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WHEAT AS A SOURCE FOR THE ISOLATION OF β-GLUCANASE PRODUCING BACTERIA

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ABSTRACT
Beta glucanase is most important enzyme that offer significant benefits, including improved brewing efficiency and product clarity in the food and beverage industry, enhanced nutrient absorption and gut health in animal feed. The highest extracellular activity was observed at pH 8 (0.95±0.11 IU/ml), while the highest intracellular activity was observed at pH 9 (0.8±0.73 IU/ml). Both activities peaked at 24 hours (0.51±0.20 IU/ml) and 48 hours (1.16±0.13 IU/ml) of incubation for extracellular and intracellular respectively. Glucose produced both the highest extracellular (1.51±0.92 IU/ml) and intracellular activity (1.13±0.98 IU/ml). MnSO4 enhanced both extracellular (1.16±0.14 IU/ml) and intracellular (1.08±0.50 IU/ml) activity. The best nitrogen sources were Beef extract (0.98±0.22 IU/ml), and peptone (0.45±0.14 IU/ml) shows highest activity for intracellular and extracellular respectively. This research work has investigated bacteria which is producing beta glucanase enzymes with greater activity. For determining 3D structure of enzyme Phyre2 and Swiss model was used that predicted the structure of beta glucanase.

KEYWORDS: beta glucanase, enzyme assay, highest activity, SEM, phyre2

INTRODUCTION

Beta-glucanase is a valuable enzyme with significant ecological implications and utilized in various industrial sectors that include food, feed, healthcare, environment, and energy. It primarily focuses on environmental necessities and economic factors (Bhatia, 2018).

Beta-1,3-glucanases are found in many different organisms such as bacteria, fungi, plants, and some invertebrates, and play a role in various physiological functions. These processes encompass energy production, cellular regeneration and growth, protection against fungal pathogens, seed sprouting, digestion, and reproduction. β-glucanases can break down β-glucans into small oligosaccharides or glucose, leading to various biotechnological uses. Common applications include converting lignocellulosic biomass into fermentable sugars for bioethanol production, reducing antinutritional effects of cereal β-glucans as feed additives, using as biocontrol agents against pathogenic fungus in crops, managing invasive Candida albicans infections, clarifying wine, accelerating germination in malting, and preventing the buildup of barley β-glucans in brewing (Casiero *et al*., 2022).

Beta-glucanase enzymes primarily work by breaking down glycosidic bonds in β-glucans, converting them into shorter oligosaccharides or single glucose molecules. The process involves a double-displacement mechanism in which the enzyme creates a covalent glycosyl-enzyme intermediate and then releases the free sugar. The general sequence of actions of beta-glucanases is as follows: The enzyme interacts with the substrate, leading to structural alterations in the enzyme active site. The enzyme's serine or histidine residue initiates a nucleophilic attack, resulting in the formation of a covalent bond with the substrate's carbonyl. Water functions as a nucleophile by attacking the acyl-enzyme intermediate, leading to the release of the free sugar.

The enzyme releases the newly formed product, reverting to its initial conformation and becoming available to bind another substrate molecule (Chen et al., 2020).

MATERIALS AND METHODS

Isolation of organism

Wheat samples were used for isolation of bacteria. CMC agar media was used for isolation and screening of glucanase bacteria. In a flask, CMC agar media was prepared. In 100ml distilled water, 0.05g of $MgSO_4$, 0.005g of $CaCl_2$,0.005g of $NaNO_3$, 0.23g of KCl, 0.009g of $FeSO_4$, 0.002g of $ZnSO_4$, 0.012g of $MnSO_4$, 0.23g of KH_2PO4, 19g of Agar, 2g of peptone and 1g of CMC were added for media preparation. After shaking and dissolving the media components with stirrer, the flask was tightly closed with cotton plug and then covered with aluminum foil. The flask was then placed in an autoclave for 45 minutes (Heinze and Pfeiffer, 1999).

