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PLANT ROOT AS A SOURCE FOR THE ISOLATION AND CHARACTERIZATION OF β-GLUCANASE PRODUCING BACTERIA

Azka Asim
Department of Biotechnology, Lahore College for Women University, Lahore, Pakistan
azkaasim52@gmail.com
Muqaddas Saleem
Centre for Applied Molecular Biology, University of the Punjab, Lahore, Pakistan
muqaddassaleem444@gmail.com
Hafsa
Centre for Applied Molecular Biology, University of the Punjab, Lahore, Pakistan
hafsarazaullah@gmail.com
Mahnoor Jan
Centre for Applied Molecular Biology, University of the Punjab, Lahore, Pakistan
mahnoorjan3540@gmail.com
Hafiz Anas Saeed
Centre for Applied Molecular Biology, University of the Punjab, Lahore, Pakistan
anassaeed04@gmail.com
Arslan Ullah Khan
Centre for Applied Molecular Biology, University of the Punjab, Lahore, Pakistan
arslankhan11115@gmail.com
Farheen Aslam
Department of Biotechnology, Lahore College for Women University, Lahore, Pakistn
farheenpu@gmail.com
Rabia Arooj
Centre for Applied Molecular Biology, University of the Punjab, Lahore, Pakistan
rabiarooj4@gmail.com
Imdadullah
Centre for Applied Molecular Biology, University of the Punjab, Lahore, Pakistan
imdadzoology@uop.edu.pk
***Nazim Hussain**
Centre for Applied Molecular Biology, University of the Punjab, Lahore, Pakistan
nazim.camb@pu.edu.pk

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Corresponding E-mails & Authors*:

Nazim Hussain

nazim.camb@pu.edu.pk

Abstract

Background: β-glucanase is an important enzyme, which is found in abundance in plant roots. This enzyme hydrolyzes cellulose into a simple sugar i.e. glucose. It finds numerous applications in numerous industries such as brewing industry, cosmetics, medicine etc.

Purpose: The following study is set with the goal of isolating the bacterial β-glucanase-producing isolates of plant roots. It also further aims to identify and examine the ideal parameters in which the β-glucanase producing bacteria exhibit the greatest growth. This may assist the researchers in developing a satisfactory base in extending the applications of β-glucanase enzyme to different sectors and industries such as brewing, cosmetics, medicines etc.

Methodology: The samples of plant roots were taken from the premises of Lahore College of Women University (LCWU), Lahore. Bacterial isolates were obtained and purified, followed by screening for β-glucanase activity using Congo red staining. Gram staining and endospore staining were performed to characterize the morphological features of the isolates. Once identified, DNS assay was done at different temperatures and pH levels, at different carbon and nitrogen sources and at different incubation periods to determine the optimum extracellular and intracellular activity of the enzyme. Some softwares including messsager tool, ExPASy tool and predict protein etc. were used for bioinformatical analysis.

Results: It was found that the isolated bacteria were *Bacillus* species. The β-glucanase enzyme had the highest extracellular and intracellular activity of 0.82 ± 0.2500U/ml and 0.70 ± 0.2500U/ml respectively at an incubation time of 96 hours. The findings implied that the most suitable carbon source to the enzyme activity is glucose (0.28 ± 0.19519 U/ml) and the most suitable nitrogen source is the yeast extract (0.32 ± 0.2500 U/ml) and peptone (0.68 ± 0.2500 U/ml). The enzymatic activity was highest in the pH of 8.0 (0.42 ± 0.300 U/ml) and 7.0 (0.85 ± 0.2200 U/ml). $MnSO_4$ (Manganese

sulfate) was the most observed source of metal ions with the extracellular and intracellular activities of 1.08 ± 0.0500 U/ml and 1.01 ± 0.1500 U/ml, respectively. 217 triplets and codons were obtained in bioinformatic analysis.

Conclusion: To conclude, the current research shows that *Bacillus*-associated β-glucanase has an industrial value (which can be possibly applied in brewing, cosmetics, and medical industry) and gives a general understanding of the characteristics of this enzyme.

