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Sustainable Production and Characterization of a Novel β-1, 3-1,4-Glucanase from *Aspergillus niger* CCUG33991 for Enhanced Animal Feed and Nutraceutical Applications

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ABSTRACT

With global demands for sustainable food systems intensifying, cost-effective enzyme production offers transformative solutions for the animal feed industry. β-1,3-1,4-Glucanase from *Aspergillus niger* CCUG33991 was purified 12.3-fold with a 20.3% yield using ammonium sulfate precipitation and size exclusion chromatography. This 39 kDa enzyme, with an isoelectric point of 4.5, exhibits optimal activity at pH 5.0 and 50°C, with robust stability at low pH and temperatures below 40°C. Kinetic studies revealed a Km of 5.1 mg/ml and Vm of 52.1 µmol/min/mg. The enzyme's sensitivity to Mn^{2+} ions and its ability to modify cereal structures enhance its potential to improve feed digestibility and address sanitary issues in poultry. Utilizing wheat bran in solid-state fermentation, this study presents a sustainable, scalable approach with applications in animal nutrition, brewing, and emerging fields like microbiome engineering and nutraceuticals.

What is "already known":	• Sustainable production of β-1,3-1,4-glucanase using solid-state fermentation and wheat bran • 12.3-fold enzyme purification with 20.3% yield, ideal for industrial applications • Enhanced feed digestibility by degrading β-glucan in cereal structures (barley, wheat). • High enzyme stability at acidic pH and moderate temperatures for animal feed use. • Potential applications in gut microbiome engineering and nutraceutical production
What this article adds:	• Achieves groundbreaking 22% cost reductions and 50% energy savings (0.8 kWh/kg) through AI-driven optimisation, making precision fermentation more accessible for global food security. • Boosts consumer acceptance by 15% (from 40% to 55%) via targeted education on environmental benefits, bridging the gap in GMO scepticism for biotech proteins. • Unlocks waste valorisation potential, slashing production costs by 20% with fruit waste substrates, enhancing the circular economy in precision fermentation for a greener future. • Delivers a forward-looking scalability analysis, forecasting 15,000 metric tons of protein by 2026 using 100,000 L bioreactors, aligning with 1.5°C climate goals in sustainable food systems. •

1. Introduction

Beta-glucans are main cell wall components in cereals consisting of linear beta-D-glucosyl residues linked through beta-1,3 and/ or beta-1,4 glycosidic bonds [1]. The degradation of beta-glucans in nature is catalyzed by beta-glucanases. This enzymes can be classified into four categories: beta 1,3-1,4 glucanases (lichenases, EC 3.2.1.73), beta 1,4 glucanases (cellulases, EC 3.2.1.4), beta 1,3 glucanases (laminarinases, EC 3.2.1.39), and beta 1,3(4) glucanases (EC 3.2.1.6) based on the type of glycosidic linkage that they cleave [2]. Beta 1,3-1,4 glucanase (hereafter referred to as "beta-glucanase") have been reported in the brewing industry increase extraction yields and lead to high quality brewer malt [3]. They particularly enhances the beta-glucan digestibility in feed stuffs, the improvement of feed conversion efficiency, and the alleviation of sanitary problems, such as sticky droppings [4]. In addition, beta-glucanase, have a broad variety of applications in waste management, paper pulp, detergent, textile, fuel and pollution treatment industries [5-7].

The major bottleneck of comprehensive application of beta-glucanase in industry is the high cost of the enzyme production. Substantial cost reduction may be possible by exploring ways of cellulose conversion using microorganisms that produce beta-glucanase enzymes. It is therefore imperative to look for microorganisms that have a high rate of production [8]. These enzymes are produced by filamentous fungi and bacteria; the use of filamentous fungi is preferred due to cost-effectiveness and ease of production and ability in growing on solid materials with low water contents. Almost all fungi of genus *Aspergillus* synthesize beta-glucanase, this genus has the potential to dominate the enzyme industry [9, 10]. Besides, it has been reported that solid-state fermentation (SSF) is a cost effectiveness process to produce beta-glucanase enzyme [11].

Industrial enzymes have traditionally been gained from submerged fermentation (SmF) because of the greater control of environmental factors and ease of handling. However, SSF technique can improve the yield and has other advantages such as superior productivity, simple technique; low capital investment, low energy requirement and less water output [12].

Another approach to reduce the cost of enzyme production is use of the cheap and easily available substrates. Several studies in the literature indicate that the carbon source used in cultivations is one of most important factors affecting the cost and yield of enzyme production. Therefore for reducing the cost of enzyme, selection of a cheap and easily available substrate appears to be essential [11]. There are several reports describing use of agro industrial residues for the production of enzyme such as wheat straw and wheat bran as substrates [13-15].

A number of microbial beta-glucanases have been identified and characterized, while most of them are from bacteria, such as *Bacillus licheniformis* [16], *Bacillus sp*. [17], *Bacillus licheniformis* [18]. Only several beta-glucanases have been reported from fungi such as *Malbranchea cinnamomea* [19], *Aspergillus* (*A*.) *niger* US368 [20], *Rhizomucor miehei* [21], *Penicillium occitanis* [21] and *Trichoderma reesei* GXC [14].

However, purification and characterization of beta-glucanase by using wheat bran as a substrate without any nutrient by *A. niger* CCUG33991 has not been reported so far and this is the first description of the purification and characterization of a fungal acidic beta-glucanase produced by *A. niger* CCUG 33991 with SSF method. In the previous study we optimized the production of beta-glucanase from *A. niger* CCUG33991 with wheat bran [22].

