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SOIL AS A SOURCE FOR THE ISOLATION AND CHARACTERIZATION OF BETA-GLUCANASE PRODUCING BACTERIA AND ITS INSILICO ANALYSIS

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ABSTRACT

Beta-glucanases are crucial enzymes that hydrolyze beta-glucans, polysaccharides of D-glucose linked by beta-glycosidic bonds. This research focuses on the isolation, purification, and *in-silico analysis* of beta-glucanase producing *Bacillus species*. This enzyme has potential in applications such as biofuel production, food and feed industry, and bioremediation. The bacterial strain was sourced from soil samples. Beta-glucanase was purified from the *Bacillus* culture obtained from the soil using repeated streaking techniques. This research determines that the bacterial beta-glucanase exhibited optimal activity at pH 2, with an incubation period of 120 hours. The study revealed that for the extracellular and intracellular enzyme assay, beef extract and $MnSO_4$ were identified as the most effective sources of nitrogen and metal ions, respectively. Glucose was identified as the best carbon source, yielding the highest extracellular and intracellular enzyme activity. To determine the secondary and 3D structure of the enzyme, bioinformatics tools such as Phyre2 and Predict Protein were utilized. Bioinformatics analysis revealed 3D structure of the protein and depicted the presence of 24% alpha helix and 32% beta strands. This study concentrates on isolating and characterizing bacteria from soil samples that are capable of producing beta-glucanase.

KEYWORDS: Beta-glucanases, *Bacillus species*, 3D structure, soil, Insilico analysis.

INTRODUCTION

A class of carbohydrate enzymes known as beta-glucanase is found in different types of plants, fungi, animals, and bacteria. It breaks down the beta-1,3 and beta-1,4 glycoside linkage found in beta-glucans. Many *Bacillus species* secrete glucanases; *Bacillus amyloliquefaciens, Bacillus macerans, Bacillus circulans, Bacillus polymyxa, Bacillus licheniformis, Bacillus brevis,* and alkalophilic *Bacillus sp.* N137 are among the species whose genes have been characterized (Addington et al., 2020).

The stacking of several β-sheets, composed of multiple anti-parallel strands that bend and form a cleft crossing the enzyme's active site, is responsible for the secondary and tertiary structures of β-glucanases. We refer to this kind of structure as the "jelly roll fold." (Edison et al., 2022).

The amount of reducing sugars, expressed as glucose, released under test conditions by 1g (or 1 mL) of enzyme per minute is known as a unit of beta-glucanase (beta-Glu-U). (Perrot et al., 2022) The enzymatic activity releases glucose which, in an alkaline salt solution, reduces 3,5-dinitrosalicyclic acid to 3-amino-5-nitrosalicyclic acid. The addition of phenol increases the sensitivity of the reaction. Sodium bisulphite serves to stabilize colour (Cruz et al., 1995).

MATERIALS AND METHODS

Collection of Sample

Approximately 100 grams of soil samples was collected from a damp garden from Johar Town area of Lahore. Placed in a polythene bag and taken to laboratory.

Bacterial Isolation

1g of sample was thoroughly mixed with 9ml of distilled water. Serially diluted and was then spread on agar plates that included 0.05g $MgSO_4$. 7 H_2O, 0.005g $CaCl_2$, 0.005g $NaNO_3$, 0.009g $FeSO_4$. $7H_2O$, 0.23g KH_2PO_4, 0.012 g $MnSO_4.7H_2O$, 0.23g KCl, 2g peptone, 1% CMC in distilled water 1.0 liter. These plates were incubated at 37°C for 48 hours. The colonies appeared were sub-cultured to obtain the pure isolates.

Screening of Bacteria

A sterilized loop was employed to pick up bacterial isolates, which were then streaked onto a new CMC media plate using the zigzag streaking technique. These plates were then incubated at 37°C for 48 hours within an incubator. After incubation plated were flooded with congo red dye for 30 minutes to check the beta-glucanase production and destained with 0.1% NaCl. Formation of

clear zones indicted the starch hydrolysis around the colony.

Morphological testing of Bacteria

Each pure colony was identified morphologically using a light microscope. Characteristics such as colony shape, number, margin, elevation, opacity, color, fluorescence qualities, and any notable zones around the colony were examined. This comprehensive profiling aided in the accurate identification and classification of the colonies.

Biochemical Testing of Bacteria

These methods were utilized to differentiate between bacterial species based on their distinct biochemical activities. Freshly prepared chemicals and stains were used for accuracy. The biochemical tests conducted included Gram staining, endospore staining, and catalase tests. The bacteria was identified using these biochemical identification tests.

