AB680855        .......... ...AC..... .........C -G........ .......... .......... .......... ..........

EG5_JX462644                        AACGGCTCAC CAAGGCAACG ATGCGTAGCC GACCTGAGAG GGTGATCGGC CACACTGGGA CTGAGACACG GCCCAGACTC
Bacillus_licheniformis_JX112647     .......... .......... .......... .......... .......... .......... .......... ..........
Bacillus_subtilis_EU257431          .......... .......... .......... .......... .......... .......... .......... ..........
Bacillus_sonorensis_JQ712166        .......... .......... .......... .......... .......... .......... .......... ..........
Bacillus_pumilus_AY211128           .......... ......G... .......... .......... .A........ .......... .......... ..........
Bacillus_tequilensis_JQ619484       .......... .......... .......... .......... .......... .......... .......... ..........
Bacillus_amyloliquefaciens_DQ389755 .......... .......... .......... .......... .......... .......... .......... ..........
Bacillus_vallismortis_NR_024696     ..T....... .......... .......... .......... .......... .......... .......... ..........
Bacillus_atrophaeus_AB680855        .......... .......... .......... .......... .......... .......... .......... ..........

EG5_JX462644                        CTACGGGAGG CAGCAGTAGG GAATCTTCCG CAATGGACGA AAGTCTGACG
Bacillus_licheniformis_JX112647     .......... .......... .......... .......... ..........
Bacillus_subtilis_EU257431          .......... .......... .......... .......... ..........
Bacillus_sonorensis_JQ712166        .......... .......... .......... .......... ..........
Bacillus_pumilus_AY211128           .......... .......... .......... .......... ..........
Bacillus_tequilensis_JQ619484       .......... .......... .......... .......... ..........
Bacillus_amyloliquefaciens_DQ389755 .......... .......... .......... .......... ..........
Bacillus_vallismortis_NR_024696     .......... .......... .......... .......... ..........
Bacillus_atrophaeus_AB680855        .......... .......... .......... .......... ..........
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Figure 2. Alignment of *16S rDNA* gene of the isolate EG5 (accession no. JX462644) and 8 *Bacillus* sequences using ClustalW program resulting in 370 positions including the gaps.