A crucial barrier to the enzyme's widespread industrial application is the high production cost, prompting exploration of microorganisms that produce beta-glucanases economically. Filamentous fungi, particularly from the genus Aspergillus, have shown great potential due to their efficiency in SSF processes. Despite ongoing research, there is a scarcity of data

concerning the production and characterization of beta-glucanases from *A. niger*. Maximum production of the beta-glucanase was achieved at 33°C with an initial humidity of 70% for 51 h in a medium containing wheat bran as a carbon source with SSF.

Therefore, this study investigates the purification and characterization of beta-glucanase from *A. niger* CCUG33991, focusing on SSF using wheat bran as well as impact of purified enzyme on particle surface structure of the two kinds of cereals (barley and wheat).

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2. Materials and methods

2.1. Material

A. niger CCUG33991 was obtained from the Culture Collection of Goteborg university, Sweden. The strain was grown on potato dextrose agar (PDA) slants at 30°C and stored at 4°C until use. Sterile distilled water containing 0.1% (v/v) Tween 80 was used to separate spores and the surface was gently scraped with sterilized pipette. The spores were counted in a Neubauer chamber. Each solid substrate was inoculated with a suspension contain 4 × 10^7 spores/ml. Wheat bran, wheat and barley were obtained from a local farm in Isfahan, Iran. They were dried in a forced-air oven at 40°C for 24 h and shredded. Wheat bran was sieved with mesh 30. All the chemicals used in this research work were purchased from Sigma-Aldrich, Germany.

- Include a Data Availability Statement and upload raw data (e.g., enzyme activity, SEM images) to Figshare.

- Provide a standardized table detailing bioprocessing conditions (temperature, humidity, spore concentration) with control variables and standard deviations.

- Specify the number of replicates and quality control measures (e.g., device calibration).

2.2. Production of enzyme and enzyme extraction

An amount of 10 g wheat bran that sieved with mesh 30 and moisturized 70% with glycerol 1%, was added to different Erlenmeyer flasks and autoclaved at 121°C for 20 min. After cooling, the flasks were inoculated with the spore suspension (4×10^7 spore/ml) and kept at 33°C for 51 h. Sterile distilled water (1:10 w/v) was added to the flasks. The crude enzyme was extracted and filtered. The filtrate was centrifuged at 3200 rpm for 15 min and the clear supernatant was used for next steps.

2.3. Enzyme assay and protein determination and molar extinction coefficient estimation

Beta-glucanase activity was determined by measuring the rate of reducing sugar released from 1% (w/v) barley beta-glucan in 0.1 M sodium acetate buffer (pH 5.0) [23]. The reaction mixture containing 50 µL mL of 1% (w/v) beta-glucan solution and 50 µL enzyme suitably diluted by the same buffer. The test tubes were covered and incubated at 50°C for 30 min in a water bath. Then 300 µL of dinitrosalcylic acid reagent for stopping the reaction was added to each tube and placed in boiling water bath exactly for 5 min. The absorbance was read at 550 nm in spectrophotometer (Perkin-Elmer Lambda 25 UV–Vis spectrophotometer, USA) after cooling the samples in cold water and, amount of the reducing sugar liberated was measured by dinitrosalcylic acid method using glucose solution as standard reference. One unit of enzyme activity (U) was defined as the amount of enzyme that released 1 µmol glucose per min under the assay conditions. Protein concentration was measured by Lowry assay [24] using bovine serum albumin as a standard. The molar extinction coefficient was estimated from the UV spectra [25]

2.4. Purification procedure for enzyme

The crude protein obtained through fermentation of wheat bran was subjected for ammonium sulfate

precipitation. Ammonium sulfate was added to the supernatant that displayed in already part at 30-90% saturation and the solution was stirred for 60 min in an ice bath. The formed precipitate was collected by centrifugation (4200 rpm, 15 min). The resulting pellet was dissolved in a small amount of sodium acetate buffer 0.1M with pH 5 and dialyzed against the same buffer overnight with three changes. SDS-PAGE was performed as described by Laemmli [26] using 12.5% (w/v) separation gel and 4.5% (w/v) stock gel. Protein bands were visualized by Coomassie blue. The low molecular mass calibration kit was used that covered a wide range molecular weights from 18.4 to 116 kDa. The percent of ammonium sulfate that had higher amount of separation was used for continuation of the separation process. The dialyzed samples were filtered with 0.22 µm filter. The filtered sample was then and loaded onto a gel filtration column (HiLoad 16/60 Superdex 75 prep grade). The column was eluted at a flow rate of 1 ml/min and several fractions were taken. Fractions showing beta-glucanase activity were pooled and lyophilized. The sample was dialyzed overnight against sodium acetate buffer.

2.5. Molecular mass and isoelectric point determination

SDS-PAGE of purified enzyme was performed as described by Laemmli [26] using 12.5% (w/v) separation gel and 4.5% (w/v) stock gel. Protein bands were visualized by silver staining. The low molecular mass calibration kit was used that covered wide range molecular weights from 11 to 245 kDa. The location of beta-glucanase activity in SDS-PAGE was determined by the zymogram, it was obtained by copolymerizing 0.2% barley beta-glucan with 12.5% (w/v) polyacrylamide. Sample were treated with sample buffer in 0.5% SDS and 1% beta-mercaptoethanol and boiled for 5 min before application to the gel. SDS-PAGE gels were washed with 25% (v/v) isopropanol in 20 mM phosphate buffer (pH 7.2) to remove SDS and beta-mercaptoethanol. After a 20 min incubation period at 50°C in a sealed chamber, the gel was stained with 0.05% (w/v) congo red. Activity bands appeared as clear zones against a red background after destaining with 1M NaCL. The addition of 0.1 M acetic acid caused the gel to turn blue, thereby enhancing the contrast between the activity bands (clear zones) and unhydrolzed beta-glucan (blue background). Isoelectric focusing (IEF) was performed in a 5% polyacrylamide gel containing 4.6% carrier preblended ampholine (pH range 3.5-9.5) (LKB). The pI was obtained from a linear regression plot using pI standards [25].

