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ORIGINAL ARTICLE

# Screening of Chitinolytic Microfungi and Optimization of Parameters for Hyperproduction of Chitinase Through Solid-State Fermentation Technique

**Muhammad Adnan**[1] · **Muddassar Zafar**[1] · **Zahid Anwar**[1]

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**Abstract**
This study is intended for the production of chitinase enzyme from locally isolated fungal strains. Out of 10 isolated fungal strains from district Gujrat, Punjab, Pakistan, *Aspergillus terreus* SB3 (accession number ON738571) was found with maximum chitinolytic potential (80.8 U/mL/min). By applying central composite design (CCD) through response surface methodology (RSM) under solid-state fermentation (SSF), eight nutritional and physical parameters were optimized. Among these, temperature, substrate concentration, and pH were found as significant factors toward chitinase production in the first phase. Moisture and nitrogen source were found as significant factors during second phase of chitinase production. The effect of incubation period, inoculum size, and magnesium source was observed as non-significant. The chitinase activity was successfully enhanced more than 2 folds up to 198.5 U/mL/min at optimized conditions of 35 °C temperature, 4.5 pH, 20 g substrate concentration, 4-day incubation period, 55% moisture content, 4.5 mL inoculum size, 0.25 g ammonium sulfate, and 0.30 g magnesium sulfate using RSM design. It was also found that *Ganoderma lucidum* (bracket fungus) has more potential to be used for the production of chitinase compared to fish scales. The present study exhibited *Aspergillus terreus* SB3 (ON738571) as a potential indigenous strain capable for hyperproduction of chitinase through cheap fermentation technology that might be employed for the eradication of chitin-based sea waste to remove the marine pollution.

**Keywords** Chitinase · *Aspergillus terreus* · Optimization · Solid-state fermentation (SSF) · Response surface methodology (RSM)

## Introduction

Enzymes are biological catalysts which are central to biological process and are basis of biochemical live. They are protein in nature, and due to their naturally perfected specificity and substrate bias, they are the most efficient natural substrate converters. Enzymology is

✉ Muddassar Zafar
muddassar.zafar@uog.edu.pk

[1] Department of Biochemistry and Biotechnology, University of Gujrat, Hafiz Hayat Campus, Gujrat, Punjab, Pakistan

central to biotechnology at basic, applied, and industrial levels. Their applications are vast arranging from crude formulations to highly value-added, sophisticated biotransformation applications for use in environmental sciences, textile, pharmaceuticals, diagnostics, leather manufacturing, genetic engineering, and healthcare industries. With continuous advances in genetic and protein engineering sciences, the field of enzymology has also transformed its landscape. With more stable, wide spectrum, and rugged enzymes available, it is now possible to engineer enzymes according to application with additional expanding or specifying their pH, temperature, and environmental conditions. However, despite tailoring of enzymes at the molecular level, optimization of production parameters for a single enzyme is sometimes limited due to temperature and surrounding environment, especially in the presence of small toxic molecules which tend to inhibit enzymes in reversible and non-reversible manners. These problems are in continuous considerations of research community and are being addressed as a rationalized approach. The primary application areas of enzymes at industrial scale are textile, food, and healthcare industry.

Different techniques utilizing fungi and bacteria at cellular and nanoscale level are being practiced by various researchers to explore the ways to reduce environmental pollution through green synthesis [1–5]. However, enzymes are preferred for being substrate specific. Living organisms produce enzymes naturally and artificially which are vital for the development and maintenance of life [6]. Enzymes are highly efficient biological molecules that perform a lot of chemical reactions and gained the attention of researchers, scientists, and industrialists because of their wide range of analytical, physiological, and commercial applications with several distinct advantages such as working in mild reaction conditions, lesser energy requirements, simplified product routes, and low physiological and environmental toxicity [7–10]. With advances in biotechnology, chiefly in the areas of protein engineering, genetics, and sequenced data accessibility, enzymes have exciting opportunities for industrial use [11]. However, enzymes have been bioprocessed from microbe, plant, and animal sources but microbes are the most typical source for enzyme production due to their ease of mass culture production and vast biochemical diversity. Apart from industrial use, enzyme application in catalysis of a chemical process is restricted by the instability at high temperatures as well as in toxic solvents [12–14]. Therefore, scientists and researchers are focusing on the production of novel, stable, and robust enzymes that are suitable for broader range of commercial settings [15]. Enzymes gained much more attention due to use in industries like textiles, animal feed, pharmaceuticals, surfactants, and leather processing. The most abundant enzymes which are present in nature are lactases, amylases, chitinases, invertases, cellulases, and lipases [16].

Chitinases (EC 3.2.1.14) are chitin-degrading enzymes that cleave $\beta$-1,4 glycosidic linkage between N-acetyl D-glucosamine units to convert them into oligomers and monomers (N-acetyl D-glucosamine) [17, 18]. Chitinase(s) were first identified by Bernard in 1911, from a fraction derived from orchid pulp. Since then, it has received continuous research focus with more efficient characters of chitinases being reported, e.g., exhibiting thermostable and diffusible characters [19]. Chitinases belong to glycosyl hydrolase family, and molecular mechanism involves cleavage of the 1,4-glycoside linkage in N-acetyl D-glucosamine. They are further subclassified into families of 18 and 19 based on primary stricture; therefore, the tertiary structural features are not identical between members of family 18 and family 19. The family 18 chitinases of family 18 have ($\alpha/\beta$) 8 barrel fold structure in catalytic domain and use substrate-assisted catalytic mechanism, which preserves the anomeric configuration of the product, while chitinases of family 19 consist mainly of high $\alpha$-helical content and use general acid–base catalysis mechanism which inverts the anomeric arrangement of the hydrolyzed GlcNAc residue. Family 18 consists of chitinases from viruses, bacteria, fungi, some plants,

and animal while family 19 contains chitinases from some *Streptomyces* and plant chitinases [20].

The whole living kingdom produces the chitinases but organisms like fungi, bacteria, plants, and mammals produce extensively while insects, viruses, actinomycetes, and vertebrates also synthesize chitinases [20]. These organisms produce chitinase for different purposes like defense against pathogens, parasitism, cell differentiation, digestion and morphogenesis, etc. [17, 20–22]. Naturally, fungi and bacteria majorly degrade the chitin by chitin hydrolyzation [23]. In recent times, chitinases have wide range of applications like pollution management, in medical sciences, single cell protein production, bioremediation, and plant defense against pathogens. This enzyme is mostly produced by bacteria, fungi, plants, animals, and humans. Now, different low-cost and high-yielding techniques are used for the production of efficient chitinases: such as monoculture and coculture, by immobilizing the cells and by using recombinant cells in laboratories and industries. Of which immobilized and recombinant cell techniques are desired for scientist for the production of enriched amount of chitinases [24]. Microbes and their activities transform nutrients into metabolites through the process of fermentation. Fermentation may be categorized as solid-state fermentation (SSF) and submerged fermentation (SmF) [25]. Recent research has found that SSF has a greater influence than SmF on the high yield productivity of improved products [26]. SSF has been recognized as a potential biotechnological process for the production of industrially important pigments, enzymes, bioethanol, antibiotics, mycotoxins, and aroma compounds. Apart from this, SSF has a big advantage of using agro-industrial waste as substrate. This helps in the reduction of environmental pollution produced by the accumulation of such substances [27]. Enzymes are one of the industrially important metabolites produced by the SSF. Enzyme production offers several benefits, including use of agro-industrial wastes, resemblance to natural growth environment, lower energy requirements, high volumetric productivity, facilitated secretion of extracellular bioproducts, and easier downstream processing [26]. The production of chitinase is influenced by many physiochemical parameters like temperature, pH, incubation period, moisture, and nitrogen source [28].