INTRODUCTION

It has been found that many microorganisms such as bacteria, fungi, yeast and algae have the ability to produce β glucanase enzyme. It is a significant enzyme, which is highly found in plant root. Such enzymes are used in many industries such as cosmetics, medicine, brewing industry, textile industry etc. They degrade the β-1,3 and β-1,4 glycosidic bonds in β glucans. Bacteria and fungi produce this enzyme through fermentation.

The available literature widely classifies the β-glucanases in four key types: β-1,3 glucanases; β-1,3,4 glucanases; β-1,3-1,4 glucanases; and β-1,4 glucanases (Goldenkova-Pavlova *et al.*, 2018). They are also subdivided into endoglucanases (breaks internal bonds) and exoglucanases (breaks external bonds) based on their mechanism of action (Srivastava *et al.*, 2018).

They are usually present in large amounts in the endosperm cell walls of cereals that have great commercial value, such as wheat, barley, rye, sorghum, and rice. These polysaccharides are also essential in the structure of the grain of cereal which ensures its integrity. The β-glucans are made up of about 1200 L-D-glycosyl units that are linked with different kinds of glycosidic bonds, including β-(1-3) and β-(1-4) bonds. The solubility, viscosity, and biological characteristics of β-glucans depend on these bonds. β -glucanase has a specificity and cleavage activity against these glycosidic linkages, cleaving β-glucans to monomeric and smaller units, oligosaccharide and glucose. Therefore, the major substrate of the β-glucanase activity in systems based on cereals is β-glucans (Planas *et al.*, 2000; Miljakovic D. *et al.*, 2020).

The active biochemical pathway involved in the action of β glucanase is also a factor of critical methodology. The mechanism of action of the enzyme is a two-step displacement mechanism

involving two key glutamate residues (Glu134 and Glu138). This is a two-step process, namely, glycosylation, during which Glu134 serves as a nucleophile to form a covalent glycosyl-enzyme intermediate, and deglycosylation, during which a water molecule is directed to liberate the free sugar without changing the anomeric configuration (Kaur *et al.*, 2019). The research methodology that the study will use is the isolation of bacteria using a novel source (plant roots) and sequential characterization. The β-glucanase has multi-sectoral benefits. Among them are the ability to increase the quality and shelf-life of items in the brewing sector (Niu *et al.*, 2018; Liu, X *et al.*, 2020), the ability to make fabrics soft and finishing in the textile sector (Edison and Pradeep, 2022) and as a crucial digestive aid in animal feed to increase nutrient absorption without any adverse effect on livestock (Ojha *et al.*, 2022). Moreover, it has great health advantages because of its anti-inflammatory and wound-healing effect, applied in pharmaceutical supplements and in skincare products (Zhu *et al.*, 2016; Venkatachalam *et al.*, 2021).

Its industrial and ecological significance is highlighted by its use in biofuel production using biomass (Srivastava *et al.*, 2018) and enhancing the performance of detergent. Improving the quality of paper (Kumar *et al.*, 2016), and the process of wastewater treatment and its environmental biodegradation (Annamalai *et al.*, 2016; Chavanke *et al.*, 2022) are all spheres in which glucanases might come in handy. The contribution of the present study corresponds to the UN Sustainable Development Goals (SDGs) of Good Health and Well-being, Affordable and Clean Energy, and Industry, Innovation, and Infrastructure.

The main purpose of the research is to isolate the β-glucanase producing bacteria in the plant roots and to determine β-glucanase production by performing some histological tests and enzyme assay. The purpose extends to determine the activity of enzyme in various conditions (temperature, pH, sources of carbon, sources of nitrogen, presence of metal ions) and then, the bioinformatic analysis of the enzyme.

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METHODOLOGY

Laboratory environment and Sample Collection

The current research was conducted at the Department of Biotechnology, LCWU, Lahore. Chemicals were of analytical grade, and all equipment was sterilized and work was done in laminar air flow cabinet. Rhizomes were detached off the plant and weighed up to 0.5 gm. The stock solution was made in 5ml of distilled water (Koshy *et al.*, 2019).

Media Preparation

$MgSO_4$ (0.05 g), $CaCl_2$ (0.005g), $NaNO_3$ (0.23g) KCl (0.009g), peptone (2g), $FeSO_4$ (0.009g) and $ZnSO_4$ (0.012g) were added to 100 ml of distilled water and autoclaved to make CMC media (Dawarakanath *et al.*, 2021).