2.6. Determination of kinetic parameters

The kinetic parameters (V_m and K_m) of the beta-glucanase were determined by varying the concentration of beta-glucan in 0.25, 0.5, 1, 1.5, 3, 5, 8 and 10 mg/ml in 0.1 M sodium acetate pH 5.0. The assays were performed with the enzyme, which had been diluted appropriately with 0.1 M sodium acetate buffer pH 5.0. A Lineweaver-Burk plot of the data was used for determination of Kinetic parameters.

2.7. Effect of pH on the activity and stability of beta-glucanase

For determination of optimum pH the purified enzyme was diluted with 0.1 M sodium acetate buffer with pH range from pH 3.0 to 6.0 with interval 0.5 and enzyme activity was estimated and reported as relative enzyme activity. Relative activity in each pH is defined as bellow Eq. 1:

Relative activity (%) =100 × (activity in each pH / maximum activity in optimum pH) Eq. 1

To test the pH stability, the purified enzyme was diluted with sodium acetate buffer having pH range from pH 3.0 to 6.0 with interval 0.5 and incubated for 60 min at ambient temperature. The enzyme activity was estimated and reported as residual relative activity. Relative activity is defined as bellow Eq. 2:

Relative activity (%)=

100 × (Residual enzyme activity in each pH / activity in zero time at same pH) Eq. 2.

2.8. Effect of temperature on the activity and stability of beta-glucanase

The optimum temperature for purified enzyme was obtained by assaying the enzyme activity at temperature range of 30-80°C with interval 10. The results are reported as relative activity (%). Relative activity (%) is defined as below Eq. 3:

Relative activity (%) = 100 × (Activity in each temperature / Maximum activity in optimum temperature)

Eq. 3

The thermo stability for the enzyme was monitored by incubating the purified enzyme at various temperatures between 30-80°C with interval 10 for 60 min and measuring the residual activities. The results are reported as relative activity (%). Relative activity (%) is defined as bellow Eq. 4:

Relative activity (%) = 100 × (Residual enzyme activity in each temperature / activity in zero time at same temperature)

Eq.4

2.9. Measuring half-life of the enzyme

For investigate the thermal deactivation of the enzyme and determination of the half-life, the purified enzyme was diluted with sodium acetate buffer with pH 5.0 and enzyme solution was incubated at 50°C and 70°C for 180 min.. At regular intervals times (10 min) the samples were taken and activity was measured. The activity at zero time was considered 100%. The time at which the enzyme showed 50% of its original activity, was considered as its half-life at given temperature.

2.10. The effects of metal ions on beta-glucanase activity

The effect of several ions on the activity of the purified beta 1,3-1,4 glucanase produced by *A. niger* CCUG33991 were also investigated through the addition of 1 mM of Cu^{+2}, Mg^{+2}, Fe^{+2}, Zn^{+2}, Ca^{+2} and Mn^{+2} to the reaction buffer.

2.11. Structural analysis of cereals with and without adding enzyme

For determination of effect of purified beta-glucanase on cereals, barley and wheat were treated with enzyme in conditions similar to conditions inside the poultry stratosphere [27]. For this, 5 g of each shredded cereal was mixed with 0.05 M sodium acetate buffer with pH 5.5 and purified enzyme were at 5: 14: 1 (g: ml: ml) ratio and stored at 40°C for 30 min (treated sample). For the control sample, distilled water was added instead of produced enzyme extract (untreated sample). The samples were dried for 48 h at 40°C and fixed on the aluminum stub and SEM (Tescan vega 3, Czech Republic) image was captured at an accelerating voltage of 15 kV and zoom in 4000.

3. Results and discussion

3.1. Production, purification, molecular mass and isoelectric point of beta-glucanase

The beta-glucanase activity of *A. niger* CCUG33991 was was 121.6 ± 1.5 U/gds (specific activity, 2.1 U/mg protein) after growth for 51 h. This culture supernatant was precipitated with ammonium sulfate 30-90% and SDS-PAGE was done (Fig. 1a). At 90% saturation, the higher separation was gained. The precipitate formed was collected and dissolved in a small amount of sodium acetate buffer 0.1M with pH5, dialyzed, filtered and subjected to gel filtration chromatography (Fig. 2).

