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Thermostable chitinase from *Cohnella* sp. A01: isolation and product optimization

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ABSTRACT

Twelve bacterial strains isolated from shrimp farming ponds were screened for their growth activity on chitin as the sole carbon source. The highly chitinolytic bacterial strain was detected by qualitative cup plate assay and tentatively identified to be *Cohnella* sp. A01 based on 16S rDNA sequencing and by matching the key morphological, physiological, and biochemical characteristics. The cultivation of *Cohnella* sp. A01 in the suitable liquid medium resulted in the production of high levels of enzyme. The colloidal chitin, peptone, and K_2HPO_4 represented the best carbon, nitrogen, and phosphorus sources, respectively. Enzyme production by *Cohnella* sp. A01 was optimized by the Taguchi method. Our results demonstrated that inoculation amount and temperature of incubation were the most significant factors influencing chitinase production. From the tested values, the best pH/temperature was obtained at pH 5 and 70 °C, with K_m and V_{max} values of chitinase to be 5.6 mg/mL and 0.87 μmol/min, respectively. Ag^+, Co^{2+}, iodoacetamide, and iodoacetic acid inhibited the enzyme activity, whereas Mn^{2+}, Cu^{2+}, Tweens (20 and 80), Triton X-100, and EDTA increased the same. In addition, the study of the morphological alteration of chitin treated by enzyme by SEM revealed cracks and pores on the chitin surface, indicating a potential application of this enzyme in several industries.

Introduction

Chitin, a β-1,4 polymer of N-acetyl-D-glucosamine (GlcNAc), which is widely distributed among fungi, crustaceans, molluscs, coelenterates, protozoan, and green algae, is the second-most abundant biopolymer found in nature after cellulose.[1,2] Several million tons of chitin is synthesized and degraded each year in the biosphere.[3] This natural resource is relatively easily accessible, e.g., from sources such as shrimp, crab, and krill, which are considered as waste; chitin accounts for 20–58% of the dry weight of these wastes.[4] Chitinous wastes are also produced in large amounts in industries

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such as seafood processing industry, which produces prawn waste (containing 23% chitin).[5] These wastes may pose as an environmental threat on their accumulation and due to extremely slow decomposition.[6] Therefore, organisms that produce chitin-degrading enzymes can be useful in bioremediation and waste management as well as help release nutrients and maintain the carbon, nitrogen, and other biogeochemical cycles in the environment.[5,7,8]

Chitinases (EC 3.2.1.14) are present in a wide range of organisms, including viruses, bacteria, fungi, insects, higher plants, and animals; these enzymes are capable of catalyzing the hydrolysis of chitin.[9] Chitinase participates in a variety of functions, including defense, nutrient digestion, morphogenesis, and pathogenesis.[3] Most chitin-degrading prokaryotes are the gliding bacteria, pseudomonad, vibrio, enterobacteria, actinomycete, bacilli, and clostridia.[10] Bacterial chitinases have a size range of 20–60 kDa.[11,12] Chitinases have potential applications in various areas of biotechnology, biomedicine, agriculture, and nutrition.[13,14]

Microorganisms adapt to the condition in which they have to live and survive. Thermophiles synthesize proteins that are thermostable and resist denaturation and proteolysis. Due to their ecological role and growing interests of their application in biotechnology, a large number of chitin-degrading bacteria have been isolated and their respective genes have been cloned and characterized. However, only few thermostable chitinases have been reported in microorganisms.[15,16] The thermostable chitinolytic enzymes can hydrolyze their substrates at high temperatures and represent important advantages against their mesophilic counterparts, for example, chemical and thermal stability, decreased viscosity, increased solubility, and significantly reduced contamination risk.[16] Therefore, researches have been focused on microorganisms capable of producing such enzymes that can tolerate extreme environmental conditions.