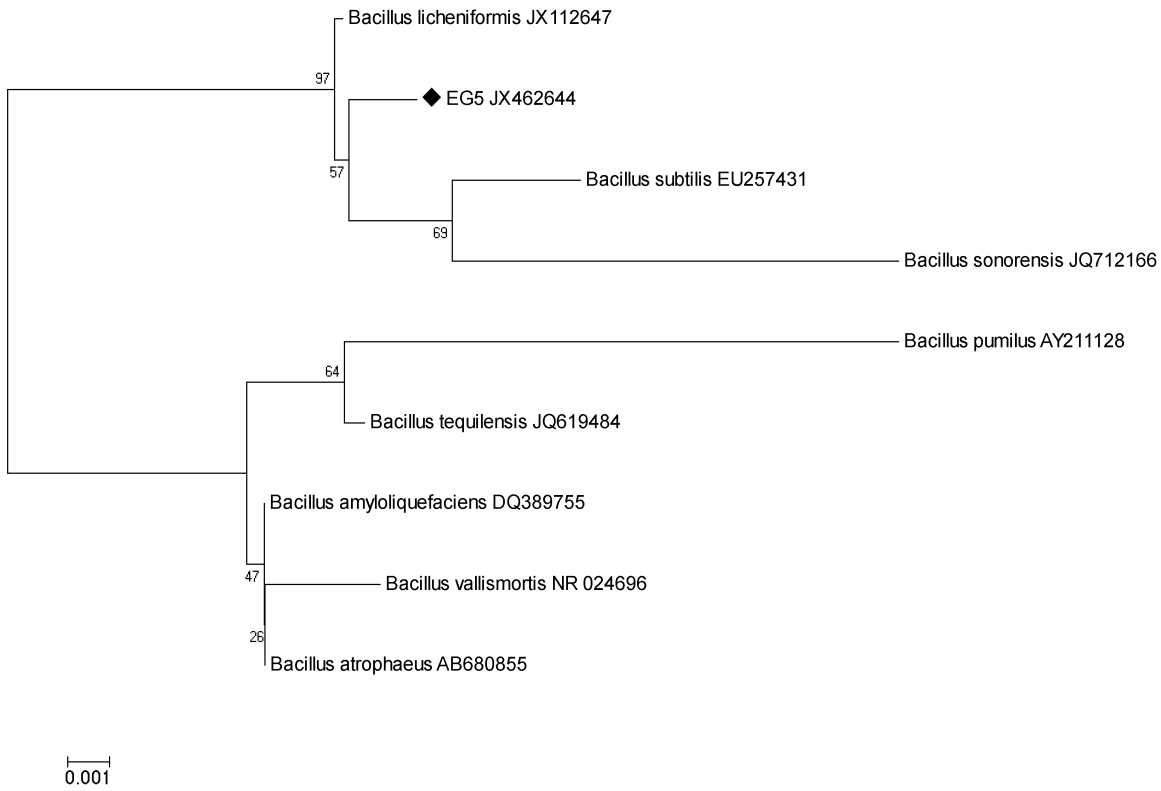


Figure 3. Neighbour-joining tree of *16S rDNA* gene of the isolate EG5 (accession no. JX462644) and 8 *Bacillus* sequences published in GenBank. Numbers represent bootstrap percentage values based on 1000 replicates.

Table 3. Comparison between bases composition of *16S rDNA* gene of the isolate EG5 (accession no. JX462644) and 8 *Bacillus* sequences published in GenBank.

| Isolates | Total (bp) | A | | C | | G | | T | | G+C | | A+T | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | No. | % | No. | % | No. | % | No. | % | No. | % | No. | % |
| EG5 | 370 | 94 | 25.4 | 89 | 24.1 | 118 | 31.9 | 69 | 18.6 | 207 | 56 | 163 | 44 |
| *B. licheniformis* | 370 | 93 | 25.1 | 89 | 24.1 | 118 | 31.9 | 70 | 18.9 | 207 | 56 | 163 | 44 |
| *B. subtilis* | 370 | 94 | 25.4 | 90 | 24.3 | 117 | 31.7 | 69 | 18.6 | 207 | 56 | 163 | 44 |
| *B. sonorensis* | 370 | 92 | 24.9 | 92 | 24.9 | 118 | 31.9 | 68 | 18.3 | 210 | 56.8 | 160 | 43.2 |
| *B. pumilus* | 369 | 93 | 25.2 | 89 | 24.1 | 121 | 32.8 | 66 | 17.9 | 210 | 56.9 | 159 | 43.1 |
| *B. tequilensis* | 369 | 92 | 24.9 | 90 | 24.4 | 120 | 32.5 | 67 | 18.2 | 210 | 56.9 | 159 | 43.1 |
| *B. amyloliquefaciens* | 369 | 92 | 24.9 | 91 | 24.7 | 119 | 32.2 | 67 | 18.2 | 210 | 56.9 | 159 | 43.1 |
| *B. vallismortis* | 369 | 92 | 24.9 | 90 | 24.4 | 119 | 32.2 | 68 | 18.5 | 209 | 56.6 | 160 | 43.4 |
| *B. atrophaeus* | 369 | 92 | 24.9 | 91 | 24.7 | 119 | 32.2 | 67 | 18.2 | 210 | 56.9 | 159 | 43.1 |

**3.4 Enzyme production optimization**

Effect of different incubation factors on the production of chitinase enzyme by the isolate under study was conducted. The variables tested were the incubation period, aerobic incubation under static and shaking conditions, incubation temperature, the pH of the culture medium, and different concentrations of the used substrate under study. The above cultivation medium (colloidal chitin) was used and at the end of incubation, both enzyme activity and the protein concentrations, for each culture under the tested variable were determined, and hence, the specific activities were calculated. In each case, for testing a different variable the initial state used for isolation was kept constant. The incubation under static and shaking conditions (100 & 200 rpm) at 30ºC for five days was tested first. Then, the incubation period was conducted for 1, 2, 3, 5 and 7 days. The effect of different pH values (5, 6, 7, 8 & 9), and temperature range (10, 20, 30, 40 & 50ºC) on chitinase production was conducted. Also, the substrate concentrations tested of the used substrate (colloidal chitin) were 0.1, 0.3, 0.5, 0.7 & 0.9 % (w/v).

Results revealed that the incubation under static state was best where the recorded chitinase activity was 5.30 $UmL^{-1}$ and the protein concentration was 14.70 mg $mL^{-1}$ (Table 4). Results also revealed that the incubation period for three days was best where the recorded chitinase activity was 5.55 $UmL^{-1}$ and the protein concentration was 14.90 mg $mL^{-1}$ (Table 5). On the other hand, while, results in table (6) revealed that incubation at pH value 8.00 was best where the recorded chitinase activity was 5.15 $UmL^{-1}$ and the protein concentration was 14.40 mg $mL^{-1}$, results in table (7) revealed that the incubation at temperature 40ºC was best where the recorded chitinase activity was 5.50 $UmL^{-1}$ and the protein concentration was 13.15 mg $mL^{-1}$. Finally, results revealed that growth of the isolate under study was best on colloidal chitin concentration of 0.7 % (w/v) where the recorded chitinase activity was 5.45 $UmL^{-1}$ and the protein concentration was 13.85 mg $mL^{-1}$ (Table 8).

