PROSPECTION OF CHITINOLYTIC BACTERIA AND EVALUATION OF ENZYME ACTIVITY.

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ABSTRACT

Chitin is the second most abundant renewable biomass resource in nature and can be enzymatically degraded by chitinases. These hydrolytic enzymes have vast potential for industrial biotechnological applications. Consequently, the search for new chitinolytic bacterial strains with high enzyme production and activity is constantly required to optimize the acquisition of this enzyme. The present study aimed to prospect chitinase-producing bacteria and evaluate their enzyme activity profiles. The isolation of chitinolytic bacteria was carried out from various samples collected from natural environments in the state of Sergipe, Brazil. The bacteria were isolated on minimal salt medium supplemented with 1% colloidal chitin using a swab. Enzyme activity was evaluated by quantifying reducing sugars using the DNS (3,5-dinitrosalicylic acid) method, at two incubation times (1 hour and 24 hours) and two temperatures (37°C and 25°C). Ten bacterial isolates with distinct morphologies and chitinolytic potential were obtained. Among the bacterial isolates, V3 exhibited the highest enzymatic activity (4.06 U/mL) and FL the lowest (3.13 U/mL) after 1 hour of incubation at 37°C. Under the condition of 24 hours of incubation at 25°C, V2 showed the highest activity (6.21 U/mL), while V3 showed the lowest (3.18 U/mL). The conditions of 25°C and 24 hours of incubation were optimal for chitinolytic activity in all bacterial isolates.

Keywords: Bacterial chitinase. Biotechnology. Enzymatic activity. Bioprospection.

1 INTRODUCTION

Chitin is an aminopolysaccharide composed of a linear chain of N-acetyl-D-glucosamine (GlcNAc) units linked by 1,4-ß-glycosidic bonds. It is widely found in the environment and serves as the primary structural component of a wide variety of unicellular and multicellular organisms. [1] This biopolymer is degraded by chitinases (EC 3.2.1.14) enzymes, which are naturally produced by various organisms in nature, such as bacteria, fungi, plants, and animals. These enzymes catalyze the hydrolysis of the glycosidic bonds of chitin, resulting in low molecular weight products such as chitooligosaccharides and GlcNAc. [2, 3]

Bacterial chitinases are promising for use in various biotechnological applications due to their advantages, which include better adaptation to extreme pH and temperature conditions, rapid production, and ease of manipulation through genetic engineering.[3] Furthermore, bacterial chitinases have the potential for use in different fields such as agriculture, food and pharmaceutical industries and environmental management.[4,5] Prospecting chitinolytic bacteria in different environments could result in the discovery of new chitinases with increased stability or catalytic efficiency for industrial applications.[6,7,8] Therefore, the aim of this study was to prospect bacteria in different natural environments to identify and select isolates that produce chitinase, and to evaluate their enzymatic activity profile.

2 MATERIAL & METHODS

Sampling and isolation

Samples from different natural environments (including mushrooms, arthropods (bee and wasp), lobster breeding water, lizard droppings and shrimp shells) collected in Sergipe/Brazil were used to prospect for chitinolytic bacteria. The criterion for choosing isolation sources was based on the presence of chitin in the collected samples. Minimum Salt Medium (MSM)[9] was used for isolation of chitinolytic bacteria, with adaptations. MSM contained $Na_2HPO_4 \cdot 12H_2O$ (6 g/L), K_2HPO_4 (3 g/L), NH_4Cl (1 g/L), bacteriological agar (15 g/L), yeast extract (0.05 g/L), NaCl (0.5 g/L), plus chitin colloidal 1% (w/v) as only carbon source, also acting as an enzyme inducer. The samples were homogenized in 0.9% saline and plated on the selective media using a swab. The plates were incubated at 30°C for 7 days. The isolates that presented clear zones around their colonies were further analysed by Gram staining and isolates showing different morphology were selected for further studies.