Congo red staining
Plates show growth at the marked areas on the plate.one of the plate was taken for Congo red staining to determine the zones or holes showing active enzyme. Clear zones were formed that confirmed that the glucanase producing enzyme converted cellulose in CMC agar into glucose (Apuun *et al*., 2000).

Gram staining
Gram staining was used for the biochemical tests. A light microscope was used to examine the glass slide at both high and low magnifications (Pukhrambam, 2019).

Endospore stain procedure
Endospore staining was done and the slide was examined under the oil immersion lens (1,000X) for the presence of endospores (Hussey and Zayaitz, 2007).

Characterization of glucanase producing bacteria
For determining the best activity of glucanase producing bacteria from wheat grain, optimization was done at different parameters such as

Effect of pH
The pH of culture medium was changed that range from 4 pH,5 pH,7 pH, 8 pH and 9 pH on glucanase was observed.

Time of incubation
The effect of the time of incubation on production of bacterial growth was checked at 24hrs, 48hrs, 72hrs and 96hrs and 120hrs.

Effect of metal ions
Effects of different metal ions like magnesium sulphate, sodium chloride, manganese sulphate, iron sulphate and calcium chloride were checked by growing isolates in the production of medium.

Effect of carbon sources
Effect of different carbon sources like glucose, maltose, lactose, sucrose and starch was checked by growing isolates in the production of medium.

Effect of nitrogen sources
The effect of different nitrogen sources like beef extract, yeast extract, sodium hydrogen oxalate, ammonium hydrogen citrate and peptone were checked by growing isolates in the production of medium (Dwarakanath, 2021).

Intracellular glucanase enzyme assay
The incubated culture was centrifuged for 15 minutes to separate the pallet and supernatant. After centrifugation supernatant was taken out and then added 0.5ml chloroform, shake it well on vortex for 5 minutes. Then take 0.5ml of pallet and add 0.5ml of citrate buffer solution as a substrate. The solution was incubated in water bath for 10 minutes at 50°C. After incubation, add 2ml DNS to it, again test tubes were placed in water bath at 92°C to boil. Change in color was observed (Bisswanger, 2014).

Extracellular enzyme assay
0.5ml of supernatant was added in a test tube. 0.5ml of citrate buffer solution was added into the test tubes. The solution was incubated for 10 minutes at 50°C. After incubation, add 2ml of DNS to it, again test tubes were placed in a water bath for 92°C to boil. Change in color was observed (Bisswanger, 2014).

Calculation of OD on spectrophotometer
At 540 nm, the absorbance was adjusted. First, the optical density (OD) of the blank solution which was made up of one milliliter of water and one milliliter of DNS was determined in a cuvette. This measurement of OD was made.

RESULTS

Isolation of bacteria
Wheat grains were obtained from a local store in shadman, Lahore which was used to extract the bacteria. Media prepared for the isolation of bacteria contain $MgSO_4$, $CaCl_2$, $NaNO_3$, KCl, agar and peptone. Bacteria was obtained after 48 hours of incubation. Figure 1 showed the growth of bacteria on the petri plates.

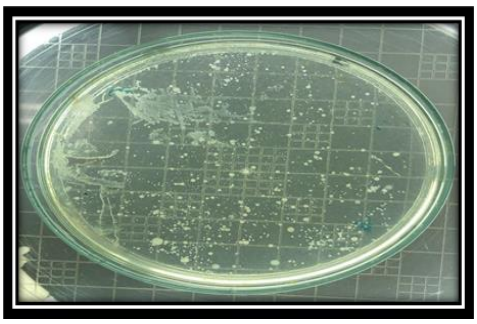

Figure 1: Bacterial colonies produced on CMC agar media after 48 hours of incubation.

Screening of bacteria by gram iodine
The screening of bacteria was done by gram iodine solution on nutrient agar plates. The plate was flooded with Congo red solution and then destained with NaCl solution, which forms clear zones on the petri plate. The zones of hydrolysis are shown in Figure 2.

Figure 2: The bacteria producing beta glucanase showed clear zone of hydrolysis on the plate dyed with Congo red stain.