Enzyme Assay

Enzyme assays were performed using DNS reagent and a colorimetric method with starch as the substrate. The enzyme assays were conducted to determine the presence and amount of β-glucanase enzyme in the prepared samples. For the intracellular assay, the culture was centrifuged, treated with chloroform, and vortexed, followed by another centrifugation to separate the supernatant and pellet. The pellet was then incubated with 1% CMC medium in citrate buffer and DNS, leading to a color change. In the extracellular assay, culture filtrate was incubated with CMC in citrate buffer and DNS, with a subsequent boiling step, also resulting in a color change, indicating the presence of β-glucanase enzyme in both assays.

Characterization of Bacteria

Enzyme characterization was conducted by evaluating various parameters: incubation time (48, 72, 96 and 120 hours), pH levels (2,4, 6, 8 and 10), different carbon sources (glucose, maltose, lactose, saccharose, dextrose, and starch), nitrogen sources (yeast extract, beef extract, peptone, urea, and ammonium hydrogen carbonate), and metal ions (NaCl, CaCl2, MnSO4, MgSO4, and FeSO4).

Bioinformatics Analysis

The secondary and tertiary structure of (*Bacillus amyloliquefacians* beta-glucanase gene) was predicted by different online tools of bioinformatics including NCBI, phyre2, predict protein and expasy.

RESULTS

A 50X dilution of the soil sample was spread on petri plates and incubated at 37°C for 48 hours, resulting in bacterial colony growth as shown in Figure 1(A). For purification, the streaking plate method was used under sterilized conditions, followed by another 48-hour incubation period, leading to pure bacterial isolates depicted in Figure 1(B).

(A) **(B)**

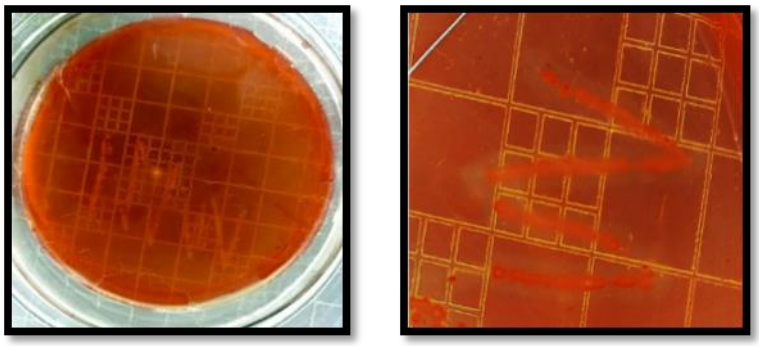
Figure 1: (A) Bacterial colonies produced on CMC media after 48hrs of Incubation. (B) Bacterial pure isolates after incubation of 48 hours by streaking.

Table 1: Morphological Results of Bacterial colony.

Sr. No.	Features	Bacteria 1.1	Bacteria 1.2
1	Size	0.2mm × 0.2mm	0.1mm × 0.1mm
2	Shape	Round	Round
3	Surface	Dull	Dull
4	Color	Light yellow	White
5	Elevation	Raised	Raised
6	Consistency	Sticky and thick	Sticky and thick
7	Odour	Null	Null
8	Opacity	Translucent	Opaque
9	Pigmentation	No	No

Screening of beta-glucanase Producing Bacteria

The screening of glucanase producing bacteria was performed by using Congo red indicator on CMC media plates. The clear wide zone indicated the breakdown of cellulose into glucose by bacteria. The zone of hydrolysis are shown in Figure 2 (A) and (B).



(A) **(B)**

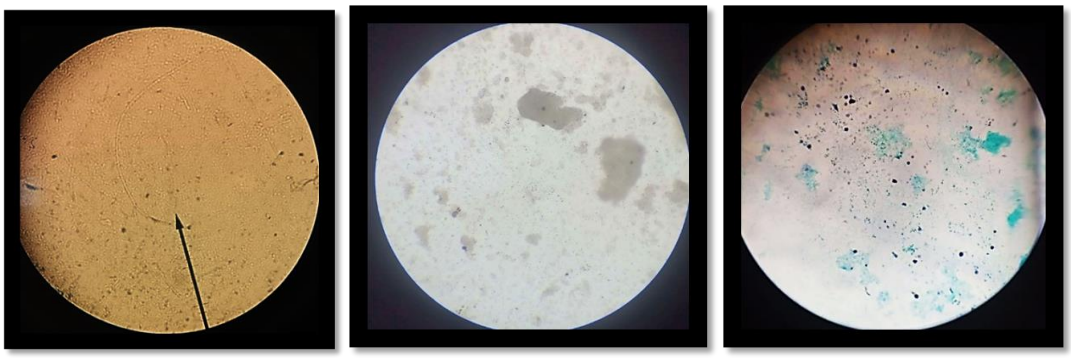
Figure 2: (A) Petri plate flooded with Congo red indicator. (B) Beta-Glucanase producing bacteria showed clear zone of hydrolysis on CMC media plate.