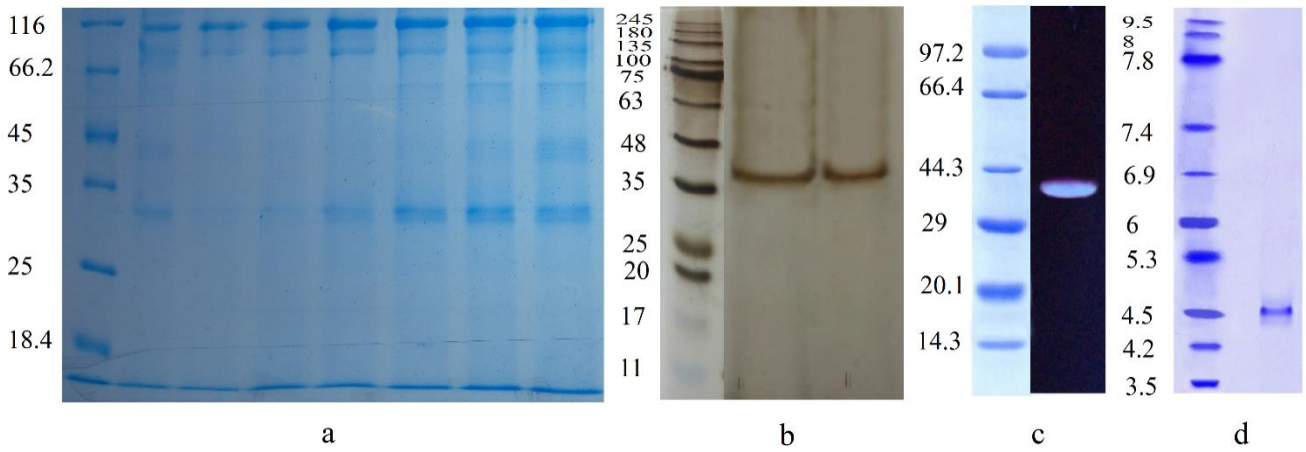

Figure 1. (a) SDS-PAGE of different ammonium sulfate precipitates of crud enzyme solution (2.1 U/mg protein), Lane 1 molecular weight of standard proteins lane 2-8 amount of ammonium sulfate (30-90%) with interval 10; (b) SDS-PAGE, Lane 1 molecular weight of standard of proteins, lane 2 purified enzyme after gel chromatography (25.6 U/mg protein); (c) Zymogram, lane 1 molecular weight of standard proteins, lane 2 purified enzyme after gel chromatography (25.6 U/mg protein); (d) Isoelectric focusing, lane 1 pH of standard proteins, lane 2 purified enzyme after gel chromatography (25.6 U/mg protein)

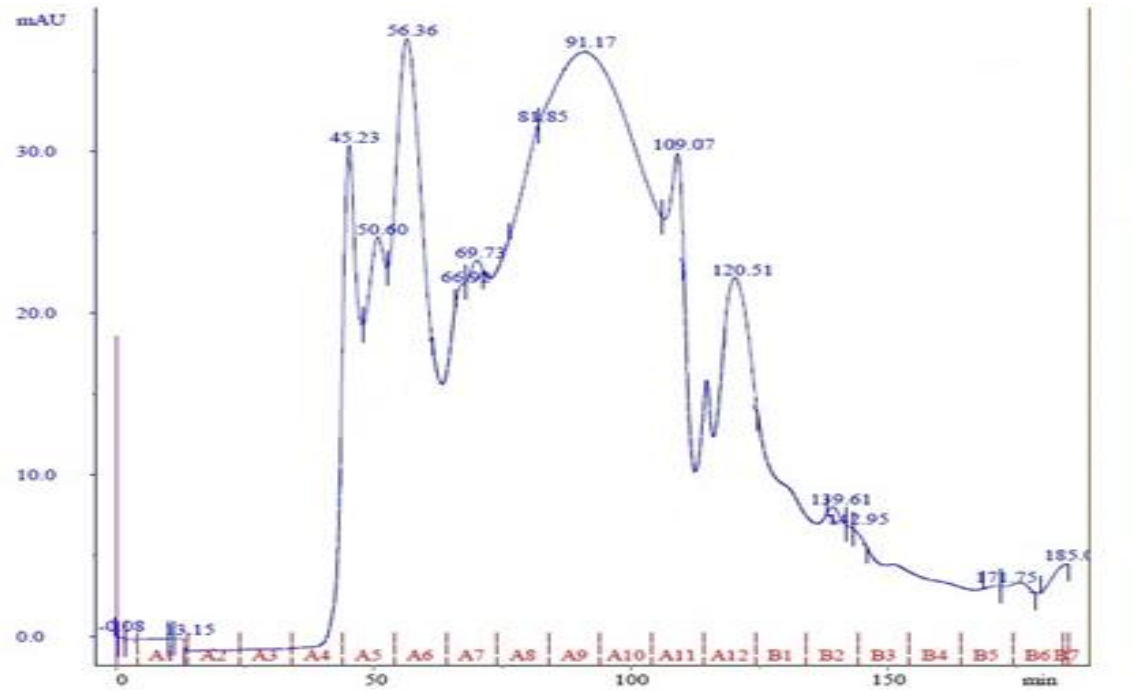

Figure 2. Elution profile of gel filtration chromatography of dialyzed enzyme

At chromatography several fractions (10 mL) were taken. A6 Fraction showing beta-glucanase activity were pooled and lyophilized (Fig. 2). The beta-glucanase was purified with a factor of 12.2 and a yield of 20.3%. The purified enzyme gained specific activity of 25.6 ± 1.1 U/mg (Table 1) and showed a single protein band on SDS-PAGE (Fig. 1b, lanes 2), with a molecular mass of 39 kDa.

Table 1. Summary of purification of a beta-glucanase from culture medium extract

	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude enzyme	605.5 ± 3.1	288.3 ± 3.9	2.1 ± 0.1	100.0	1.0
Ammonium sulfate dialyzed	205.0 ± 2.5	42.4 ± 1.3	4.8 ± 0.3	33.9	2.3
Gel filtration	123.0 ± 1.2	4.8 ± 0.4	25.6 ± 1.1	20.3	12.2

This value is higher than that of most bacterial beta-glucanases with molecular masses in the range of 25–30 kDa [28]. It is also higher than those of beta-glucanases from other fungi, including *P. occitanis* (20 kDa) [21], *Rhizopus microspores* var. *microsporus* (33.7 kDa) [3], *Thermoascus aurantiacus* (37 kDa) [29], *A. niger* US368 (32 kDa) [20], but lower than that of a beta-glucanase from an alkalothermophilic actinomycete, *Thermomonospora sp.* (64.5 kDa) [30]. Zymogram analysis (Fig. 1c, lane 2) of the pure enzyme showed a single clear band of beta-glucanase activity corresponding to a minor protein band of 39 kDa that it was subsequently identified as beta-glucanase. The molar extinction coefficient is 38000 M cm^{-1} at 550 nm.