Bacteria Identification and Isolation

In petri plates, the inoculation of bacteria samples was done using spreading method followed by incubation of 24 hours at 37 ° C. Purification was done through quadrant streaking. Once clear colonies were obtained, they were subjected to different ways of identification, both morphological and phenotypical. Calculation of Colony Forming Units was done (Guan *et al.*, 2020). The phenotypical tests included gram staining, congo red staining and endospore staining (Islam *et al.*, 2018).

DNS Assay

The identification and isolation of the bacteria was followed by DNS assay that was conducted to investigate extracellular and intracellular activity of the β-glucanase enzyme secreted by the bacteria. Standard glucose curve was developed, and DNS reagent was prepared.

To monitor the extracellular activity, 1% carboxymethyl cellulose (CMC) medium in 0.1 M citrate buffer were taken. After incubating a mixture of medium and supernatant in a water bath at 40°C, 2 mL of DNS solution was added and boiled for five minutes. The change in color was noted, and the optical density was determined at a wavelength of 540nm (Dawarakanath *et al.*, 2021).

To see intracellular activity, chloroform was added (500ml) to pallet and vortexed. This was then mixed with 5ml medium and incubated in a water bath at 40 ° C for a period of 30 minutes. The

test tubes were removed out of water bath after 30 minutes and 2ml of DNS solution was added to terminate the reaction. The test tubes were then boiled for another 5 minutes and change in color was noted. Optical density was taken at 540nm (McCleary *et al.*, 1987).

Characterization of β -glucanase producing bacteria

Optimization of the enzyme was done to determine the maximum enzymatic activity by providing different variations of pH, temperatures, time of incubation, carbon sources and nitrogen sources (Islam *et al.*, 2018). The enzyme responded differently to different carbon sources, nitrogen sources, metal ions, pH and times of incubation. The conditions given to the bacteria are mentioned in the table below.

Table 1: Different sources and conditions given to the bacteria

Carbon Sources	Glucose, maltose, dextrose, lactose and sucrose
Nitrogen Sources	Beef extract, yeast extract, urea, peptone and ammonium hydrogen citrate
Metal Ions	$MgSO_4$, $MnSO_4$, NaCl, $CaCl_2$, and $FeSO_4$
pH	4, 5, 7,8 and 9
Time of incubation	24, 48, 72, 96, and 120 hours

SEM and FTIR Structural and Functional Characterization.

Scanning Electron Microscopy (SEM) was used to examine the morphology of the *Bacillus* bacteria to provide a detailed view of the cell shape, surface texture, and structure of the cell. The specimen samples were fixed with glutaraldehyde, dried in an ethanol series, critical point dried and imaged under the Secondary Electron (SE) mode at working distance of 20 mm to study the surface at a high level of resolution (Bozzola and Russell, 1998).

To determine the molecular structure of the starch, Fourier Transform Infrared (FTIR) spectroscopy was applied, whereby KBr starch pellets were prepared and the analysis performed on a spectral range of 10,000100 cm^{-1}. The spectral data were measured using the assistance of the OPUS software and characteristic absorption bands were determined which were ascribed to hydroxyl (OH), carbonyl

(C=O) and glycosidic linkage vibrations which confirmed the presence of vital functional groups in the starch structure (Agarwal, 2014).

Bioinformatic Analysis

Bioinformatic analysis was conducted to forecast the structure of the enzyme synthesized by bacteria. It included NCBI to retrieve the nucleotide sequence of the gene encoding β glucanase in *Bacillus*, messager tool to obtain triplets of nucleotide sequence, ExPASy tool to translate and obtain codons, "predict protein" to predict the protein structure and phyre^2 to predict the 3D structure of enzyme (Singh and Oberoi, 2022).

RESULTS

Isolation and Purification of Bacteria

Bacteria are isolated and purified by numerous different methods depending on the type of bacteria and its specific properties. The bacteria that were isolated on the incubated agar plates were circular and white in color. Quadrant streaking was used to pick and purify one colony. On agar plates, pure isolated colonies of the bacteria were seen (Fig 1).