Gram staining

Gram staining was used to classify bacteria into gram-positive and gram-negative types. The bacteria showed purple color which determined that it was gram-positive bacteria.

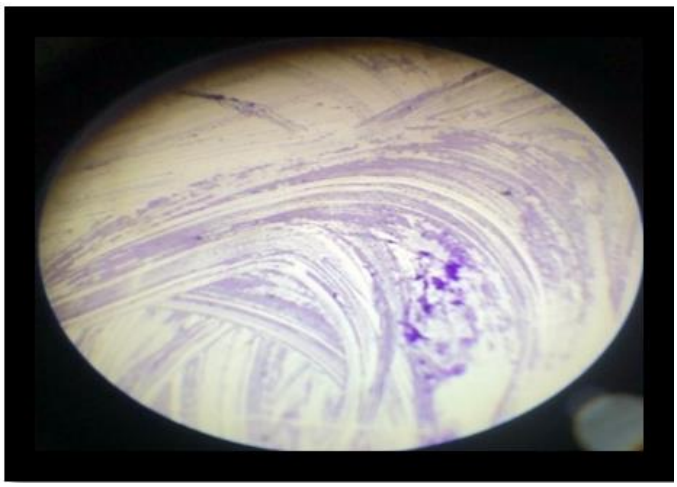

Figure 3: Glass slide showing the gram-positive bacteria under microscope.

Endospore test

In endospore staining, endospores appeared green because they retained the malachite green stain.

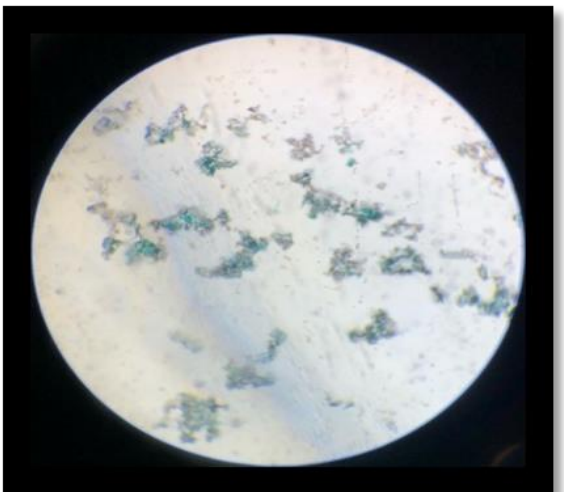

Figure 4: Endospores in bacterial colonies shown under the microscope.

Scanning Electron Microscope

The Scanning Electron Microscope (SEM) produced a high-resolution image of the bacterial surface. The observed characteristics revealed that the bacteria were rod-shaped and measured approximately 1 µm in length. These findings confirmed the presence of Bacillus sp. in the sample.

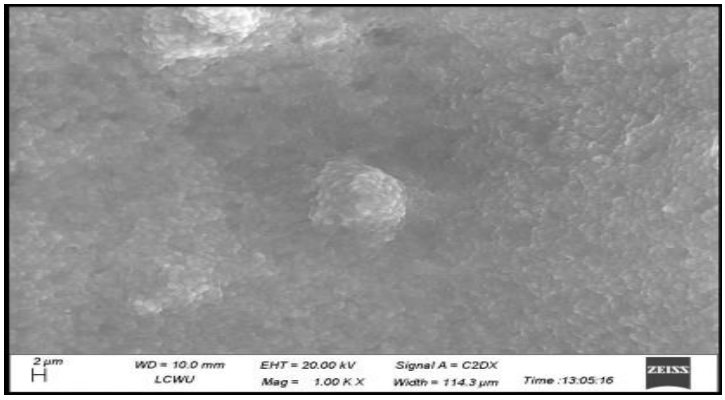

Figure 5: SEM of Bacillus sp.

Characterization of glucanase enzyme

The glucanase enzyme was characterized at various incubation time, pH levels, carbon sources, nitrogen sources, metal ion and substrate concentrations and their effect on enzyme activity was determined. The activity of enzyme was determined by Glucose standard curve.