**3.5 Enzyme production and purification**

Chitinase enzyme from the isolate under study was then produced under the determined optimum conditions. These conditions were static incubation for three days at initial pH 8.00 & incubation temperature 40ºC with chitin concentration 0.7 % (w/v). Under these conditions, the enzyme activity in culture supernatant was 6.18 $UmL^{-1}$ & the protein concentration was 12.75 mg $mL^{-1}$ with specific activity 0.48. Purification of the produced enzyme was then performed (Table 9). The enzyme was subjected to precipitation with ammonium sulphate then collected by centrifugation and dialysis. The enzyme concentrate was loaded on Sephadex G-100 column and *in-vitro* chitinolytic active fractions were recovered and concentrated. This partially purified enzyme was then subjected to enzyme activity and protein concentration determinations. The results obtained revealed that the specific activity, 0.97 calculated for the partially purified chitinase produced by the isolate under study had increased by about factor of two, where, the recorded enzyme activity was 11.35 $UmL^{-1}$ while the protein concentration determined reached to 11.70 mg $mL^{-1}$.

**3.6 Assay of chitinolytic activity of the purified chitinase**

Chitinolytic activity of the partially purified enzyme was examined here by agar well diffusion method again. Chitinolytic zones around the wells were developed on CC agar plates and recorded diameters reached in mean to 23 mm after 12-24 h of incubation at 30ºC. In contrast, controls maintained with heat inactivated enzyme did not record any chitinolytic zones at all.

**3.7 Inhibition of fungal growth by the purified chitinase**

For final assessment of antifungal activity of chitinase produced by the isolate EG5, agar well diffusion test was used also here against *Fusarium* spp. only cultured on PDA plates. Controls inoculated and maintained (with heat inactivated enzyme) for three days did not show any activity against *Fusarium* spp. tested where they showed full growth *i.e.* no inhibition zones were recorded at all. In contrast, inhibition of fungal growth against *Fusarium* spp. tested maintained with normal enzyme produced were recorded in mean diameters of inhibition zones (Table 10).

**3.8 Molecular weight determination of the purified chitinase**

The partially purified chitinase of the isolate EG5 exhibited a distinct protein band about 63 KDa in size (Figure 4).

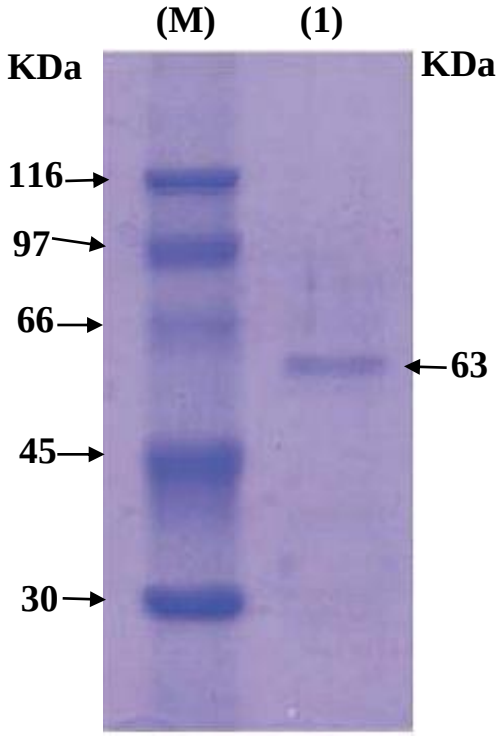


Figure 4. SDS-PAGE of chitinase produced by *B. licheniformis* EG5 (1) & the protein marker (M).