Preparation of colloidal chitin

Colloidal chitin was prepared using a standard protocol[9] with adaptations. 20g chitin powder (Chitin from shrimp shells - Sigma-Aldrich®) was dissolved in 400 mL of hydrochloric acid (37%) and kept under stirring on a magnetic stirrer for 2h. Subsequently, the solution was filtered through an 8-layered gauze glass funnel to retain the undissolved chitin fraction. The filtrate was transferred to a beaker, where 800 mL of ice-cold 50% ethanol was added and homogenized on a magnetic stirrer for twenty minutes. To obtain the precipitate, the solution was centrifuged at 10,000 rpm for 20 minutes. The precipitated chitin was subjected

to successive washes with distilled water until it reached pH 7, where it was subjected to the drying process in an oven at 37°C (overnight) and was subsequently macerated with the aid of a porcelain crucible until obtaining a thin powder.

Preparation of crude enzyme extract

The bacterial isolates were inoculated into 50 mL Falcon tubes containing 6 mL of liquid MSM medium supplemented with 0.3% (w/v) colloidal chitin, resulting in a bacterial suspension with an initial cell concentration equivalent to 0.5 on the McFarland scale. The tubes were incubated on an orbital shaker at 30°C and 180 rpm for 5 days. Following the incubation period, samples were centrifuged at 10,000 × g and 4°C for 20 minutes to collect the supernatant, constituting the crude enzymatic extract.

Assessment of chitinase activity

The evaluation of enzymatic activity was performed by determining reducing sugars using DNS method[10], with adaptations. To accomplish this, 1 mL of crude extract from each bacterium was mixed with 1 mL of chitinolytic solution (0.3% (w/v) colloidal chitin in 50 mM phosphate-buffered saline (PBS), pH 7). The mixture was incubated at two different temperatures, 37°C and 25°C, and for two distinct periods, 1 hour and 24 hours, to induce the enzyme-substrate reaction. After incubation, 200 µL of the reaction mixture was withdrawn and added to glass test tubes containing 1 mL of DNS reagent. The mixture was then incubated at 96°C for 10 minutes, and absorbance was measured using a spectrophotometer (Epoch 2, Biotek) at a wavelength of 550 nm. The chitinolytic solution described earlier was used as a blank, and a standard curve with N-acetylglucosamine (GlcNAc - Sigma-Aldrich®) was constructed to quantify enzymatic activity following the same reaction model described above. GlcNAc concentrations used in the curve were: 50, 100, 200, 300, and 400 µg/mL. One unit of enzyme activity is defined as the amount of reducing sugar produced per mL of enzyme used over time and was stated as U/mL^{-1}.[13] All experiments were conducted in triplicates and were subjected to t-test or one-way analysis of variance (ANOVA).

3 RESULTS & DISCUSSION

Ten isolates exhibited a clear zone surrounding the colony on the selective media, indicating chitinase activity associated with the breakdown of chitin compounds in the medium. Colloidal chitin served as the sole carbon source in the media, which was degraded to release nutrients, thereby supporting the isolates' life cycle. These ten isolates were replated on the selective media to obtain pure colonies, and their morphological characteristics were observed. Additionally, the isolates were analyzed for their Gram character (all results presented in Table 1). Gram staining identification revealed that only isolate V2 is Gram-positive, while the remaining isolates are Gram-negative. Regarding cellular morphology, 50% of the isolates exhibited coccal morphology, 40% displayed bacillary morphology, and 10% were classified as coccobacilli, indicating an intermediate morphology. Chitinolytic bacteria have been isolated from various environments, including the gastrointestinal tract of the Asian common toad (*Duttaphrynus melanostictus*)[7] and the feces of Goeldi's monkey[1] , associated with the digestion of chitin present in the diets of these animals.

Table 1. Information on prospected bacterial isolates.

Isolate	Sample	Morphology on solid media	Gram Character
ABL1	Bee	Shiny white colonies, irregular margin	Gram-negative, Coccus
ABL2	Bee	Milky translucent colonies, regular margin, white	Gram-negative, Diplococcus
AQ1	Lobster breeding water	Shiny translucent colonies with white center, regular margin	Gram-negative, Coccus
AQ2	Lobster breeding water	Shiny white colonies, regular margin	Gram-negative, Streptobacillus
FL	Lizard dropping	Yellow and opaque colonies, irregular margin	Gram-negative, Streptobacillus
COGP	Mushroom	White and opaque colonies with center, regular margin	Gram-negative, Coccobacillus
V1	Wasp	Translucent and dry colonies, regular margin	Gram-negative, Bacillus
V2	Wasp	Shiny white colonies, regular margin	Gram-positive, Streptobacillus
V3	Wasp	Yellow and opaque colonies, regular margin	Gram-negative, Streptococcus
CM	Shrimp shell	White opaque colonies with center, regular margin	Gram-negative, Coccus