Time of incubation

Characterization was done at different times of incubation in shaking incubator in which highest extracellular activity was shown at 24 hours which was 0.51±0.20 IU/ml and intracellular activity was shown at 48 hours which was 1.16±0.13 IU/ml. The detailed values are mentioned in the table and represented by the graph.

Table 1: The glucanase bacteria at different times of incubation showing its extracellular and intracellular activity.

Serial no.	Time of incubation	Extracellular enzyme activity (IU/ml) Mean ±(SD)	Intracellular enzyme activity (IU/ml) Mean ±(SD)
1	24hrs	0.51±0.20	0.06±0.02
2	48hrs	0.25±0.02	1.16±0.13
3	72hrs	0.36±0.18	1.13±0.02
4	96hrs	0.15±0.14	1.06±0.03
5	120hrs	0.03±0.02	0.48±0.14

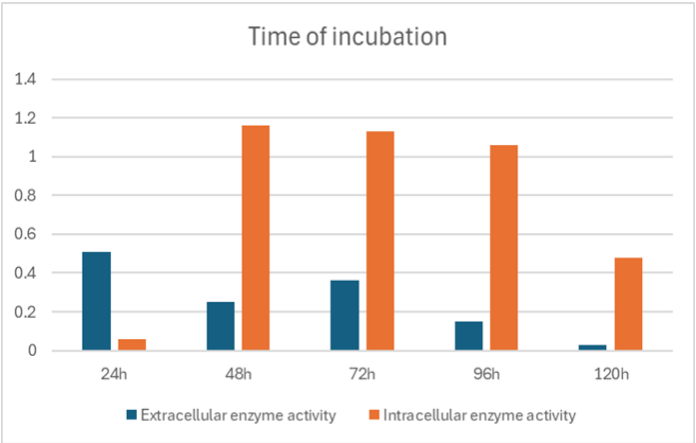

Figure 6: Graphical representation of different time of incubation. In these, bacteria at 48 hours shows high enzyme activity as compared to other time periods.

Effect of different carbon sources

Characterization was done by using different sources of carbon where the highest extracellular and intracellular activity was shown at glucose which were 1.51±0.92 IU/ml and 1.13±0.98 IU/ml respectively.

Table 2: The glucanase bacteria grows on different carbon sources showing its extracellular and intracellular activity.

Serial no.	Carbon sources	Extracellular enzyme activity (IU/ml) Mean ±(SD)	Intracellular enzyme activity (IU/ml) Mean ±(SD)
1	Maltose	0.75±0.40	0.65±0.26
2	Glucose	1.15±0.92	1.33±0.98
3	Lactose	0.33±0.12	1.23±0.54
4	Dextrose	0.35±0.23	1.13±0.44
5	Sucrose	0.65±0.26	0.55±0.23

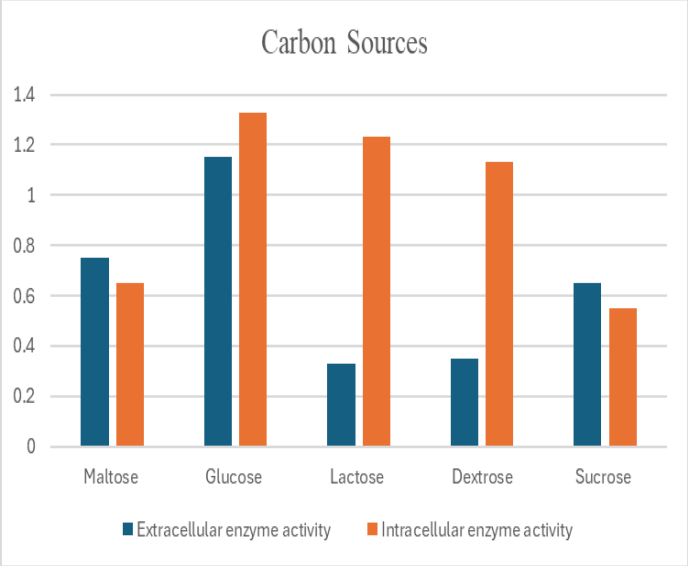

Figure 7: Graphical representation of different carbon sources. In these sources, bacteria at high glucose concentration show high enzyme activity as compared to other sources.