Production of chitinase on industrial scale is a major problem as chitin has complex structure and insoluble in water while production is much costly while chitinase requirement is increasing day by day as it has promising antifungal activity. To solve this problem, industries are using many optimized processes. So, there is an emerging demand for production and characterization of much effective and more stable chitinase by using different optimization conditions [29]. This in turn incites the researchers to divert their attention toward the use of agricultural and industrial waste as substitute for producing chitinase which may also be very helpful to reduce sea waste which is majorly due to the amassing of chitin at sea bed. The objective of the present study is to screen local chitinase producing fungi, molecular identification of high chitinase producing fungal strain, and optimization of cultural conditions through solid-state fermentation (SSF) using response surface methodology (RSM). It will not only fulfill the industrial requirement of Pakistan but also helps in eradication of sea waste.

## Material and Methods

### Sample Collection

Fungal strains were isolated from different decaying materials such as rotten fruits, stems, and branches collected from different areas of district Gujrat, Punjab, Pakistan, and were

kept in sterilized plastic bags and brought to the laboratory for further processing. Potato Dextrose Agar (PDA) solid media was used for the growth of different fungal strains. To achieve maximum growth of different fungal strains, plates were incubated at 37 °C for 7 days. After attaining maximum growth, spores of different fungal strains (based on different colony color) were transferred to slants. The slants were prepared to get the pure fungal culture.

For inoculums of different fungal isolates, liquid PDA media (2 g D-glucose, 0.05 g $CaCl_2$, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.02 g $(NH_4)_2SO_4$, and 0.02 g $KH_2PO_4$ per 100 mL) was prepared. All the ingredients were mixed and autoclaved for 20 min at 15 lb pressure and 121 °C. In laminar air flow (LAF) hood, fungal spores of different fungal isolates from stored slants were transferred to autoclaved flasks containing growth media by using sterilized loop. Inoculated flasks were kept in shaking incubator (150 rpm) at 37 °C for 7 days.

## Substrate Collection

*G. lucidum* (basidiomycete white rot macrofungus) and fish scales were used as raw substrates for production of chitinase enzyme. The macrofungus was collected from different localities of district Gujrat while the fish scales were collected from a local fish market. The collected substrate was washed with distilled water and air dried. The substrate was ground to get fine powder. Pure chitin flakes were commercially purchased from Sigma-Aldrich (USA).

## Preparation of Colloidal Chitin

In order to perform enzyme assay, pure chitin flakes were converted to soluble form known as colloidal chitin. For this purpose, chitin flakes were ground into fine powder. Chitin powder (2.5 g) was mixed gently into 45 mL of conc. HCl with continuous rapid stirring. Absolute ethanol (250 mL) was added with continuous stirring. The mixture was placed overnight at 25 °C. Subsequently, the mixture was centrifuged at 4400 rpm at 4 °C for 10 min. Supernatant was discarded while pellet containing colloidal chitin was washed with distilled water and phosphate buffer (pH 7.0). For further use, colloidal chitin was placed in the dark at 4 °C as described [30].

## Screening of Chitinolytic Fungal Isolates Using Solid-State Fermentation (SSF)

Chitinase-producing fungal isolates were screened through SSF using *G. lucidum* and fish scale powder as substrates. In order to perform SSF, 5 g of each substrate was mixed with 5 mL distilled water in flasks and inoculated with 3 mL of inoculum of different fungal isolates. The flasks were incubated for 7 days at 37 °C. The experiments were carried out in triplicates.

## Enzyme Extraction

After 7 days of incubation, all trial flasks were collected from an incubator. Each flask was filled with 50 mL of distilled water and placed in a shaking incubator at 37 °C for an hour. The enzymes produced by various fungal strains were first separated by centrifugation at

4400 rpm for 10 min at 4 °C after being filtered through a cheese cloth. The supernatant was collected for enzyme activity test, and pellet was discarded.

### Enzyme Assay

For this purpose, colloidal chitin solution was prepared by mixing 1 g of colloidal chitin in 100 mL of phosphate buffer [30]. To observe the chitinase activity, 0.9 mL of colloidal chitin solution was mixed with 0.1 mL of crude enzyme. All test tubes containing enzyme substrate mixture were incubated at 45 °C for 30 min. After incubation, 3 mL of dinitrosalicylic acid (DNS) was added to each test tube to stop the reaction. The test tubes were kept in water bath at 100 °C for 5 min. The test tubes were later placed in cold water for 3 min. DNS reacted with reducing sugar N-acetyl D-glucosamine (NAG) monomers produced by the hydrolysis of chitin by chitinase and formed complexes during boiling. The concentrations of these complexes were observed at 540 nm using a spectrophotometer [31].

### Determination of Protein Content

Standard Bradford assay was used to assess the total protein contents present in enzyme extract by Bovine Serum Albumin (BSA) as a standard protocol, and absorbance was noted at 595 nm using a UV/visible spectrophotometer (model T80 + , PG instruments, UK) [32].

### Identification of Chitinolytic Fungal Isolate

18S rRNA sequence analysis was employed for the molecular identification of isolated strain. The universal primers NS1 5′ (GTA GTC ATA TGC TTG TCT C) 3′ and NS8 5′ (TCC GCA GGT TCA CCT ACG GA) 3′ were employed for the amplification of 18S rRNA gene from fungal DNA using Polymerase Chain Reaction (PCR). Total 30 µL reaction mixture was prepared using 20 ng of genomic DNA for PCR by employing *EF-Taq*. Initial denaturation was performed at 95 °C for 2 min. Followed by 35 cycles of 95 °C, 55 °C, and 72 °C (1 min each temperature), respectively, with a 10 min finishing step at 72 °C in purification step, the amplified products were subjected to multi-screen filter plate. For sequencing, a PRISM BigDye Terminator v3.1 Cycle sequencing kit was used followed by subjection of DNA extension products to Hi-Di formamide. DNA evaluation was performed on ABI Prism 3730XL DNA analyzer followed by incubation at 95 °C and ice cooling for 5 min each. The elucidated sequence of 18 s rRNA gene of fungus was subjected to BLAST tool at NCBI website for evaluation followed by generation of phylogenetic tree using MEGA 7.0 version [33].

### Optimization of Fermentation Parameters by RSM

The different factors used to assess their relationship in SSF included amount of substrate, temperature, pH, incubation period, moisture content, inoculum size, nitrogen source, and magnesium source. To optimize all these factors, central composite design (CCD) of RSM was used. As multiple factors were involved to be addressed for enzyme production, therefore, experiment was performed in two stages to increase the accuracy of results.

During first stage, physical parameters of substrate concentration, temperature, pH, and incubation days were optimized to harvest maximum amount of enzyme. For this

purpose, substrate (1–15 g) was mixed with 1–15 mL of buffer solutions (to achieve 100% humidity); moreover, pH variations, e.g., acetate buffer (3.0–5.0 pH) and phosphate buffer (5.0–8.0 pH), in each flask were also used to assess enzyme activity. After sterilization, the flasks were inoculated with 5 mL inoculum aseptically and placed in an incubator for different incubation periods (1–8 days) at different temperatures (15–55 °C).

After the optimization of initial four parameters, the second stage of RSM was performed. During this stage, four secondary physiochemical parameters including moisture content (5–125%), inoculum size (0.5–9.5 mL), nitrogen source (0.05–4.25 g), and magnesium source (0.05–0.7 g) were optimized with first four optimized parameters.