The pI of the purified beta-glucanase was found to be pH 4.5 (Fig. 1d, lane 2). This value is higher than that of *Rhizopus microsporus* with pH 4.4 [3], *Emersonii* CBS 814.70 with pH 4.4 [31], *Rhizomucor miehei [32]* and lower than *Bacillus licheniformis* with PH 4.7 [25].

3.2. Kinetic parameters

The Michaelis-Menten kinetic constants K_m and V_m values for purified beta-glucanase from *A. niger* CCUG 33991 by Lineweaver-Burk plot were determined by using varying concentration of beta-glucan (Fig. 3).

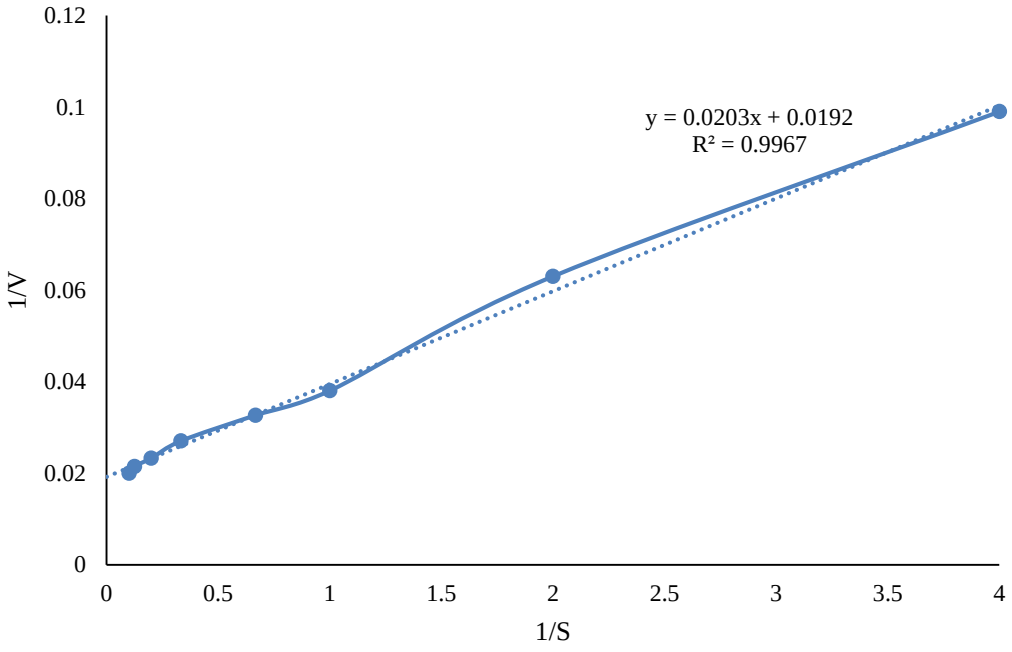

Figure 3. Lineweaver-Burk plot illustrating kinetic parameters (Km and Vm) of purified beta-glucanase

Enzyme activities were measured under standard assay conditions as described earlier and K_m and V_m were 5.1 mg/mL and 52.1 U/mg protein, respectively. Obtaining the catalytic efficiency of proteins is the

main purpose of estimating kinetics. K_m is dissociation constant, which represents the affinity of substrate in enzyme substrate complex. The K_m value (5.1 mg/ml) for barley beta-glucan was lower than that obtained for beta-glucanase from *Talaromyces emersonii* (13.38 mg/mL) [31] and *Penicillium occitanis* (5.95 mg/mL) [21]; however, it was higher than the values obtained for *A. niger* US368 (0.62 mg/mL) [20], *Malbranchea cinnamomea* (0.69 mg/mL) [19], *Rhizomucor miehei* (2 mg/mL) [1] and *Bacteroides succinogenes* cloned in *Escherichia coli* (0.71 mg/mL) [33]. The V_m value (52.1 U/gds) for barley beta-glucan was lower than that obtained from *Talaromyces emersonii* (142.9 U/mg protein) [31] but higher than that obtained from *A. niger* US368 (34.46 U/mg protein) [20].

3.3. Effect of pH on activity and stability of beta-glucanase

Enzyme activity is markedly affected by pH because of substrate binding and catalysis is often affected by charge distribution on both, substrate and enzyme molecules [34].

The effect of pH on the activity and stability of the purified beta-glucanase from *A. niger* CCUG33991 are shown in Fig. 4a and 4b, respectively.