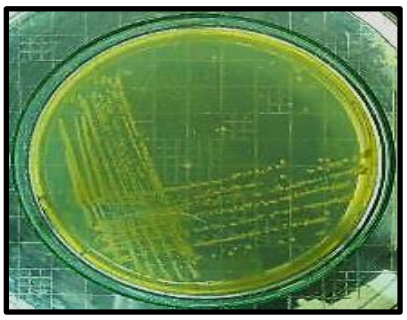


Fig. 1: Purified colony obtained by quadrant streaking after 24 hours of incubation

Screening of β-Glucanase producing Bacteria

The isolated colony was screened to obtain β-glucanase producing bacteria using congo red staining. Different replicas of CMC medium plates that had the purified bacterial isolates were flooded using congo red solution. The bacteria had clear margins around them signifying their zones.



Fig. 2: Clear zones of hydrolysis by β-glucanase producing bacteria shown on plate dyed with congo red stain

Morphological observation gave different features of the isolates. These attributes are as explained in the table below:

Table 2: Morphology of isolated bacterial colonies

Sr. No.	Features	Bacteria 1.1	Bacteria 1.2
1	Size	0.2*0.8 cm	0.1*0.5 cm
2	Shape	Irregular	Round
3	Surface	Bright	Dull
4	Color	White	Beige
5	Elevation	Slightly raised	Flat
6	Consistency	Slightly Sticky	Non-sticky
7	Odour	Null	Null
8	Opacity	Opaque	Opaque
9	Pigmentation	No	No

Phenotypical Characterization of Bacterial Colonies

To preliminary identify the species of bacteria in the isolates, two phenotyical tests were conducted. Gram-staining showed that the identified bacteria were gram-positive since they were stained purple. The staining of endospores demonstrated the presence of the endospores in the isolated bacteria.

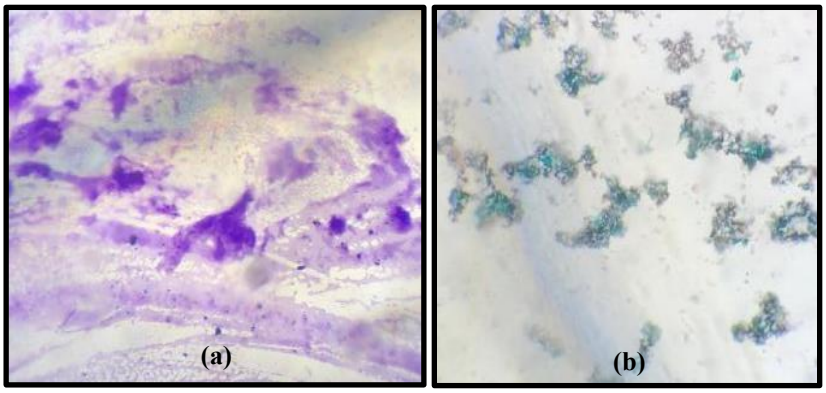


Fig. 3: (a) Gram-positive bacteria observed under microscope after staining (b) Endospores in bacterial colonies shown under the microscope

Glucose Standard Curve

Various concentrations of glucose (0.5-6 mL) were used in order to prepare a standard curve. The quantity of reducing sugars released during the enzyme assays was quantified using this type of curve enabling the enzymatic activity to be calculated.

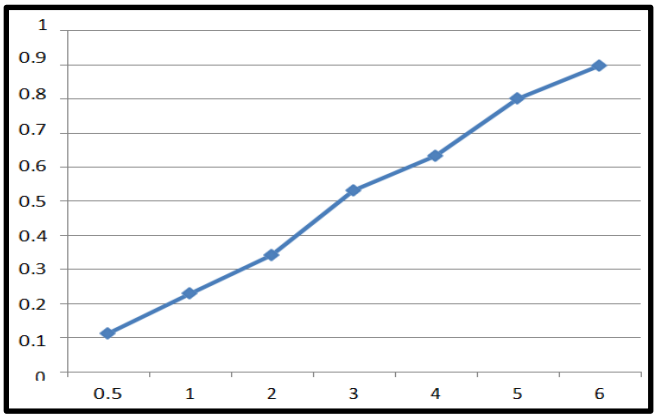


Fig. 4: Glucose concentrations calculated against OD measured at 540nm

Characterization of β-glucanase enzyme activity

β -glucanase activity was characterized and optimized to establish the conditions in which the activity is maximum. The enzyme was provided with different sources of carbon, nitrogen, metal ions, pH and incubation times and intracellular and extracellular assay were conducted and their OD measured at 540nm. It was further compared to glucose standard curve to determine the quantity of starch that the enzyme hydrolyzed.