In RSM, CCD is the most often employed experimental design, and it displays equal confidence in all directions from the center. The statistical significance of the model equation was examined using the F-test analysis of variance (ANOVA). The second-order polynomial equations were produced by Design Expert software (trial version 15, Stat-Ease Inc., USA) using RSM to examine experimental data. Regression was used to produce coefficients of second order. Multiple regressions were first used to fit the response to the variables. The coefficients of determination and the analysis of variance were used to assess how well the two models fit each other. The following equations were used to fit the two quadratic response surface models developed by central composite design:

Chitinase activity (U/mL/min) = −90.03765 + 5.29159 temperature − 3.19094 substrate concentration + 25.74608 pH + 30.38723 incubation period − 0.059375 temperature * substrate concentration − 0.006250 temperature * pH − 0.223438 temperature * incubation period + 0.687500 substrate * pH + 0.403125 substrate * incubation period − 0.864583 pH * incubation period − 0.055936 temperature$^2$ + 0.048788 substrate$^2$ − 2.98606 pH$^2$ − 2.66337 incubation period$^2$

Chitinase activity (U/mL/min) = +92.06958 + 1.69851 moisture + 16.59466 inoculum − 7.76240 nitrogen source − 38.90268 magnesium source + 0.070357 moisture * inoculum − 0.127857 moisture * nitrogen source + 0.924107 moisture * magnesium source + 0.150000 inoculum * nitrogen source + 6.31250 inoculum * magnesium source + 16.12500 nitrogen source * magnesium source − 0.020196 moisture$^2$ − 2.27420 inoculum$^2$ − 0.130269 nitrogen source$^2$ − 127.17357 magnesium source$^2$

## Results

### Screening of Fungi and Substrates for Chitinase Production

Ten different fungal strains isolated from different sources belonging to different areas of district Gujrat, Punjab, Pakistan, were screened for chitinase production (Fig. 1). The powder of *G. lucidum* (bracket fungus) and fish scales were used as substrates for chitinase production (Fig. 2). One of the strains named SB3 yielded maximum chitinase activity (80.8 U/mL/min) by using bracket fungus as substrate (Fig. 3). This strain was therefore selected to proceed for further experimentation.

### Molecular Identification of Fungal Strain

The molecular identification of the best chitinase-producing fungal strain SB3 (Fig. 4) was carried out by amplification and sequencing of 18S rRNA gene. The obtained sequence of gene was compared with closely related other sequences available in GenBank through

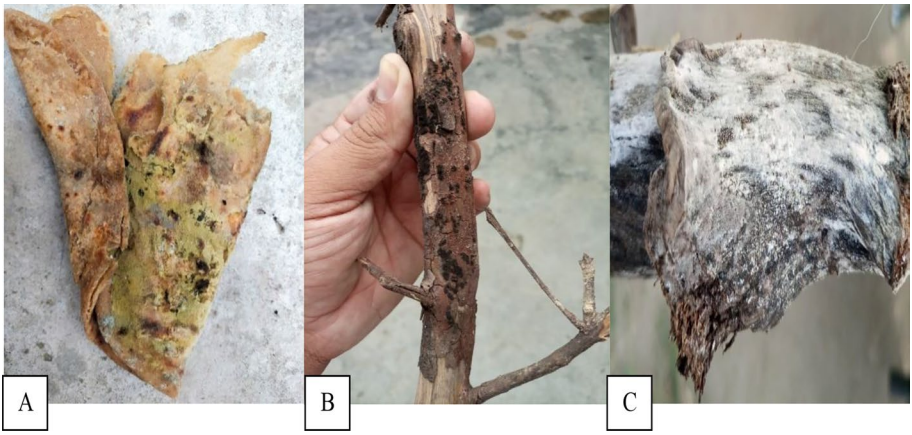


**Fig. 1** Collection of fungi from different sources **A** rotten bread, **B** guava branch, and **C** banana stalk

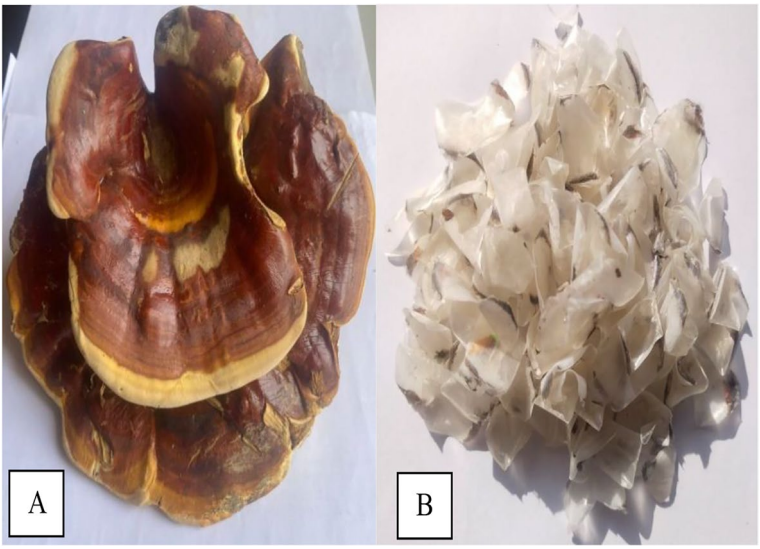


**Fig. 2** Different substrates used for chitinase production **A** *G. lucidum* and **B** fish scales

nBlast for the phylogenetic relationship analysis as shown in Fig. 5. The 18S rRNA gene sequence was also used to construct a dendrogram. The nucleotide sequence of the fungal isolate SB3 was submitted to NCBI GenBank under the accession number ON738571 as *Aspergillus terreus*.

## Optimization of Fermentation Parameters for Maximum Chitinase Production

The production of chitinase from fungi was greatly influenced by composition of growth media. From commercial prospective, the optimization of different parameters is significant for enzyme production by using low-cost media on large scale. Therefore, the effect

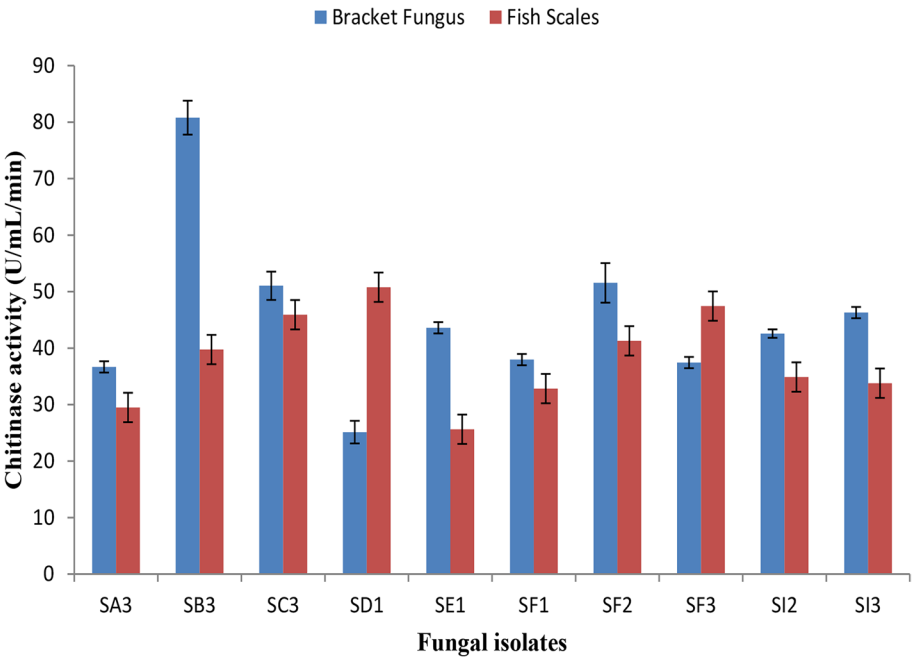


**Fig. 3** Screening of fungal strains and substrates for chitinase production. The maximum chitinase was produced by fungal strain named SB3 using bracket fungus as substrate


**Fig. 4** Colony of maximum chitinase-producing fungus (*A. terreus* SB3)

of different physiochemical parameters such as temperature, pH, incubation days, and substrate content was studied during the first stage of enzyme production. The maximum chitinase activity (125.5 U/mL/min) was found in treatment # 4 at 35 °C temperature, 20 g substrate concentration, 4.5 pH, and incubation period of 4 days (Table 1).