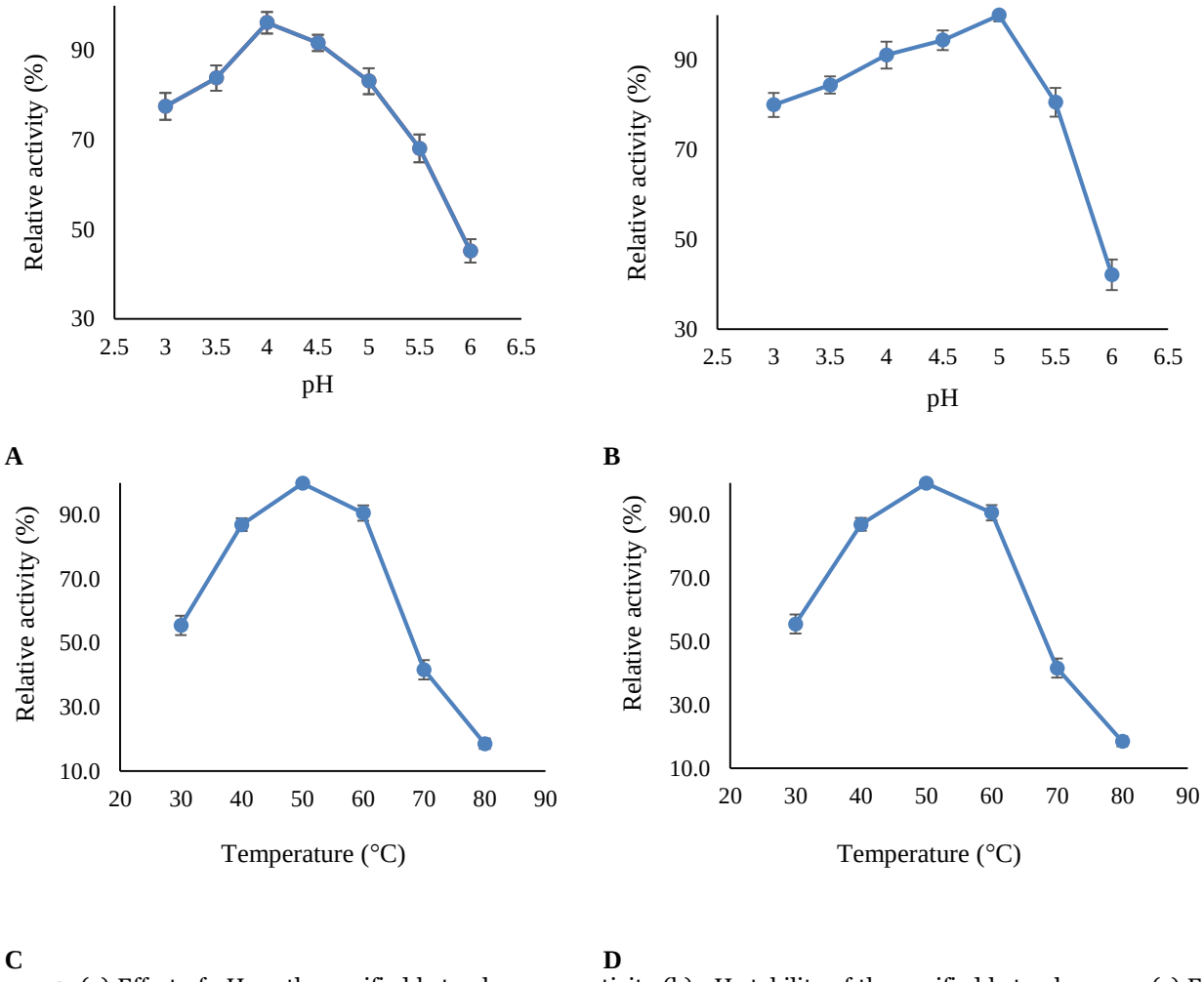

Figure 4. (a) Effect of pH on the purified beta-glucanase activity (b) pH stability of the purified beta-glucanase (c) Effect of temperature on the purified beta-glucanase activity (d) Temperature stability of the purified beta-glucanase

The activity of beta-glucanase to hydrolyze barley beta-glucan was examined at pH values ranging from 3.5 to 6.5 at 50°C. When the reactions were performed without the enzyme under the experimental condi-

tions, reducing sugar was not formed. The maximum activity was observed at pH 5.0. At pH 4.5 and 5.5, the activities were 94.4 ± 2.2% and 80.6 ± 3.2% of the maximum, respectively. The application of beta-glucanase in the feed industry to digest the hemicellulose and decrease the digesta viscosity demands good enzyme activity and stability under acidic conditions. The beta-glucanase activity was 80.0 ± 2.7% of the optimum pH at pH 3.0. The stability of the beta-glucanase at pH 3.5-6.5 was determined. As seen in Fig. 4b, the beta-glucanase activity was relatively more stable at low pHs than at high pHs. The optimum was at pH 4 when beta-glucanase activity was 96.2 ± 2.4%.

The relative activity of beta-glucanase from *Bacillus subtilis* was in the pH region of 6.5 to 6.6 [35]. The enzyme from *Rhodothermus marinus* had a pH optimum of 7.0 [36]. The optimum activity appearing in acidic condition was found to be the same as that of fungal beta-glucanases from *Trichoderma longibrachiatu*m [37], *Trichoderma reesei* GXC [14], *Rhizopus microspores* var. *microsporus* [3]. In addition, these values are comparable to those presented by enzymes currently being used in the brewing industry [30, 38].

3.4. Effect of temperature on activity and stability of beta-glucanase

The effect of temperature on the activity and stability of the purified beta-glucanase *A. niger* CCUG33991 are shown in Fig. 4c and 4d, respectively. The temperature optimum of purified beta-glucanase was measured by using the standard assay in the temperature range of 30-80°C. Fig. 4c shows that the beta-glucanase activity increased with rise of temperature, maximized at 50°C; then decreased rapidly with the temperature, and exhibited about 18.5 ± 1.6% of the maximal activity at the temperature of 80°C; and was about 87.0 ± 2% of the maximal activity at 40°C. For determining the thermostability, the enzyme solution in sodium acetate buffer (pH 5) was incubated at various temperatures for 60 min, the enzyme was cooled and residual activity was determined under standard enzyme assay conditions. Fig. 4d shows that the beta-glueanase was almost stable during 60 min incubations at temperature of 30-40°C; that loss of enzyme activity was countinued above 50°C; and the loss rate of activity increased with rise of temperature. However, after incubation of the enzyme at 60 °C and 70 °C for 60 minutes, the relative enzyme activities were 57% and 28%, respectively, indicating that the enzyme has a good thermal stability.