Several articles have been published on the classical method of medium optimization by changing one independent parameter while fixing the others fixed.[9] This process can be extremely time consuming, expensive, and unmanageable when involving a large number of variables as well as it cannot describe the combined effect of all the factors involved. Several factors have been reported to influence enzyme production by bacteria.[17] Optimizing all of these affecting factors by statistical experimental designs can address these limitations. The methods of Taguchi have been used extensively in experiment designing.[18] However, the application of Taguchi method in biological science is scarce.[17]

The genus *Paenibacillus* was originally defined in 1993 by Ash *et al.* after an extensive comparative analysis of 16S rRNA gene sequences of approximately 50 species of the genus *Bacillus*.[19–21] They were reported to possess inhibitory effect on bacteria or fungi owing to their cell wall-degrading enzymes.[22] *Cohnella* is a member of the Paenibacillaceae family.[23] Earlier, we had reported the Taguchi method of chitinase production optimization from *Serratia marcescens* B4A[9] and polygalacturonase production from *Macrophomina phaseolina*.[24] In this study, we attempted to isolate and characterize the thermostable chitinase from the novel thermophilic strain, *Cohnella* sp. A01. Moreover, with the objective of obtaining accurate data and economizing the use of time and materials, we decided to use the Taguchi method for the optimization of culture medium instead of using traditional method. Only limited studies have reported statistical optimization for the production of chitinase.[25] The present report is an attempt to formulate a suitable production medium by using statistical optimization that can substantially enhance chitinase production by *Cohnella* sp. A01.

Materials and methods

Materials

Flake crab shell chitin, 3,5-dinitrosalicylic acid (DNS), N-acetyl-D-glucosamine, and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, Mo. USA). Colloidal chitin was prepared by the modified method of Roberts and Selitrennikoff.[26] *Taq* DNA polymerase, 1-kb DNA ladder, standard proteins for molecular weight determination, T4 DNA ligase, IPTG, and X-Gal were purchased from Fermentas (Burlington, Canada). DNA extraction kit was purchased from Metabion (Martinsried, Germany). The High-Pure PCR Purification Kit was sourced from Roche (Indianapolis, USA). All other chemicals were purchased from Merck (Darmstadt, Germany) and were of the highest analytical grade available.

Isolation of microorganisms

Samples collected from shrimp farming wastewater located in Choebdeh-Abadan (southwestern of Iran) and used for isolation studies in our laboratory. The climate in Abadan is arid. Summers are dry and hot with temperatures of >45 °C (average 55 °C); the soaring temperatures may advance to >65 °C.

Screening of thermophilic chitinase-producing microorganism

For the direct screening of chitinase activity from bacterial colonies, clear zone production was monitored at 60 °C.[27] The isolated microorganisms were cultured on agar plates containing 0.5% colloidal chitin, 0.07% K_2HPO_4, 0.03% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 2% agar, 0.2% NH_4NO_3, 0.1% NaCl (w/v), and 0.1% trace elements (pH 7.8). The cultures were incubated for 3 days at 60 °C. Only one chitinolytic bacterial strain (detected by a colony producing a halo around itself) was transferred into fresh chitin containing nutrient broth medium and incubated at 60 °C, following which, the strain was preserved as cell suspensions in 10% glycerol at −80 °C.

Culture and growth conditions

For chitinase production, the strain selected from the primary screening was cultured in a preculture medium (trace element 0.1%, tryptone 1%, yeast extract 0.5%, NaCl 0.5%, agar 0.2%, peptone 0.03%, K_2HPO_4 0.07%, KH_2PO_4 0.03%, $CaCl_2 \cdot 2H_2O$ 0.013%, NH_4NO_3 0.1%, glucose 0.2%, colloid chitin 0.5%, $MgSO_4 \cdot 7H_2O$ 0.05%) for 24 h at 60 °C on a shaker incubator (180 rpm). Then, 4 mL of the preculture (3.5×10^8 cells/mL) was added to 100 mL of the production medium (preculture medium without glucose). The resultant inoculated medium