Table 4. Effect of aerobic incubation under static and shaking conditions of the culture medium on chitinase production by the isolate *B. licheniformis* EG5

| Incubation condition | Enzyme activity U $mL^{-1}$ | Protein concentration mg $mL^{-1}$ | Specific activity U $mg^{-1}$ |
|---|---|---|---|
| **Static** | 5.30 | 14.70 | 0.36 |
| **Shaking (100 rpm)** | 3.25 | 15.40 | 0.21 |
| **Shaking (200 rpm)** | 2.50 | 15.50 | 0.16 |

Table 5. Effect of the incubation period of the culture medium on chitinase production by the isolate *B. licheniformis* EG5

| Incubation period days | Enzyme activity U $mL^{-1}$ | Protein concentration mg $mL^{-1}$ | Specific activity U $mg^{-1}$ |
|---|---|---|---|
| **1** | 3.70 | 17.40 | 0.21 |
| **2** | 4.85 | 16.70 | 0.29 |
| **3** | 5.55 | 14.90 | 0.37 |
| **5** | 3.45 | 17.30 | 0.20 |
| **7** | 2.50 | 16.50 | 0.15 |

Table 6. Effect of different initial pH values of the culture medium on chitinase production by the isolate *B. licheniformis* EG5

| Initial pH value | Enzyme activity U $mL^{-1}$ | Protein concentration mg $mL^{-1}$ | Specific activity U $mg^{-1}$ |
|---|---|---|---|
| **5** | 1.15 | 16.35 | 0.07 |
| **6** | 3.90 | 15.20 | 0.26 |
| **7** | 4.65 | 14.10 | 0.33 |
| **8** | 5.15 | 14.40 | 0.36 |
| **9** | 1.95 | 15.90 | 0.12 |

Table 7. Effect of different incubation temperatures of the culture medium on chitinase production by the isolate *B. licheniformis* EG5

| Incubation temperature $^{o}C$ | Enzyme activity U $mL^{-1}$ | Protein concentration mg $mL^{-1}$ | Specific activity U $mg^{-1}$ |
|---|---|---|---|
| **10** | 0.40 | 13.40 | 0.03 |
| **20** | 1.80 | 14.20 | 0.13 |
| **30** | 4.70 | 15.10 | 0.31 |
| **40** | 5.50 | 13.15 | 0.42 |
| **50** | 4.50 | 15.30 | 0.29 |

Table 8. Effect of different chitin concentrations of the culture medium on chitinase production by the isolate *B. licheniformis* EG5

| Chitin concentration % (w/v) | Enzyme activity U $mL^{-1}$ | Protein concentration mg $mL^{-1}$ | Specific activity U $mg^{-1}$ |
|---|---|---|---|
| **0.1** | 2.35 | 16.70 | 0.14 |
| **0.3** | 3.25 | 17.65 | 0.18 |
| **0.5** | 4.45 | 15.10 | 0.29 |
| **0.7** | 5.45 | 13.85 | 0.39 |
| **0.9** | 3.90 | 14.60 | 0.27 |

Table 9. Purification steps of chitinase produced by the isolate EG5

| Purification step | Total Volume (ml) | Total Activity (U) | Total proteins (mg) | Specific activity (U $mg^{-1}$) | Purification Fold (%) | Yield (%) |
|---|---|---|---|---|---|---|
| **Culture Supernatant** | 500 | 3090 | 6375 | 0.48 | 0 | 100 |
| **$(NH_4)_2 SO_4$ Precipitation** | 419 | 2590 | 4888 | 0.53 | 1.10 | 83.82 |
| **Sephadex G-100** | 130 | 1476 | 1521 | 0.97 | 2.02 | 47.77 |

Table 10. Inhibition of fungal growth by the purified chitinase of the isolate EG5

| Test Strain | *Diameter of inhibition zone (mm) |
|---|---|
| *Fusarium graminearum* (NRRL 5883) | 23.00 |
| *Fusarium* sp. (NRRL 37262) | 24.00 |
| *Fusarium pseudograminearum* (NRRL 28062) | 19.00 |
| *Fusarium acaciae-mearnsii* (NRRL 26752) | 20.00 |
| *Fusarium cerealis* (NRRL 25491) | 19.00 |
| *Fusarium acuminatum* (NRRL 29154) | 18.00 |
| *Fusarium asiaticum* (NRRL 6101) | 17.00 |
| *Fusarium virguliforme* (NRRL 34552) | 16.00 |
| *Fusarium mesoamericanum* (NRRL 25797) | 16.00 |
| *Fusarium tucumaniae* (NRRL 34546) | 15.00 |

*****Zone diameters not include the diameter of the agar well (10 mm)

**4. Discussion**

*Bacillus licheniformis* is a Gram-positive, spore-forming bacterium widely distributed as a saprophytic organism in the environment. This species is a close relative of *Bacillus subtilis*. Experiments with cultured bacterial strains suggest that chitinase activity, actively produced during exponential growth phase, mainly produces chitin dimmers (diNAG), but also chitin monomers (NAG) are being released (Horn *et al*. 2006). Beier and Bertilsson (2011) have investigated to what extent chitinolytic bacteria subsidize bacterial populations that do not produce chitinolytic enzymes but still use the products of chitin hydrolysis.