After optimizing the first four parameters, additional four parameters including moisture, inoculum size, nitrogen source, and magnesium source were optimized and their effect on enzyme production was observed during the second stage of experimentation.

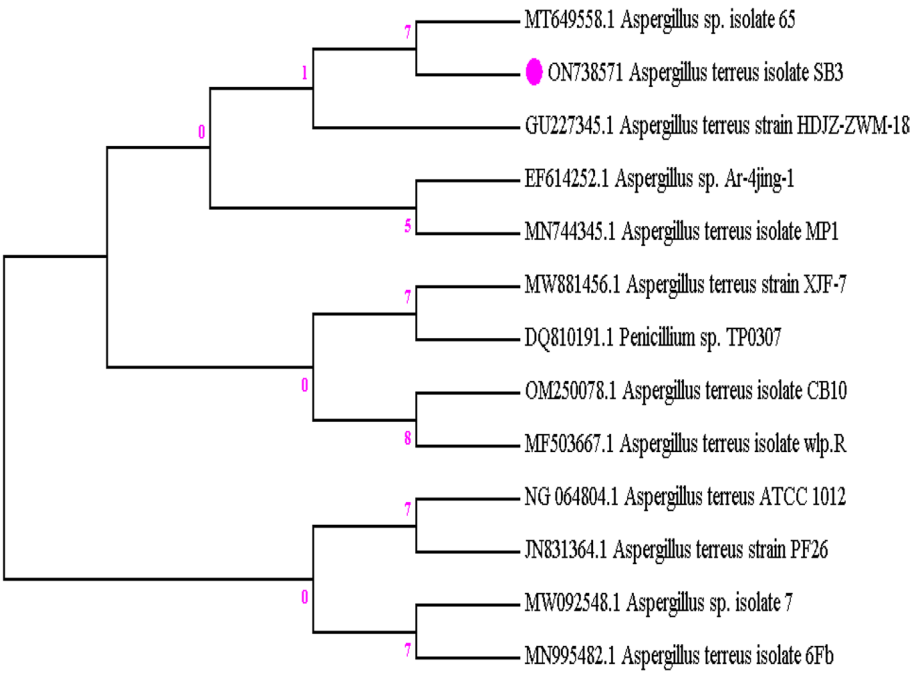


**Fig. 5** Phylogenetic tree of *Aspergillus terreus* SB3 deduced by neighborhood joining method

The maximum chitinase activity (198.5 U/mL/min) was found in treatment # 10 at 55% moisture, 4.5 mL inoculum size, 0.25 g nitrogen source, and 0.30 g magnesium source (Table 2). The optimization of these fermentation parameters was performed using response surface methodology under SSF. All the parameters were analyzed using Design Expert [34].

### Effect of Temperature

Different temperature ranges (15, 25, 35, 45, and 55 °C) were designed by RSM and applied to SSF medium. The results displayed that the maximum activity of chitinase was observed at 35 °C.

### Effect of pH

Different pH values (1.5, 3, 4.5, 6, and 7.5) were designed by RSM and applied in SSF. The maximum chitinase activity was observed at pH 4.5, indicating that chitinases are naturally acidic.

### Effect of Incubation Period (Days)

Different time intervals 1, 2, 4, 6, and 8 days were applied for chitinase production by using RSM. The maximal chitinase activity was observed in fermented medium after 4 days of incubation.

**Table 1** Optimization of different fermentation parameters for chitinase production using RSM (phase I)

| Treatment # | Temp. (°C) | Substrate conc. (g) | pH | Incubation (days) | Chitinase activity (U/mL/min) |
|---|---|---|---|---|---|
| T1 | 35 | 10 | 3.0 | 8 | 74 |
| T2 | 35 | 10 | 4.5 | 2 | 79.5 |
| T3 | 35 | 10 | 7.5 | 4 | 89.5 |
| T4 | 35 | 20 | 4.5 | 4 | 125.5 |
| T5 | 35 | 10 | 4.5 | 4 | 113.5 |
| T6 | 35 | 5 | 4.5 | 4 | 99.5 |
| T7 | 15 | 10 | 4.5 | 4 | 90.5 |
| T8 | 35 | 10 | 1.5 | 4 | 77.5 |
| T9 | 35 | 10 | 4.5 | 4 | 113.5 |
| T10 | 55 | 10 | 4.5 | 4 | 85.5 |
| T11 | 45 | 5 | 6.0 | 2 | 99 |
| T12 | 25 | 5 | 3.0 | 6 | 97.5 |
| T13 | 25 | 5 | 3.0 | 2 | 86 |
| T14 | 45 | 15 | 6.0 | 6 | 95 |
| T15 | 25 | 5 | 6.0 | 6 | 88 |
| T16 | 35 | 10 | 4.5 | 4 | 113.5 |
| T17 | 25 | 15 | 3.0 | 6 | 101.5 |
| T18 | 35 | 10 | 4.5 | 4 | 113.5 |
| T19 | 35 | 10 | 4.5 | 4 | 113.5 |
| T20 | 45 | 5 | 3.0 | 2 | 98.5 |
| T21 | 45 | 15 | 3.0 | 2 | 74.5 |
| T22 | 25 | 15 | 3.0 | 2 | 74.5 |
| T23 | 25 | 15 | 6.0 | 6 | 113.5 |
| T24 | 45 | 5 | 6.0 | 6 | 82.5 |
| T25 | 45 | 15 | 3.0 | 6 | 84 |
| T26 | 45 | 15 | 6.0 | 2 | 96.5 |
| T27 | 35 | 10 | 4.5 | 4 | 113.5 |
| T28 | 45 | 5 | 3.0 | 6 | 91.5 |
| T29 | 25 | 5 | 6.0 | 2 | 88 |
| T30 | 25 | 15 | 6.0 | 2 | 96 |

## Effect of Substrate Concentration

In current experimental study, bracket fungus *G. lucidum* was found as the best substrate and different substrate concentrations (2, 5, 10, 15, and 20 g) were applied by RSM in the fermented media to observe its effect on chitinase production. The maximum activity was observed using 20 g of fermented substrate.