Table 3: Extracellular activity of enzyme at different given parameters

Carbon Sources		Nitrogen Sources		Metal Ions		pH		Time of Incubation	
Maltose	0.66±0.10000	Urea	0.20±0.0200	$MgSO_4$	1.03±0.0600	4	0.03±0.0100	24	0.65±0.2200
Sucrose	0.65±0.3000	Beef Extract	0.18±0.0500	$CaCl_2$	1.06±0.0200	5	0.25±0.2000	48	0.48±0.1500
Glucose	1.03±0.21517	Yeast Extract	0.32±0.2500	$MnSO_4$	1.08±0.0500	7	0.33±0.1400	72	0.38±0.0700
Lactose	0.60±0.15000	Peptone	0.11±0.0100	NaCl	0.90±0.0100	8	0.42±0.0300	96	0.70±0.2500
Dextrose	1.02±0.25000	Ammonium Hydrogen Citrate	0.25±0.2000	$FeSO_4$	0.96±0.3818	9	0.36±0.1600	120	0.18±0.0600

Table 4: Intracellular activity of enzyme at different given parameters

Carbon Sources		Nitrogen Sources		Metal Ions		pH		Time of Incubation	
Maltose	0.20±0.0500	Urea	0.13±0.0800	$MgSO_4$	0.95±0.2500	4	0.53±0.2500	24	0.40±0.2500
Sucrose	0.56±0.19000	Beef Extract	0.28±0.2000	$CaCl_2$	0.82±0.2300	5	0.21±0.4000	48	0.32±0.1000
Glucose	0.28±0.19519	Yeast Extract	0.32±0.0100	$MnSO_4$	1.01±0.1500	7	0.85±0.2200	72	0.36±0.0900
Lactose	0.38±0.25000	Peptone	0.68±0.2500	NaCl	0.80±0.500	8	0.36±0.1500	96	0.82±0.2500
Dextrose	0.13±0.05000	Ammonium Hydrogen Citrate	0.43±0.2200	$FeSO_4$	0.20±0.100	9	0.08±0.0500	120	0.12±0.0500

Structural and Functional Characterization by SEM and FTIR

SEM provided results in the form of the observation of rod-shaped cells and surface textures and configurations, which provided data on the organization of cells and their structural changes. Moreover, analyses of biofilm structure and endospore create and sustain suggested the flexibility of *Bacillus* to diverse environmental conditions.

Fourier Transform Infrared Spectroscopy (FTIR) was used to investigate the molecular structure of starch. FTIR spectra were accurate in both the structure and composition of starch as they gave clear absorption peaks which led to the identification of important functional groups such as hydroxyl and carbonyl groups.

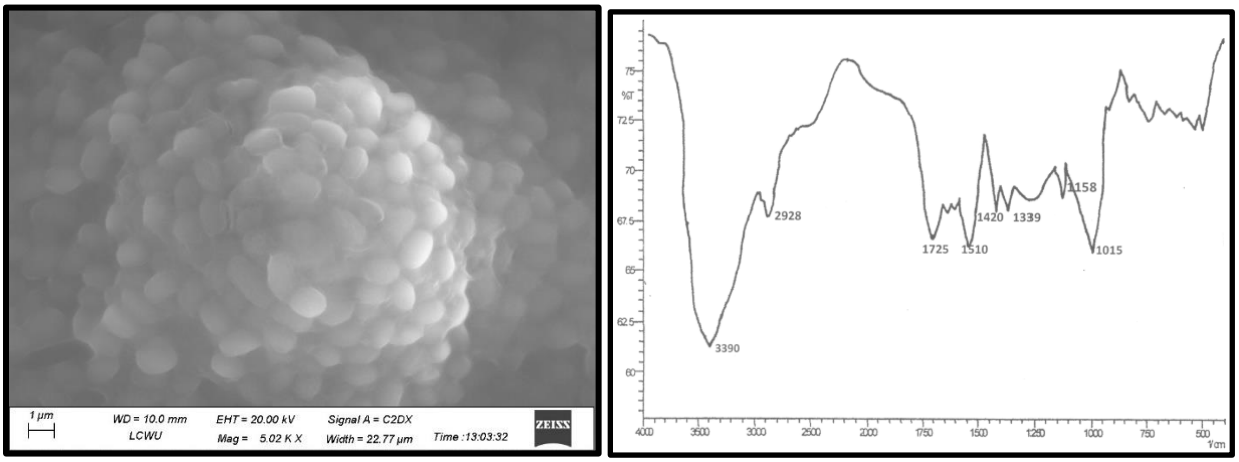
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(a) (b)