The isolate EG5 under study was selected from a number of different bacterial isolates based on chitinolytic activity on colloidal chitin agar (25 mm clearance zone on CCA). *In-vitro* chitinase activity, 18 mm clearance zone on CCA & antifungal activity 16 mm inhibition zone, mainly against *F. graminearum* (NRRL 5883) & *F.* sp. (NRRL 37262) and absence of any activities against bacteria revealed potential antifungal activity of this isolate.

Identification of the isolate EG5 based on classical bacteriological tests, Biolog metabolic fingerprinting and phylogenetic analysis of 16S rRNA gene revealed that this isolate is strongly recommended to belong to *Bacillus subtilis* group species especially *B. licheniformis* and so, it was named *B. licheniformis* EG5.

It is well known that temperature is one of the factors influence the activity of an enzyme. Also, there is a maximum rate at which a certain amount of enzyme can catalyze a specific reaction that can be achieved when the concentration of substrate is sufficiently high. Effect of different incubation factors on the production of chitinase enzyme by the isolate under study was conducted. The variables tested were the incubation period, aerobic incubation under static and shaking conditions, incubation temperature, the pH of the culture medium, and different concentrations of the used substrate, colloidal chitin.

Chitinase enzyme from the isolate under study was produced under the determined optimum conditions. These conditions were static incubation for three days at initial pH 8.00 & incubation temperature 40$^{o}$C with chitin concentration 0.7 % (w/v). Under these conditions, the enzyme activity was 6.18 $UmL^{-1}$ & the protein concentration was 12.75 mg $mL^{-1}$ with specific activity 0.48. Purification of the produced enzyme resulted in an increase in the specific activity, 0.97, by about factor of two where the recorded enzyme activity was 11.35 $UmL^{-1}$ & the protein concentration determined was 11.70 mg $mL^{-1}$.

Chitinase isolated mainly from thermophilic organisms such as *Bacillus licheniformis* and *Bacillus sp*. have found to be commercialized as they possess inherent stability (Haki and Rakshit., 2003). *Bacillus licheniformis* may produce for example 0.35 U/ml chitinase activities from colloidal chitin at 50°C (Felse and Panda, 2000). Many other studies were previously conducted and reported more or less similar activities from *Bacillus subtilis* group species such as those of San-Lang *et al*. (2002) and Yan *et al*. (2011), *B. amyloliquefaciens* V656 and *B. subtilis* SL-13 antifungal chitinase activities, respectively.

Finally, chitinolytic activity of the partially purified enzyme produced by the isolate under study was examined & chitinolytic zones developed on CCA plates recorded diameters reached in mean to 23 mm after 12-24 h of incubation at 30ºC, while, controls maintained with heat inactivated enzyme did not record any chitinolytic zones at all. Final assessment of antifungal activity of chitinase produced by this isolate against *Fusarium* spp. only was recorded in this case too; (23 mm inhibition zone against *F. graminearum* NRRL 5883 & 24 mm against *F*. sp. NRRL 37262) against controls inoculated and maintained with heat inactivated enzyme that did not show any activity against *Fusarium* spp. The chitinase produced by the isolate EG5 exhibited a distinct protein band, about 63 KDa which owned the antifungal activity, in agreement with many other similar studies.

Similar studies, previously conducted, include that of Xiao *et al*. (2009) who isolated a bacterial strain secreted high levels of extracellular chitinase (4.645 U/ml) when chitin powder existed as an inducer. This strain was identified as *Bacillus licheniformis* using the Biolog MicroLog microbial identification system and sequence analysis of *16S rDNA*, *gyr*A and *rpo*B genes. This strain was able to inhibit the growth of *Gibberella saubinetii* and *Aspergillus niger*. The chitinase was proved to play an important role in this strain as antifungal activity. Also, further studies were conducted by Xiao *et al*. (2010) and revealed that the wild-type produced chitinase (55 KDa) of *Bacillus licheniformis* MY75 owned the antifungal activity.

## 5. Conclusion

The results obtained here addressed the potential of using chitinases as safe antifungal agents to reduce the effects of fungal pathogens on some crops. As an endospore-forming bacterium, the ability of the organism to survive under unfavorable environmental conditions may enhance its potential as a natural biocontrol agent. It can be inferred also from growth conditions of this isolate, which capable to grow under anaerobic conditions (Facultative anaerobe; unlike most other bacilli), that it can be used in biotechnological applications for chitinous wastes bioconversions under solid state fermentation and high degrees of pH and temperatures too.

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