**Table 2** Optimization of different fermentation parameters for chitinase production using RSM (phase II)

| Treatment # | Moisture (%) | Inoculum (mL) | Nitrogen source (g) | Magnesium source (g) | Chitinase activity (U/mL/min) |
|---|---|---|---|---|---|
| T1 | 55 | 9.5 | 1.75 | 0.30 | 112 |
| T2 | 55 | 4.5 | 1.75 | 0.30 | 150 |
| T3 | 5 | 4.5 | 1.75 | 0.30 | 112 |
| T4 | 125 | 4.5 | 1.75 | 0.30 | 50.5 |
| T5 | 55 | 0.5 | 1.75 | 0.30 | 113 |
| T6 | 55 | 4.5 | 1.75 | 0.30 | 155.5 |
| T7 | 55 | 4.5 | 4.25 | 0.30 | 124.5 |
| T8 | 55 | 4.5 | 1.75 | 0.70 | 136.5 |
| T9 | 55 | 4.5 | 1.75 | 0.05 | 149.5 |
| T10 | 55 | 4.5 | 0.25 | 0.30 | 198.5 |
| T11 | 90 | 2.0 | 3.00 | 0.50 | 84 |
| T12 | 90 | 7.0 | 0.50 | 0.50 | 131.5 |
| T13 | 90 | 2.0 | 0.50 | 0.10 | 108.5 |
| T14 | 20 | 7.0 | 0.50 | 0.10 | 121.5 |
| T15 | 90 | 2.0 | 3.00 | 0.10 | 72.5 |
| T16 | 20 | 2.0 | 3.00 | 0.10 | 117.5 |
| T17 | 20 | 2.0 | 0.50 | 0.50 | 108 |
| T18 | 90 | 7.0 | 0.50 | 0.10 | 130.5 |
| T19 | 55 | 4.5 | 1.75 | 0.30 | 150 |
| T20 | 55 | 4.5 | 1.75 | 0.30 | 150 |
| T21 | 20 | 2.0 | 3.00 | 0.50 | 100.5 |
| T22 | 20 | 7.0 | 3.00 | 0.10 | 118 |
| T23 | 20 | 7.0 | 3.00 | 0.50 | 108 |
| T24 | 55 | 4.5 | 1.75 | 0.30 | 150 |
| T25 | 90 | 7.0 | 3.00 | 0.50 | 112.5 |
| T26 | 90 | 2.0 | 0.50 | 0.50 | 100 |
| T27 | 90 | 7.0 | 3.00 | 0.10 | 82 |
| T28 | 55 | 4.5 | 1.75 | 0.30 | 150 |
| T29 | 20 | 7.0 | 0.50 | 0.50 | 108 |
| T30 | 20 | 2.0 | 0.50 | 0.10 | 136.5 |

## Effect of Inoculum Size

Different inoculum sizes 0.5, 2, 4.5, 7, and 9.5 mL were designed by RSM and applied to SSF to produce fungal chitinase. Maximum production of chitinase was observed when 4.5 mL of inoculum was added to the fermented medium.

### Effect of Moisture

Different moisture content levels (5, 20, 55, 90, and 125%) were designed by RSM and applied in SSF medium. Moisture for fermentation played a vital role for the maximum production of chitinase enzyme. Maximum chitinase activity was found at 55% moisture.

### Effect of Nitrogen Concentration

Nitrogen source played a vital role in the production of chitinase. Ammonium sulfate $(NH_4)_2SO_4$ was used as nitrogen source. Different concentrations of 0.25, 0.5, 1.75, 3, and 4.25 g were applied by RSM. The maximum activity was observed at concentration of 0.25 g.

### Effect of Magnesium Concentration

Magnesium source was not found as a significant factor in the production of chitinase. Magnesium sulfate ($MgSO_4$) was used as magnesium source. Different concentrations of 0.25, 0.5, 1.75, 3, and 4.25 g were applied through RSM design. The maximum chitinase production was observed at concentration of 0.30 g. The effects of all above parameters are shown in Fig. 6A–H.

### ANOVA of Quadratic Models

The models' $F$ value of 20.11 (first phase of chitinase production) and 17.21 (second phase of chitinase production) implies that models are significant (with only 0.01% chance that the value could occur due to noise). Response surface model was used to calculate the $R^2$ value as coefficient of determination to check the fitness of model. The influence of variables and interaction terms on the response was reflected by significant test and ANOVA to evaluate the adequacy of the model. The detailed analysis of the consequence of variables and their interactions on the different response were also implemented by surface plot by using Design Expert 15 software.

Based on the significance test results as mentioned in Table 4, the $p$ values of temperature, substrate concentration, pH, moisture, and nitrogen concentration were found less than 0.05, seeing 95% as level of confidence ($\alpha = 0.05$). These were found as significant parameters for production of chitinase from *A. terreus* SB3. The square term $p$ values of temperature, pH, incubation period, moisture, inoculum size, and magnesium source variables were less than confidence levels, showing the non-linear relationship by response on chitinase production from *A. terreus* isolate SB3, while the substrate and nitrogen source concentration square term $p$ values are greater than confidence interval which showed the linear relationship for the response on chitinase production. The $p$ values of interaction between variables such as temperature and substrate, temperature and incubation period, substrate and pH, substrate and incubation period, pH and incubation period, moisture and inoculum size, moisture and nitrogen concentration, and moisture and magnesium concentration were less than confidence level which showed the significant relationship with the response, chitinase production. However, the $p$ values of interaction between variables such as temperature and pH, inoculum size and