Gram Staining

Gram staining, catalase test, and endospore staining were conducted on the isolated bacterial colony. Gram staining showed that the bacteria retained a purple color, indicating they are gram positive. The catalase test revealed no bubble formation, indicating the absence of the catalase enzyme. Endospore staining resulted in a light blue color, indicating the presence of endospores. These results are depicted in Figures 3 (A), (B) and (C).

Characterization of beta-glucanase producing bacteria

The beta-glucanase enzyme was studied under different conditions including incubation time, pH levels, metal ions, carbon sources, and nitrogen sources to understand their impact on enzyme activity. Enzyme activity was measured using a glucose standard curve.



(A) **(B)** **(C)**

Figure 3: Microscopic image of (A) purple gram stain, (B) Catalase negative bacteria and(C) Endospore staining showing endospore presence.

Time of Incubation

Characterization experiments were conducted at various incubation times, revealing that the highest extracellular and intracellular activity occurred at 120 hours with a value of 0.91 ± 0.14084 IU and 0.64 ± 0.1756 IU.

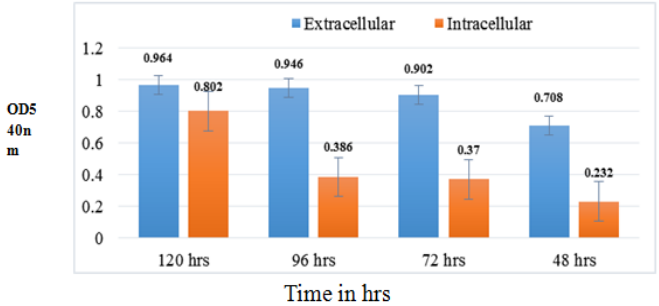


Figure 4: Graphical representation of different time of incubation.

Effect of different carbon sources

Characterization involved the utilization of various Carbon sources, with glucose displaying the highest extracellular and intracellular activity at 1.14 ± 0.03 IU and 1.64 ± 0.05 IU respectively.

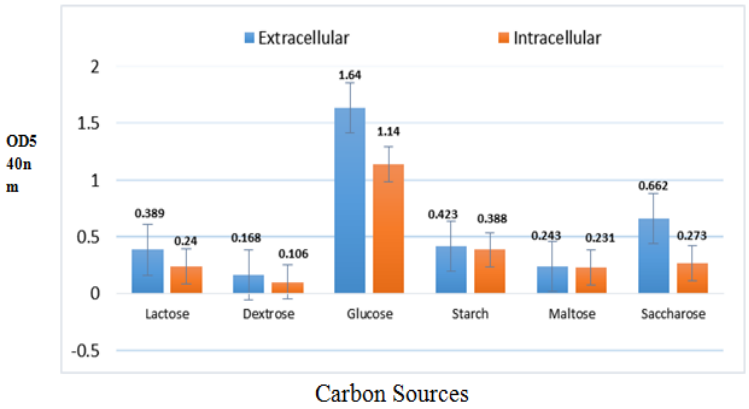


Figure 5: Graphical representation of different carbon sources.

Effect of different Nitrogen sources

Characterization involved using various nitrogen sources, with yeast extract showing the highest extracellular activity at 0.813 ± 0.1091 IU, and beef extract exhibiting the highest intracellular activity at 0.75 ± 0.021 IU.

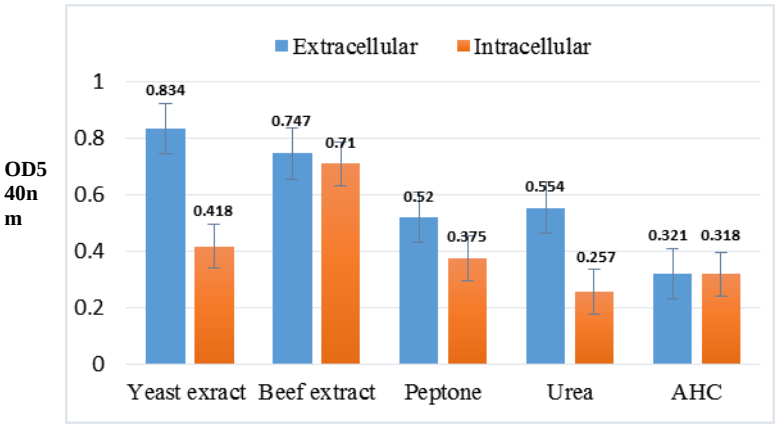


Figure 6: Graphical representation of different nitrogen sources.

Effect of different Metal ions

Characterization was conducted using various metal ions. Maganese sulphate showed the highest extracellular and intracellular activity at 0.98 ± 0.446 IU and 0.431 ± 0.012 IU.