Effect of nitrogen sources

Characterization was done by using different sources of nitrogen where the highest extracellular activity was shown in peptone which was 0.45±0.14 IU/ml and intracellular activity was shown in beef extract which was 0.98±0.22 IU/ml.

Table 3: The glucanase bacteria grows on different nitrogen sources showing its extracellular and intracellular activity.

Serial no.	Nitrogen sources	Extracellular enzyme activity (IU/ml) Mean ±(SD)	Intracellular enzyme activity (IU/ml) Mean ±(SD)
1	Peptone	0.45±0.14	0.9±0.25
2	Urea	0.35±0.14	0.9±0.25
3	Yeast extract	0.36±0.13	0.88±0.21
4	Beef extract	0.25±0.12	0.98±0.22
5	Ammonium hydrogen citrate	0.28±0.09	0.95±0.23

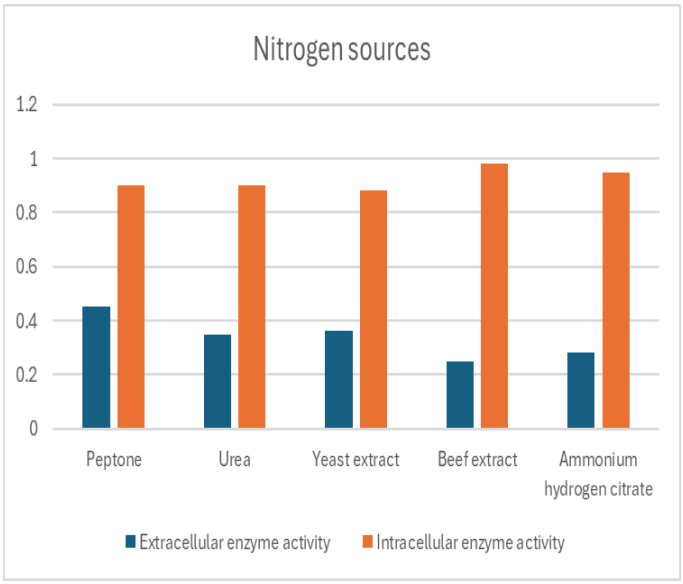

Figure 8: Graphical representation of different nitrogen sources. In these sources, bacteria at high beef extract concentration shows high enzyme activity as compared to other sources.

Effect of metal ions

Characterization was done by using different metal ions where the highest extracellular and intracellular activity was shown in $MnSO_4$ which were 1.16±0.14 IU/ml and 1.08±0.50 IU/ml respectively.

Table 4: The glucanase bacteria grows on different metal ions showing its extracellular and intracellular activity.

Serial no.	Metal ions	Extracellular enzyme activity (IU/ml) Mean ±(SD)	Intracellular enzyme activity (IU/ml) Mean ±(SD)
1	$FeSO_4$	1.13±0.11	1.06±0.03
2	$MnSO_4$	1.16±0.14	1.08±0.50
3	$MgSO_4$	1.15±0.04	1.06±0.03
4	$CaCl_2$	1.11±1.04	0.16±0.11
5	NaCl	1.10±1.02	0.06±0.04