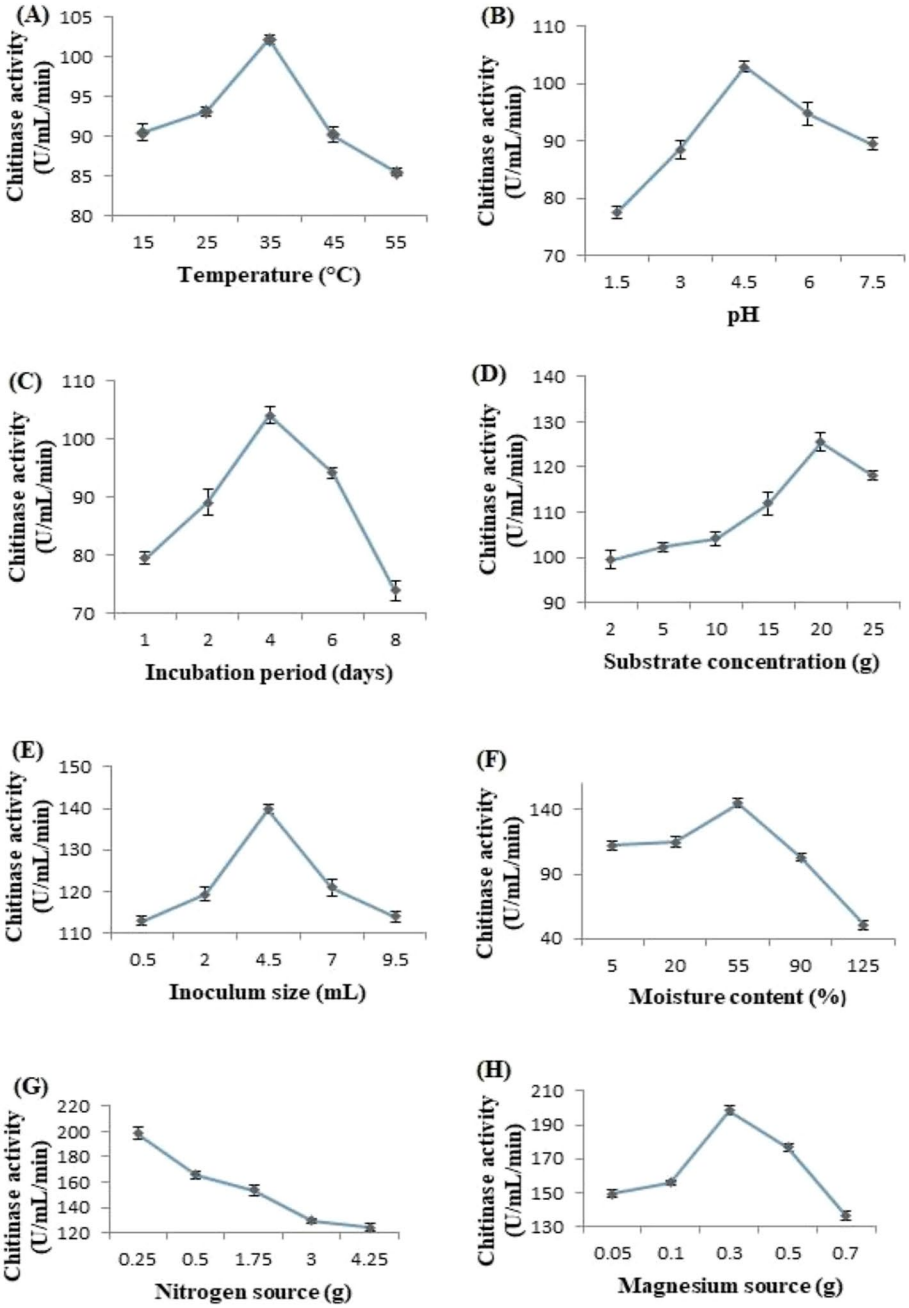


**Fig. 6** Effect of different fermentation parameters **A** temperature, **B** pH, **C** incubation period, **D** substrate concentration, **E** inoculum size, **F** moisture content, **G** nitrogen source, and **H** magnesium source on the production of chitinase from *A. terreus* SB3

nitrogen concentration, inoculum size and magnesium concentration, and nitrogen concentration and magnesium concentration were greater than $\alpha = 0.05$ which showed the non-significant relationship with the response, chitinase production. The influence of linear, square, and 2-way interaction terms was significant to the response (chitinase activity), where $p$ values of these variables were less than $\alpha = 0.05$; meanwhile, interaction terms were non-significant toward the response (chitinase activity), where $p$ values were greater than $\alpha = 0.05$ and all these had been revealed through ANOVA results as mentioned in Tables 3 and 4. The same kind of statistical models has been used by other researchers for studying such interactions among different parameters [35].

The regression square ($R^2$) of RSM model for the first phase of production of chitinase enzyme was found equal to 0.9494 and the $p$ value of this model was < 0.0001 which revealed that the established model is an acceptable predictor of the investigational conditions and had less error and established that the selected SSF progression variables significantly affect the chitinase production from *A. terreus* isolate SB3.

The regression square ($R^2$) of the RSM model for the second phase of production of chitinase enzyme was found equal to 0.9414 and the $p$ value of this model was < 0.0001which revealed that the established model is an acceptable predictor of the investigational conditions and had less error and selected SSF progression variables significantly affected the chitinase production from *A. terreus* isolate SB3. The significant interactions among multiple factors and parameters of fermentation experiment through 3-D surface plots for chitinase production are shown in Figs. 7 and 8 while the non-significant interactions are shown in Fig. 9.

**Table 3** Regression table of chitinase activity (U/mL/min) vs. temperature, substrate, incubation period (days), and pH during the first phase of chitinase production

| Source | Sum of squares | $d_f$ | Mean square | $F$ value | $p$ value |
|---|---|---|---|---|---|
| Model | 5555.53 | 14 | 396.82 | 20.11 | < 0.0001 |
| *A*—temperature | 200.78 | 1 | 200.78 | 10.18 | 0.0061 |
| *B*—substrate | 247.92 | 1 | 247.92 | 12.57 | 0.0029 |
| *C*—pH | 252.93 | 1 | 252.93 | 12.82 | 0.0027 |
| *D*—incubation period | 56.30 | 1 | 56.30 | 2.85 | 0.1118 |
| *AB* | 141.02 | 1 | 141.02 | 7.15 | 0.0174 |
| *AC* | 0.1406 | 1 | 0.1406 | 0.0071 | 0.9338 |
| *AD* | 319.52 | 1 | 319.52 | 16.20 | 0.0011 |
| *BC* | 425.39 | 1 | 425.39 | 21.56 | 0.0003 |
| *BD* | 260.02 | 1 | 260.02 | 13.18 | 0.0025 |
| *CD* | 107.64 | 1 | 107.64 | 5.46 | 0.0338 |
| $A^2$ | 875.73 | 1 | 875.73 | 44.39 | < 0.0001 |
| $B^2$ | 30.72 | 1 | 30.72 | 1.56 | 0.2312 |
| $C^2$ | 1263.41 | 1 | 1263.41 | 64.04 | < 0.0001 |
| $D^2$ | 2209.97 | 1 | 2209.97 | 112.02 | < 0.0001 |
| Residual | 295.93 | 15 | 19.73 | | |
| Lack of fit | 295.93 | 10 | 29.59 | | |
| Pure error | 0.0000 | 5 | 0.0000 | | |
| Cor total | 5851.47 | 29 | | | |

**Table 4** Regression table of chitinase activity (U/mL/min) vs. moisture, inoculum, nitrogen source, and magnesium source during the second phase of chitinase production

| Source | Sum of squares | df | Mean square | *F* value | *p* value |
|---|---|---|---|---|---|
| Model | 23,496.25 | 14 | 1678.30 | 17.21 | < 0.0001 |
| *A*—moisture | 4438.56 | 1 | 4438.56 | 45.51 | < 0.0001 |
| *B*—inoculum | 112.48 | 1 | 112.48 | 1.15 | 0.2998 |
| *C*—nitrogen source | 1907.28 | 1 | 1907.28 | 19.55 | 0.0005 |
| *D*—magnesium source | 20.82 | 1 | 20.82 | 0.2134 | 0.6507 |
| *AB* | 606.39 | 1 | 606.39 | 6.22 | 0.0248 |
| *AC* | 500.64 | 1 | 500.64 | 5.13 | 0.0387 |
| *AD* | 669.52 | 1 | 669.52 | 6.86 | 0.0193 |
| *BC* | 3.52 | 1 | 3.52 | 0.0360 | 0.8520 |
| *BD* | 159.39 | 1 | 159.39 | 1.63 | 0.2206 |
| *CD* | 260.02 | 1 | 260.02 | 2.67 | 0.1233 |
| $A^2$ | 11,580.00 | 1 | 11,580.00 | 118.72 | < 0.0001 |
| $B^2$ | 4234.78 | 1 | 4234.78 | 43.42 | < 0.0001 |
| $C^2$ | 0.7108 | 1 | 0.7108 | 0.0073 | 0.9331 |
| $D^2$ | 451.78 | 1 | 451.78 | 4.63 | 0.0481 |
| Residual | 1463.09 | 15 | 97.54 | | |
| Lack of fit | 1437.88 | 10 | 143.79 | 28.52 | 0.0009 |
| Pure error | 25.21 | 5 | 5.04 | | |
| Cor total | 24,959.34 | 29 | | | |

## Discussion

A variety of prokaryotic and eukaryotic microorganisms are capable of producing chitinases, and the composition of the medium can have a substantial impact on the production. The current study focuses on impacts of culture medium components and factors on enzyme production through application of statistical approaches based on experimental design like response surface methodology. These techniques provide for a better comprehension of potential interactions between examined factors [36]. Response surface methodology has been widely used in industry to improve the production procedures. Recent studies have suggested the use of RSM for evaluating the effects of various parameters on proteolytic activity and maximizing the enzyme production [37]. The models followed during the present research work successfully helped to analyze the impact of physiochemical inputs on chitinase production from *A. terreus*.