Fig. 5: (a) SEM results (b) FTIR shows distinctive absorption peaks

Bioinformatic Analysis

FASTA sequence of β-glucanase producing gene of *Bacillus subtilis* was retrieved. It was identified to possess 653 nucleotides. FASTA triplets of the gene that produces the β-glucanase enzyme in the *Bacillus* species were made using sequence messenger biomodel tool. JA18 endo-β-1,4-glucanase gene protein sequence was obtained and "predict protein" software was applied to predict the secondary structure of the protein.

Table 5: Predicted secondary structure composition

sec str type	H	E	L
% in protein	0	48.15	51.85

Table 6: Residue composition for protein Residue composition for protein

%A: 6.0	%C: 0.9	%D: 6.0	%E: 3.2	%F: 5.1
%G: 10.2	%H: 1.9	%I: 3.2	%K: 5.6	%L: 4.6
%M: 3.2	%N: 9.3	%P: 4.2	%Q: 3.2	%R: 2.3
%S: 6.9	%T: 8.3	%V: 4.6	%W: 3.7	%Y: 7.4

With the help of the composition of the residue, it is possible to identify amino acids and their percentage in the protein. Amino acid sequences are suggestive of functions or structural patterns in proteins. These criteria put proteins into various structural classes based on their composition of secondary structures. The following standards are the protein classification standards based on structural classes:

- "all-alpha": Percentage H greater than 45%) less than 5%.
- "all-β": %E > 45% AND %H < 5%

Phyre[2] and swiss model were used to predict the three-dimensional structure of the enzyme. The image below demonstrates that endo-β1.3-1,4 glucanase of Bacillus subtilis (strain 168) contains 15 β strands and no alpha strand and 213 residues have been modelled.

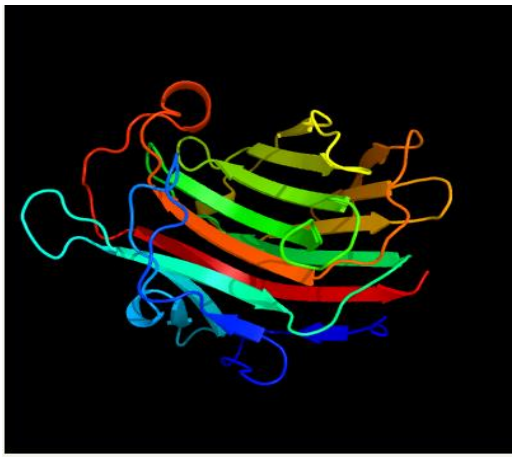


Fig. 6: 3D image of β-glucanase from phyre[2]

3-D structure prediction from Swiss Model involves the computational determination of the local structural elements within a protein sequence. By inputing the amino acid sequence in swiss model, 3d structure of β-glucanase was obtained and analyzed.

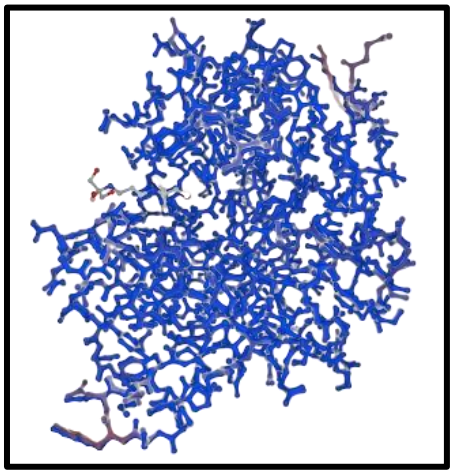


Fig. 7: 3-D structure predicted by swiss model

Table 7: Parameters depicted by swiss model

Biounit Oligo State	Monomer
QSQE	0.00
Method	X-ray, 2.20 Å
Seq Similarity	0.62
Coverage	0.99
Range	3-215

The Swiss Model of 3-D structure prediction entails the computation of the local structural components of a protein sequence. Amino acid sequence of β-glucanase was inputted in swiss model, and the 3D structure was analyzed. Secondary structure prediction in Swiss Model entails the computational definition of the local structural assemblies in a protein sequence. GMQE is a score (between 0 and 1) that indicates the perceived precision of the model in question due to the quality of the alignment and coverage, quality of the template structure, among others.