Quantification of reducing sugars, revealed chitinolytic activity ranging from 3.13 U/mL (FL) to 6.21 U/mL (V2) (Figure 1). These values indicate distinct enzymatic potential, which is crucial for selecting more efficient strains for biotechnological applications. According to the assessment of chitinolytic activity conducted after 1 hour of incubation at 37°C, isolate V3 exhibited the highest activity (4.06 U/mL), whereas isolate FL showed the lowest activity (3.13 U/mL). In contrast, after a longer incubation period (24 hours) at 25°C, all isolates demonstrated an increase in chitinolytic activity, with no significant differences observed among them. Isolate V2 exhibited the highest activity (6.21 U/mL), followed by isolate V3 with the lowest activity (3.18 U/mL) (Figure 1). These results suggest that incubation time and temperature influence the enzymatic activity of bacterial chitinases, underscoring the importance of selecting optimal conditions for chitinolytic activity. Similarly, previous studies have indicated that the optimal

temperature and incubation time for chitinolytic activity can vary significantly among microorganisms, with maximum chitinolytic activity observed at 30°C and 1 hour of incubation (0.705 U/mL^{-1} for isolate SN5 and 1.315 U/mL^{-1} for isolate SN20). [12]

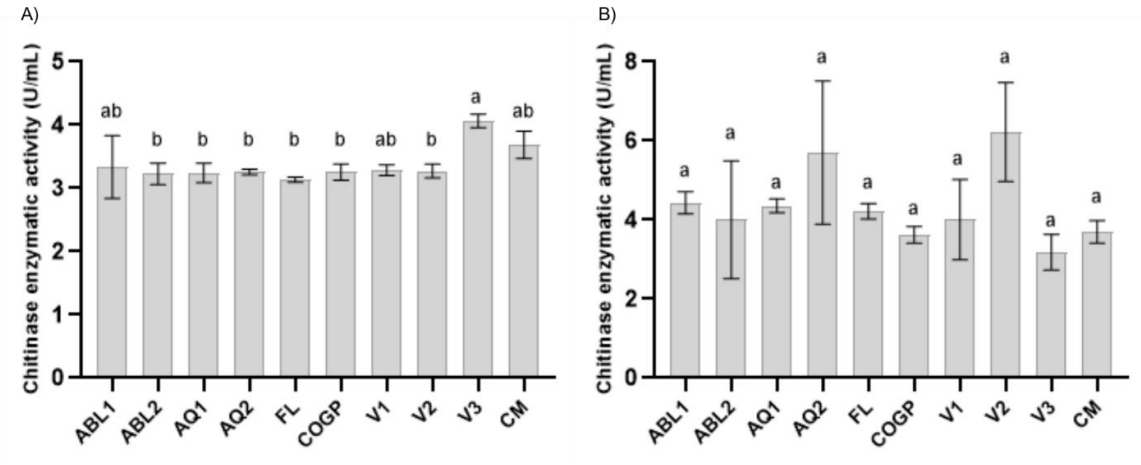

Figura 1. A) Enzymatic activity of chitinase crude extracts after 1h incubation at 37°C. B) Enzymatic activity of chitinase crude extracts after 24h incubation at 25°C. Mean values of chitinase enzymatic activity (U/mL) for different isolates. Error bars represent the standard deviation. Means followed by the same letter do not differ significantly from each other ($p < 0.05$, Tukey's test).

4 CONCLUSION

A prospection of bacteria with chitinolytic potential in all samples collected in this study underscores the importance of exploring diverse natural environments to isolate chitin-degrading microorganisms, thereby expanding the possibilities for industrial applications. The parameters of temperature and incubation time directly influenced the enzymatic activity of chitinases produced by the bacterial isolates. This study contributes to the identification of new bacterial strains with potential for biotechnological applications. Future investigations can further explore the biochemical characteristics of chitinases to maximize their potential in biotechnological processes.

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