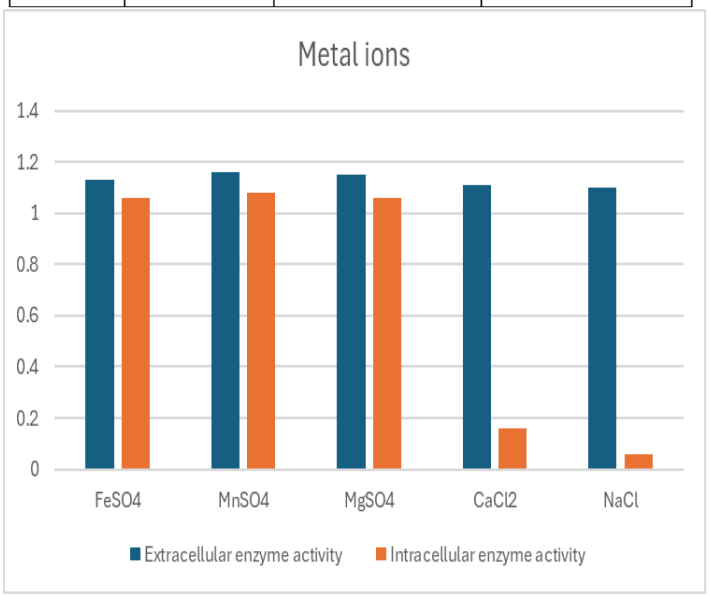

Figure 9: Graphical representation of different metal ions. In these metals bacteria at high manganese sulphate concentration shows high enzyme activity as compared to other sources.

Effect of pH

Characterization was done on different pH where the highest extracellular activity was shown at 8 which was 0.95±0.11 IU/ml and intracellular activity was shown at 9 which was 0.8±0.73 IU/ml.

Table 5: The glucanase bacteria grows on different pH showing its extracellular and intracellular activity.

Serial no.	pH values	Extracellular enzyme activity (IU/ml) Mean ±(SD)	Intracellular enzyme activity (IU/ml) Mean ±(SD)
1	4	0.02±0.01	0.03±0.02
2	5	0.21±0.04	0.02±0.01
3	7	0.25±0.06	0.4±0.15
4	8	0.95±0.11	0.16±0.12
5	9	0.38±0.13	0.8±0.73

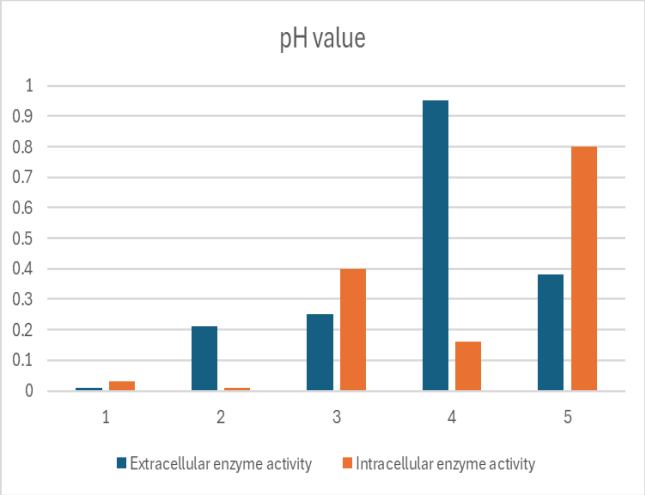

Figure 10: Graphical representation of different pH ranges. In these ranges, bacteria at pH 9 show high enzyme activity as compared to other ranges.

Bioinformatic Analysis

3D structure of enzyme Enzyme 3D structure is predicted by Phyre 2 and Swiss model.

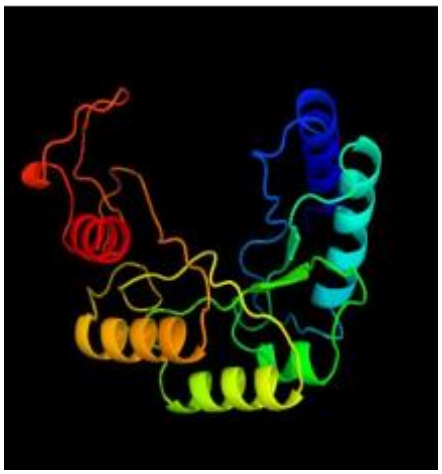

Fig. 12: 3D image of beta glucanase from Phyre2.