The 30 factorial central composite designs (CCD) of RSM were employed in the current investigation to optimize pH, temperature, substrate concentration, incubation period (first phase of chitinase production), moisture, inoculum size, nitrogen source, and magnesium source (second phase of chitinase production) utilizing Design Expert software (trial version 13, Stat-Ease, USA). The CCD made it possible to find the ideal temperature, pH level, substrate concentration, incubation period, moisture, inoculum size, nitrogen source, and magnesium source for maximum production of enzyme. The SSF technique was applied to ferment *G. lucidum* powder while CCD was effectively applied to optimize major parameters which have a significant effect to increase chitinase activity.

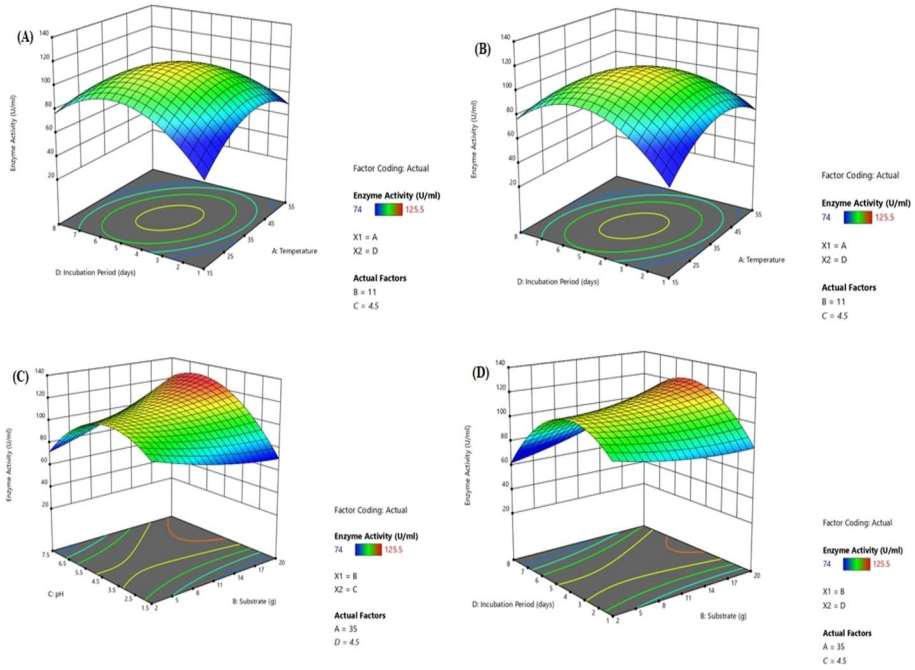


**Fig. 7** Surface plot showing significant interaction between **A** temperature vs. substrate, **B** temperature vs. incubation period, **C** substrate concentration vs. pH, and **D** substrate concentration vs. incubation period for maximum production of chitinase

The studies of different researchers have also elucidated SSF as a better technique compared to submerged fermentation for production of enzymes. SSF is a simple and economical technique wherein natural conditions of fungi are used to produce enzymes. Another advantage of using SSF is the economical substrate with huge nutrients for microbial growth that could contribute to economize the overall process cost at the industrial level. In literature, the researchers have used different fungal strains for chitinases under submerged conditions, i.e., *Alternaria alternate* on colloidal chitin (6.41U/mL) [38], *Aspergillus fumigatus* on colloidal chitosan (5.86 U/mL) [39], *Basidiobolus ranarum* (3.47 U/mL) [40] and *Colletotrichum gloeosporioides* (14.93 U/mL) [41] using colloidal chitin, *Rhizopus oryzae* on starch (3.56 U/mL) [42], *Trichoderma aureoviride* (0.036 U/mL) [43] and *T. harzianum* (14.7 U/mL) [44] using colloidal chitin, *T. harzianum* (66.5 U/mL) [45] on rice bran, *T. virens* (0.147 U/mL) [46] on colloidal chitin, and *Trichothecium roseum* (0.78 U/mL) [47] on crab shell chitin. However, compared to previous findings of researchers, our indigenous employed strain *A. terreus* SB3 (ON738571) exhibited maximum chitinase activity of 198.5 U/mL/min under SSF, revealing that the experimental strain is comparable with the previous studies and the substrate used for respective strain was ideal for hyperproduction of chitinase.

Temperature played a noteworthy role in the production of chitinase. The results inferred that the maximum activity of chitinase was observed at 35 °C. The increase of temperature above its optimum value compromised chitinase activity of isolate. The increase in temperature above 35 °C leads to loss of moisture content from substrate, and metabolism of

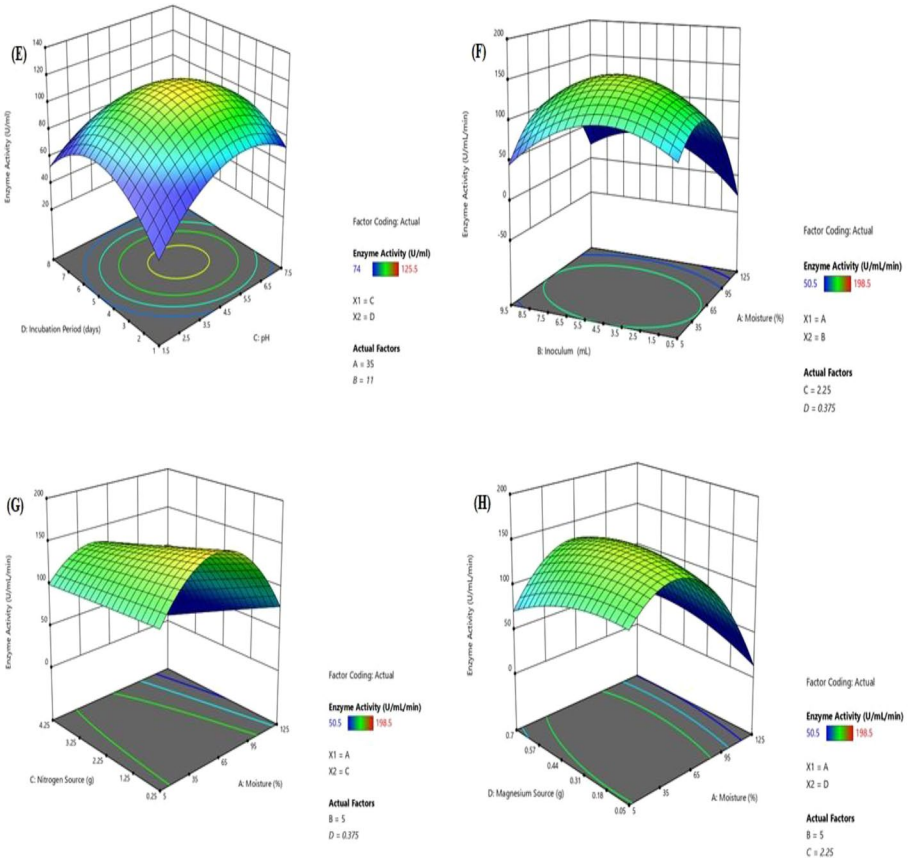


**Fig. 8** Surface plot showing significant interaction between **E** pH vs. incubation period, **F** moisture content vs. inoculum size, **G** moisture content vs. nitrogen concentration, and **H** moisture content vs. magnesium concentration for maximum production of chitinase

the organism was altered which hindered microbial growth and decrease in enzyme production. The results of the present research work are in accordance with former studies in which maximum enzyme production was achieved at temperature of 35 °C [35, 45, 48]. It has also been reported that different *Aspergillus* species, *A. niger* [49], *A. niveus* [50], *A. flavus* [51, 52], and *A. fumigatus* [39, 53], had different optimum temperature ranges from 26 to 37 °C due to their genetic variations.