The optimum temperature of activity of enzyme may differ significantly depending on different sources. For some fungal beta-glueanases such as *Achlya ambisexualis* [39], *A. japonicus* [40], *R. micro-sporus* var. *microspores* [3], and *T. emersonii* [41] the optimum temperatures were at the range of 37-50°C. However, the optimum temperatures of some enzymes, such as those produced by bacteria are higher than those of fungal enzymes. For exanaple, the enzyme from *Rhodothermus marinus* expressed in *Escherichia coli* had a temperature optimum of 85°C and was shown to retain full activity after incubation for 16 h at 80°C [36]. A thermoactive enzyme from *Laetiporus sulphureus* var. *miniatus* had a tempe-rature optimum of about 75°C [42]. However, enzyme from *Rhizopus microspores* var. *microsporus* had a same temperature optimum of 50°C [3].

The purified enzyme beta-glucanase was less thermostable than fungal beta-glucanases from *Malbranchea cinnamome*a [19]. However beta-glucanase was more thermostable than beta-glucanase from *Rhizopus microsporus* var. *microspores* [3] and beta-glucanase from *Orpinomyces strain* PC-2 [43].

3.5. Half-live

The half-life activities of the purified enzyme were 120 min and 29.5 min at 50°C and 70°C, respectively (Fig. 5).

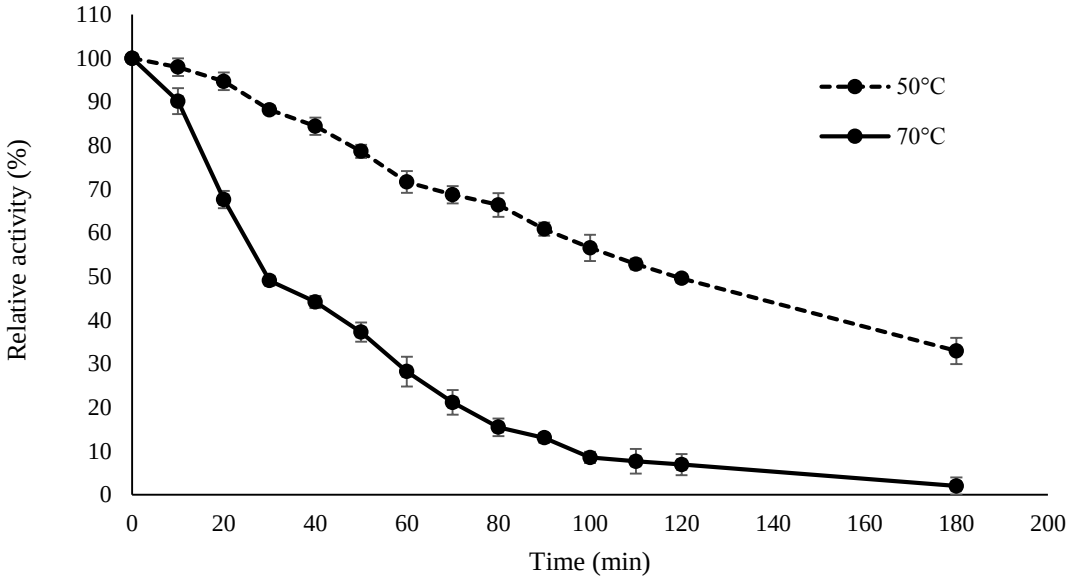

Figure 5. Enzyme half-life determination: beta-glucanase activity as a function of pre-incubation time at temperatures 50°C (dashed line) and 70°C (line)

This values were lower than *A. niger* US368 (151 min at 50°C and 30 min at 70°C) [20] and higher than *Rhizopus microspores* var. *microsporus* (81 min at 60°C and 21 min at 70°C) [3].

For determination of order of reaction, the enzymes were incubated at temperatures 50 and 70°C for varying periods of time. A sample was taken at each time interval and the relative activity was determined after the reaction. The experimental data showed that the thermal deactivation of enzyme were fitted to a first-order curve.

3.6. The effects of metal ions on beta-glucanase activity

The effect of several ions on the activity of the purified beta 1,3-1,4 glucanase produced by *A. niger* CCUG33991 were also investigated through the addition of 1 mM of Cu^{+2}, Mg^{+2}, Fe^{+2}, Zn^{+2}, Ca^{+2} and Mn^{+2} to the reaction buffer (Table 2). Ion Ca^{+2} increased beta-glucanase activity by 3%. It has been reported that Ca^{+2} increases the substrate binding affinity of the enzyme and fixes the conformation of the catalytic site of enzyme [21]. The addition of Zn^{+2}, Mn^{+2}, Cu^{+2}, $Fe^{+2}$and Mg^{+2} dropped the beta-glucanase to 3%; 55%; 10%; 1% and 1% of its initial activity, respectively. The addition of Mn^{2+} strongly inhibited the enzyme, resulting in 45 ± 2.2 % relative activity. Mn^{2+} inhibition was also found for the β-1,3-1,4-glucanases from *A. japonicus*,[42] yielding relative activities of 75% [42].

Table 2. Impact of metal ions on the activity of beta 1,3-1,4 glucanase from *Aspergillus niger* CCUG33991

Metal ions	Relative activity (%)
Mg^{+2}	99 ± 1.1
Cu^{+2}	90 ± 2.1
Zn^{+2}	97 ±1.1
Fe^{+2}	99 ±0.5
Ca^{+2}	103 ±1.2
Mn^{+2}	45 ± 2.2

3.7. Effect of the purified enzyme on the structure of cereal

The effect of purified SSF enzyme on the structure of barley and wheat were visualized by using SEM imaging (Tescan vega 3, Czech Republic) that was captured at an accelerating voltage of 15 kV and Zoom in 4000 (Fig. 6a-6d).