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DISCUSSION

The effective isolation of Gram-positive, endospore-forming, rod-shaped bacteria in the root of plants is consistent with the existing literature that the rhizosphere is a good source of industrially valuable *Bacillus* species, which is famous for its strong enzyme secretion (Islam *et al.*, 2018). The morphological and biochemical tests confirmed by SEM, which showed the presence of the rod-shaped cells with clear surface textures greatly support the classification of the isolates as those of the *Bacillus* genus. This fact is important because *Bacillus* strains are highly valued in biotechnology because of the high yield and stability which forms a strong ground in the subsequent enzyme characterization in the current research and indicates that the isolated strain is a promising candidate to be utilized in future.

The best extracellular activity with pH 8 and intracellular activity with pH 7 found in this experiment fall within the alkaline to neutral pH range of many bacterial β-glucanases. This agreed with Kaur *et al.* (2017) with *Bacillus subtilis* but was opposed to the acidic optimum of the fungal sources such as *Aspergillus niger*.

The differences that occured in the amino acid composition and the structural stability of the enzyme among the microbial kingdoms could be attributed to the underlying differences in the ionization reactions of the active site residues and hence the pH-activity profile of the enzyme. One of the most important conclusions was that it will take a long period of incubation (96 hours) to reach a peak of enzyme activity, which was different than other studies such as Singh *et al.* (2016), which found a maximum activity at 48 hours with *Bacillus licheniformis*. This delayed growth implied a reduced metabolic rate or a more complicated control method of enzyme production and release in the selected strain used in this research work. It also highlighted the importance of microbial physiology and time of incubation on the maximization of enzyme yield and as such, the standard incubation time might not necessarily be universal.

The definition of the nutritional parameters indicated that glucose and $MnSO_4$ were the best carbon sources and metal ions to increase extracellular activity respectively. This aligns with the research by Shah *et al.* (2015) and Zhao *et al.* (2018), who complained of similar improvements. The presence of

glucose as an easily metabolizable carbon source may indeed driveup metabolic activity and protein synthesis and Mn^{2+} ions have been known to serve as essential cofactors, stabilizing the tertiary structure of the enzyme, and directly involved in the catalytic reaction, therefore greatly increasing activity.

Lastly, the bioinformatic prediction of a secondary structure comprising of β strands (48%) and absences of alpha helixes is also in line with the known structure of glycosyl hydrolase families, which are supported by other related findings published by Lee *et al.* (2021). This structural observation offers a molecular explanation as to why the enzyme has the biochemical characteristics it does including stability and pH optimum and justifies the deployment of silico technology to support and interpret empirical laboratory discoveries in the study of enzymes.

CONCLUSION

This study isolated, produced, and characterizeed a β-glucanase from *Bacillus* species obtained from plant roots. In the study, it was found that the activity of the enzyme was at its peak at a long incubation period of 96 hours with significant extracellular and intracellular enzyme yields. The optimized parameters were glucose as the most efficient carbon source, yeast extract and peptone as the most efficient nitrogen sources, and $MnSO_4$ as the strengthening metal ion cofactor. Additionally, bioinformatic analysis also gave structural forecasts that underpin the functional nature of the enzyme, which further provides a greater understanding of its mechanistic activities. Collectively these findings demonstrate the industrial usefulness of this *Bacillus*-derived β-glucanase, especially in brewing, biofuel production, cosmetics, pharmaceutical industries, and other areas, where effective and stable enzymatic activity is essential. Such an all-encompassing biochemical and computer method not only strengthens the usefulness of microbial enzymes but also indicates the possibility of the further exploitation of rhizospheric bacteria in biotechnology applications.

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