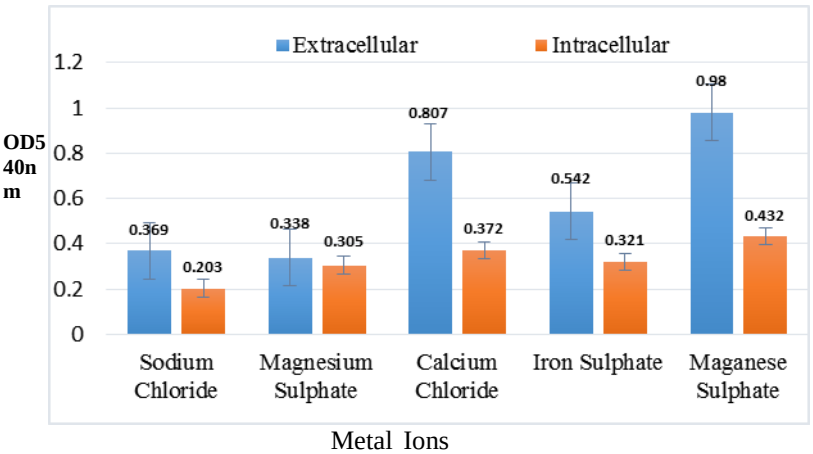


Figure 7: Graphical representation of different metal ion.

Effect of different pH

Characterization was performed across different pH ranges. The highest extracellular and intracellular activity was observed at pH 2.0, with a value of 0.917 ± 0.0826 IU and 0.805 ± 0.0524.

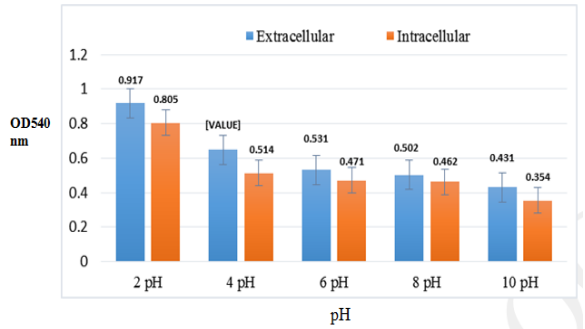
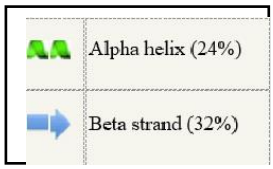


Figure 8: Graphical representation of different pH ranges.

3D Structure Predicted by Phyre2

The phyre2-generated image illustrates the predicted three-dimensional structure of the beta-glucanase enzyme. This model highlights several alpha helices depicted as spirals and beta sheets shown as arrows, indicating a complex arrangement crucial for the enzyme's catalytic function.

Figure 9: 3D Structure prediction from Phyre2.



DISCUSSION

In this research, β-glucanase-producing bacteria were successfully isolated from soil samples by employing CMC agar media, a technique widely recognized in microbial enzyme studies.

The study assessed enzyme activity under various conditions, revealing that the highest extracellular and intracellular activities were observed at 120 hours (0.91 ± 0.14084 IU and 0.64 ± 0.1756 IU, respectively), aligning with prior research indicating that extended incubation optimizes enzyme production (Singh *et al*., 2014; Lee *et al.*, 2016).

Glucose was the most effective carbon source, yielding extracellular and intracellular activities of 1.64 ± 0.357 IU and 1.14 ± 0.076 IU, respectively, consistent with findings by Kim *et al*., (2013) and Ahmed *et al*., (2015). Among nitrogen sources, yeast extract showed the highest extracellular activity (0.81 ± 0.4509 IU), while beef extract had the highest intracellular activity (0.75 ± 0.021 IU), supported by Zhao *et al*., (2012). Manganese sulphate was the most effective metal ion, with extracellular and intracellular activities of 6.73 ± 0.381 IU and 0.431 ± 0.012 IU, respectively, corroborating Patel *et al*., (2011) and Narasimhan *et al*., (2013). Interestingly, MgSO4 also enhanced enzyme activity, contrary to some studies but similar to findings by Tabssum *et al*., (2018). The optimal enzyme activity was at pH 2.0, with extracellular and intracellular activities of 0.917 ± 0.0826 IU and 0.805 ± 0.0524 IU.

CONCLUSION

In this study, we successfully isolated, screened, and characterized bacteria capable of producing beta-glucanase from soil samples. Morphological and biochemical tests were conducted to identify and characterize the bacteria, uncovering their diverse enzyme production capabilities. Optimization studies revealed that the optimal conditions for peak beta-glucanase activity differed among the isolates, underscoring the necessity of customized approaches for industrial enzyme production. Bioinformatics analysis offered further insights into the genetic and functional characteristics of the isolated bacteria, enhancing our understanding of their enzymatic mechanisms.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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