The enzyme activity was continuously increased with decrease in pH down to 4.5, followed by progressive decline in enzyme activity when the pH was increased. Maximum enzyme activity in acidic pH might be due to improved enzyme–substrate binding. The results of the current experimental work are in line with the findings of previous studies presenting pH in the range of 4–5 for fungal chitinase like *Gliocladium catenulatum* [54], *Trichoderma asperellum* [35], *T. virens* [46], *Penicillium aculeatum* [55], *Alternaria alternate* [38], and *T. harzianum* [56].

It was also observed that growth of microorganism after achieving optimum incubation period was decreased due to decline in availability of micronutrients and release of toxic metabolites in the culture. Our results showed that after 4 days of incubation

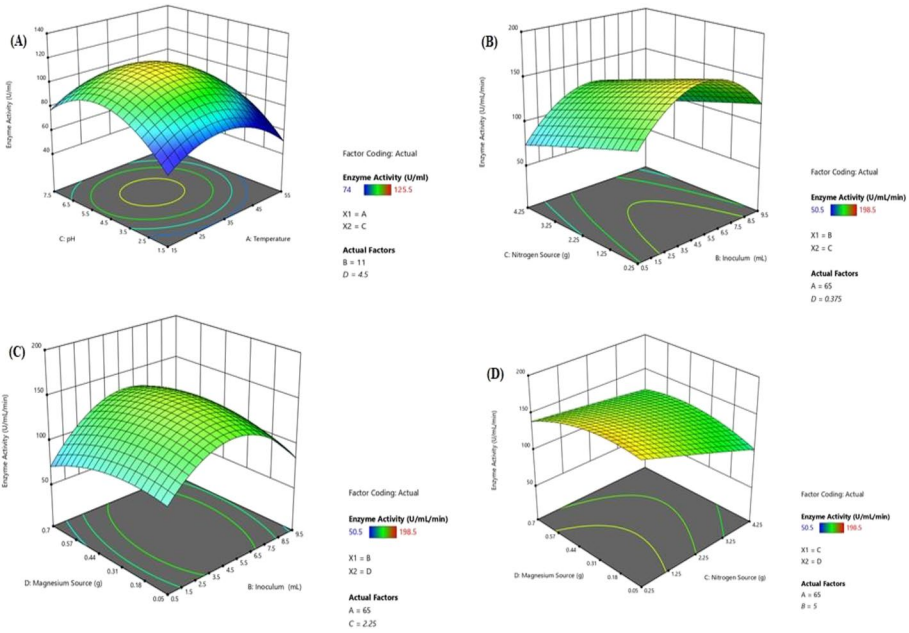


**Fig. 9** Surface plot showing non-significant interaction between **A** temperature vs. pH, **B** inoculum size vs. nitrogen concentration, **C** inoculum size vs. magnesium concentration, and **D** nitrogen concentration vs. magnesium concentration for maximum production of chitinase

period, the production of chitinase was decreased. Further increase in incubation period (days) leads to overgrowth of microorganism and non-availability of substrate. The results of optimum incubation period also support other studies presenting optimum incubation period of 4 days for fungal chitinase like *T. harzianum* [56] and *Monascus purpureus* [57]. However, it is crucial to mention that incubation period was not found as a significant factor for the growth of fungal isolate *A. terreus* SB3 in fermented environment for maximum chitinase production.

Substrate concentration was found as a significant factor for the production of chitinase from *A. terreus* isolate SB3. The maximum chitinase production was obtained using 20 g of fermented substrate. Very rare studies are reported showing the use of *G. lucidum* as substrate for the production of chitinase enzyme from *A. terreus* which contributes to the significance of the present work. In previous studies, different types of substrates have been used for fungal chitinase production like crab and shrimp shell powder of marine waste [57], sugarcane bagasse supplemented with shrimp waste silage [58], colloidal chitin and yeast extract supplemented wheat bran [56], chitin flakes containing wheat bran [55], rice bran supplemented with malt extract, urea, etc. [45], and wheat bran with chitin powder [52].

The decrease in the activity of enzyme at high inoculum size suggested the speedy lack of nutrients necessary for fungal growth. High inoculum size also increased the amount of water present, which slowed the aeration process in SSF and slowed the development of microorganisms leading to decreased production of enzymes [59]. Fungal growth and development may be hampered by the accumulation of spores; meanwhile, the optimum inoculum size showed its crucial role during fermentation process [60].

Moisture content for fermentation played a vital role for the maximum production of chitinase enzyme. Maximum chitinase enzyme was produced when the moisture was 55%. The presence of moisture above optimal moisture level required by growing microorganism to produce chitinase through SSF is capable of changing the substrate particles' structure, decreasing the porosity, and reducing the oxygen transfer. Low moisture levels jeopardize the metabolism of the fungus, nevertheless, since they raise the solid–liquid tension and decrease the solubility of nutrients in solid substrate [61]. Our findings of optimum moisture content for the production of chitinase enzyme are in accordance with previous studies presenting optimum moisture level of 55% for fungal chitinase like *T. koningiopsis* [61], while 52–56% of moisture for *A. flavus* CFR 10 and *Fusarium oxysporum* CFR 8 [52].

Ammonium sulfate $(NH_4)_2SO_4$ was used as nitrogen source which also played a vital role in the production of chitinase. The nitrogen source can also affect the synthesis of chitinase in microorganisms. As a cheap source of nitrogen, ammonium sulfate is advantageous in the low-cost fermentation process used to produce chitinase. These outcomes are quite consistent with those that have been reported for chitinase produced from *Aspergillus* sp. S.13 [62]. Magnesium sulfate ($MgSO_4 \cdot 7H_2O$) was used as magnesium source; however, it was not found as a significant factor in the production of chitinase.

## Conclusions

Screening of chitinase-producing fungi was performed out of which an indigenous strain of *A. terreus* SB3 (ON738571) exhibited hyperchitinase potential using *G. lucidum* (bracket fungus) as substrate. The maximum activity of chitinase (198.5 U/mL/min) was obtained through optimization of fermentation parameters at 35 °C temperature, 4.5 pH, 20 g of substrate, 4-day incubation period, 55% moisture, 4.5 mL inoculum size, 0.25 g of ammonium sulfate, and 0.30 g of magnesium sulfate during the first phase of RSM. During the second phase, moisture content, inoculum size, and nitrogen and magnesium concentrations were optimized successfully. The RSM model was best fitted to optimize chitinase production from *A. terreus* SB3. Fermentation parameters temperature, substrate concentration, pH, moisture content, and nitrogen concentration were found as significant factors contributing to enhance the enzyme production. The study revealed that *A. terreus* SB3 (ON738571) has potential as a novel strain to produce economical hyperchitinase that could contribute to industry if produced at commercial level. The present research work might also be employed for eradication of chitin-based marine pollutants from seawater.

**Author Contribution** Z Anwar contributed to the experimental design, M Adnan was involved in the laboratory work, and M Zafar interpreted research data. All the authors also contributed to paper writing.

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**Data Availability** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Ethics Approval** Not applicable.

**Consent to Participate** All the authors have agreed to participate in the publication of this manuscript.

**Consent for Publication** All the authors have agreed to publish this manuscript.

**Competing Interests** The authors declare no competing interests.

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