This image shows a PBD molecule of beta glucanase. 3D structure catalytic mutant of gene from bacillus subtilis shows 191 residues (54% of your sequence) have been with 100.0% confidence. Detailed structure predicted the percentage of alpha helix and beta strands. Alpha helix 16% and beta strands are 28% in sequence.

By inputting the amino acid sequence in Swiss model, the 3D structure of beta glucanase was obtained and analyzed.

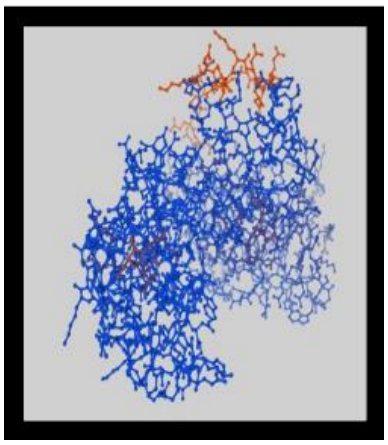

Fig. 13: 3D Structure predicted from Swiss model.

DISCUSSION

beta glucanase was extracted from wheat grains that is the best source of this enzyme. Enzyme Assay of Beta glucanase activity was measured using the dinitrosalicylic acid (DNS) method to quantify reducing sugars released from barley beta-glucan. Bacillus species showed optimal beta glucanase production at 24 hours with an extracellular activity of 0.47 IU/ml. Reported peak intracellular activity at 48 hours with a value of 1.10 IU/ml (Saha, 2006).

Bacillus sp. was cultured in a medium with peptone and beef extract at 30°C. Enzyme extraction was done through centrifugation that was used to harvest cells, and the supernatant was taken for extracellular enzyme analysis. For intracellular enzymes, cells were lysed using repeated freeze-thaw cycles, and the lysate was centrifuged. Beta glucanase activity was measured by the release of reducing sugars from lichenan using the DNS method. Reported an extracellular activity of 0.43 IU/ml with peptone and an intracellular activity of 0.95 IU/ml with beef extract (Farinas et al., 2011).

Bacillus cereus was grown in an alkaline medium at different pH levels. Supernatant was separated by centrifugation for extracellular enzyme activity. For intracellular enzymes, cells were disrupted using enzymatic lysis with lysozyme followed by centrifugation. Beta glucanase activity was measured using the DNS method to quantify reducing sugars released from wheat bran. Reported optimal extracellular activity at pH 8 with a value of 0.92 IU/ml and intracellular activity at pH 9 with a value of 0.79 IU/ml (Ahmed et al., 2017).

Enzyme shows best activity with carbon sources glucose with 24 and 48 hours of incubation and pH 8 and 9 shows maximum growth rate. The best nitrogen sources were Beef extract and peptone shows highest activity. Among metal ions MnSO4 showed a slight increase in beta glucanase activity.

For bioinformatics analysis, firstly fasta sequence of beta glucanase gene produced by bacillus subtitles was retrieved from NCBI website and their protein structure was extracted. Predict protein shows percentage of helix and loops in protein. For tertiary structure prediction Swiss model and Phyre2 tool was used. Swiss model shows the best 3D structure of beta glucanase gene.

CONCLUSION

Bacteria are an important part of life because they are present everywhere and give advantages, but some have disadvantages. Beta glucanase producing bacterial strains from wheat sources that produced a high amount of beta glucanase were found and isolated using a methodical technique. The highest extracellular activity was observed at pH 8, while the highest intracellular activity was observed at pH 9. Glucose produced both the highest extracellular and intracellular activity. MnSO4 enhanced both extracellular and intracellular activity. The best nitrogen sources were Beef extract and peptone shows highest activity. Optimization experiments revealed that beta glucanase production varied significantly under different conditions. In bioinformatics analysis, protein sequence of bacillus subtitles beta glucanase gene was used and tertiary structure was predicted, and it shows alpha helix 16% and beta strands 28% in sequence. Phyre2 and Swiss model are best tools for enzyme 3D structure. SEM results have shown that the bacteria have rod shaped morphology and they are densely packed that confirmed the presence of Bacillus specie.

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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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