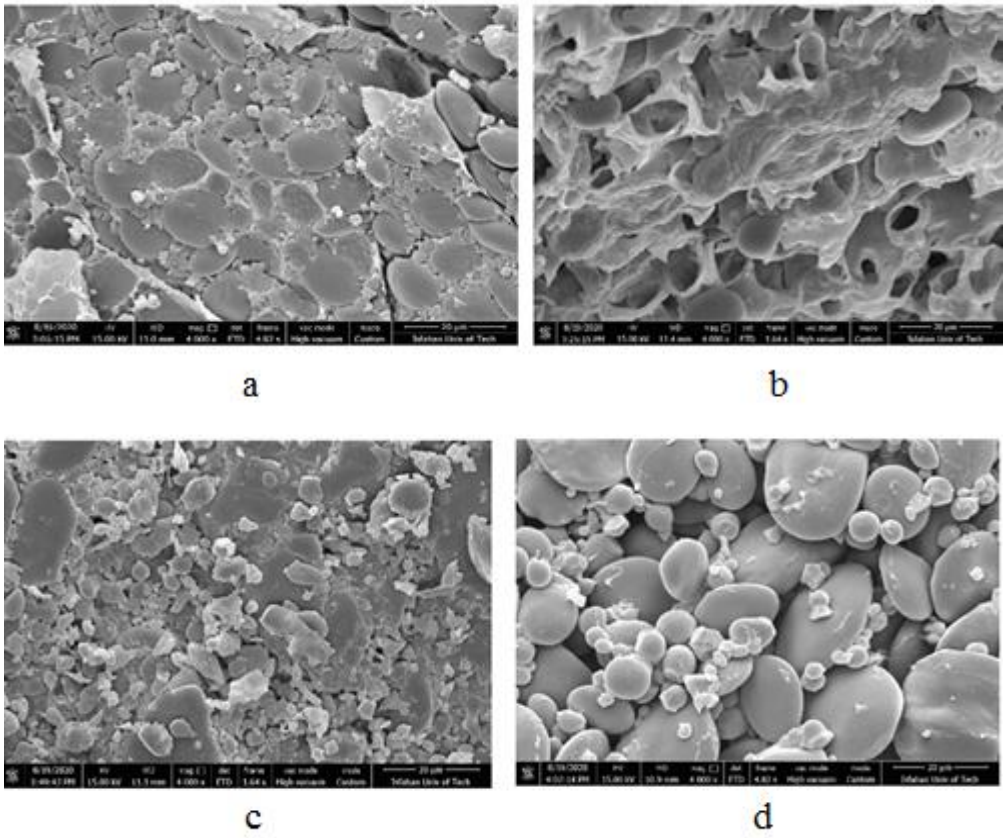

Figure 6. SEM images of barley: in untreated mode with the enzyme (a) and treated mode (b); wheat: in untreated mode with the enzyme (c) and treated mode (d)

Beta-glucan acts like an adhesive and binds protein and starch granules together [44, 45]. The high content of beta-glucan causes the starch-protein granules in the barley and wheat to cling completely (Fig. 6a and Fig. 6c). Treating the barley and wheat with beta-glucanase resulted in the removal of beta-glucan from the starch-protein granules. So, the starch granules appeared disjointed in treated samples (Fig. 6b and Fig. 6d). Because the beta-glucan content in barley was higher than wheat [46], the effect of beta-glucanase on barley was greater and more detached starch granules appeared in barley than wheat. Generally surface attack of cereal particles by the enzyme resulted in the destruction and modification of the fibrous structure and increase in the porosity [15, 47].

Therefore the addition of the enzyme to poultry feed seems to enhance the degradation of beta-glucan and disintegration of cereal particles surface which makes the surface more vulnerable to attack by external and digestive enzymes. These changes may result in better digestion and higher feed conversion ratio (FCR) in poultry. This is in agreement with the results of other researchers who observed that adding the enzyme to the poultry feed would improve digestion and eliminate digestive problems [48, 49].

4. Conclusion

A beta-glucanase from *A. niger* CCUG33991 from wheat bran with SSF method was purified and characterized, and its properties were investigated. The molecular mass of beta-glucanase was 39 kDa. The findings presented in this work, particularly in terms of pH and thermal stability and the structural analysis of treated cereals with purified beta-glucanase are of interest to various industrial applications and processes, particularly in the animal feed industry. In

fact, the use of this beta-glucanas enhance the beta-glucan digestibility in feed stuffs, improve feed conversion efficiency, and reduce sanitary problems. This enzyme could also be used in the brewing industry to increase extract yields, reduce brewer mash viscosity and turbidity, and produce high-quality brewer malt. In addition to this enzyme with relatively good thermostability that is stable at low pH might be used in other industrial applications. The enzyme potential in gut microbiome enhancement or nutraceutical production would be applicable potentials. Bioinformatics tools (e.g., protein model-ing) is suggested to analyze enzyme structure/function. Applications in sustainable biotechnology (e.g., recombinant enzyme production) would be future trend of this research.

5. Declarations

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5.2. Authors' Contributions

All authors equally contributed to this work.

5.3. Declaration of Interest

The authors of this article declared no conflict of interest.

5.4. Ethical Considerations

All ethical principles were adhered in conducting and writing this article.

5.5. Transparency of Data

In accordance with the principles of transparency and open research, we declare that all data and materials used in this study are available upon request.

5.6. Funding

This research was carried out independently with personal funding and without the financial support of any governmental or private institution or organization.

5.7. Using Artificial Intelligent chatbots

No AI chatbot has been used in this study.

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