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**ABSTRACT**

β-glucosidase enzyme produced from *Aspergillus niger* NRRL 3122 has been partially purified and characterised. Its molecular weight was 180 KDa. The optimal pH and temperature were 3.98 and 55 °C, respectively. It promoted the hydrolysis of soy flour isoflavone glycosides to their aglycone. Two-level Plackett–Burman design was applied and effective variables for genistein production were determined. Reaction time had a significant positive effect, and pH had a significant negative effect. They were further evaluated using Box–Behnken model. Accordingly, the optimal combination of the major reaction affecting factors was reaction time, 5 h and pH, 4. The concentration of genistein increased by 11.73 folds using this optimal combination. The antioxidant activity of the non-biotransformed and biotransformed soy flour extracts was determined by DPPH method. It was found that biotransformation increased the antioxidant activity by four folds.

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1. **Introduction**

Among the food eaten by humans, soybeans contain the highest level of isoflavones, which are phytoestrogen (plant-derived phenolic compounds with structural homology to human oestrogen), common in leguminous plants (Munro et al. 2003). These compounds are currently heralded to offer potential natural alternative therapies for a range of hormone-dependent conditions including menopausal symptoms (Cheng et al. 2007), cardiovascular disease (Gil-Izquierdoa et al. 2012), osteoporosis (Taku et al. 2011) as well as prostate, breast and colon cancer (Akaza 2012).

Soy isoflavones exist in the form of aglycones (daidzein, genistein and glycitein) and β-glycoside conjugates, which include glycosides (daidzin, genistin and glycitin), malonylglycosides and acetylglycosides. The content of daidzin and genistin is high in soybeans while the aglycones (daidzein, genistein) are found in trace quantities (Yamabe et al. 2007).

Numerous studies have shown that the biological effects of isoflavones are not due to the glycoside forms but mainly due to their aglycones. For example, aglycones are able to bind to the oestrogen receptor and hence mimic estradiol functions in the human body and thus prevent certain cancers (Sun et al. 2010). The anti-cancer function of soybean isoflavones was shown to be associated with genistein, which inhibits protein tyrosine kinases and DNA topoisomerase, and binds weakly to oestrogen receptors (Sunita and Pattanayak 2011). It was found that early exposure to genistein enhances cell differentiation of the mammary gland, and may confer a protective effect against carcinogenesis via this process (Lamartiniere 2000). Furthermore, in vitro studies using cultured human breast cancer cells indicate that genistein inhibited the growth of both oestrogen receptor-negative and oestrogen receptor-positive cell lines (Mai et al. 2007). Also, genistein exhibited a much greater antioxidant activity than that of genistin (Kroyer 2004).

β-glucoside enzyme EC 3.2.1.21 catalyses the hydrolysis of the β-glycosidic linkage from the non-reducing end of isoflavone glucosides, disaccharides, oligosaccharides, aryl-glucosides and alkyl-glucosides (Singh et al. 2016).

The present study reports the production, purification and characterisation of β-glucosidase from *Aspergillus niger* NRRL 3112 and its application in the hydrolysis of isoflavone glucosides in soy flour. The current work is the first time to use response surface methodology in the optimisation of the biotransformation condition of the major soy isoflavone (genestin) into its aglycone (genistein) in the soy flour extract by β-glucosidase enzyme and evaluate the effect of this biotransformation on the antioxidant activity.

2. **Results and discussion**

2.1. **Purification and characterisation of β-glucosidase enzyme**

A summary of the partial purification of the β-glucosidase enzyme is given in (Table S1). The protein precipitated in the range of 70–80% saturation contained the bulk of enzyme activity. After the purification steps mentioned above, β-glucosidase enzyme was purified by 4.44-folds and specific activity increased to 422 U/mg.

The presence of β-glucosidase enzyme was verified by native PAGE and Zymogram of partially purified β-glucosidase enzyme which revealed one band of activity (hydrolysis of MUG and release of 4-methylumbelliferone which gives blue fluorescence under UV), with apparent molecular weight of 180 KDa by comparison with a standard protein marker (Figure S1).
The optimisation of temperature and pH for activity of β-glucosidase is important as it can greatly increase the enzyme activity. In the full factorial design, we applied a sequential optimisation strategy, where the independent variables (pH and temperature) were tested at seven different levels. The average U/mL production results were used as the responses. Data shown in (Table S2) illustrate the wide variation of β-glucosidase activity from 0 to 288 U/mL, thereby reflecting the importance of temperature and pH for attaining the maximum enzyme activity. On analysing the regression coefficients for the tested variables, it was found that pH showed a significant effect on β-glucosidase activity at the low level. The results suggested that the optimal activity would be closer to the lower pH levels (Table S3).

In the central composite experimental design, the independent variables (temperature and pH) were screened in 16 runs organised according to the central composed design matrix (Table S4). In this model, the independent variables (pH and temperature) were tested at five different levels. The average U/mL production results were used as the responses. The results of this experiment showed that, the most effective variables for β-glucosidase activity were pH value and temp*temp (Table S5). The highest record for β-glucosidase activity (333 U/mL) was achieved by the trial No. 7 at pH 4 and 65 °C with an increase of 15.62%, more than that obtained by the full factorial design. The enzyme had a pH range of 3.0–6.0 and temperature range of (30–70) °C for its activity. The optimum temperature 55 °C and the optimum pH 3.98 were similar to those obtained by (Falkoski et al. 2012).

β-glucosidase was 100% active (Figure S2) at (30, 40, 50) °C, whereas 75% relative activity was retained at 60 °C after 30 min of incubation. While at 80 and 90 °C the activity was totally lost. β-glucosidase was 100% stable (Figure S3) at pH (4–5), whereas 74.0% relative activity was retained at pH (3). By increasing pH, the enzyme lost its stability gradually to reach (66, 39, 19)% at pH (7, 8, 9) respectively. The thermal stability up to 70 °C for 30 min at pH 4.8 of the partially purified enzyme is beyond the range of values reported for this enzyme from other Aspergillus species. This higher temperature is very advantageous for biomass saccharification since they can maintain a rapid reaction rate and also help in preventing contamination by allowing the reaction to proceed at a higher temperature which can be a potent supplement to the cellulases for creating better enzyme cocktails that can also act at elevated process temperatures (Reeta 2011). The pH stability (3.0–7.0) of the enzyme is beyond the range of most fungal β-glucosidases.

The action of partially purified β-glucosidase was tested over different substrates with α and β configurations. The results summarised in (Table S6) show that β-glucosidase was maximally active against cellobiose with specific activity (434 IU/mg), β-glucosidase enzyme has also high specificity towards maltodextrin (268 IU/mg). In addition, some other substrates, such as sucrose, maltose, pullulan, cellulose and starch, were also degraded but less efficiently compared with cellobiose. However, dextran was not observed to be hydrolysed. The high activity of β-glucosidase from A. niger NRRL 3122 to a great extent indicates no steric hindrance with this compound as also observed with β-glucosidase from Thermoanaerobacterium thermosaccharolyticum (Pei et al. 2012).

The kinetic parameters for cellobiose were calculated from Lineweaver–Burk plots (Figure S4). The Km and Vmax values of the enzyme for cellobiose were 2 mM and 200 IU/mg, respectively. Km (2 mM) for cellobiose is lower than half of that obtained by (Shen and Xia 2003) which was (6) mM. A low Km for the cellobiase (2 mM) indicated high affinity to the substrate. It is important in an industrial saccharification because during the saccharification it is desirable to reduce the product inhibition cellobiose exerts on the other enzymes in the system.
A specific activity (velocity) of 200 IU/mg of protein was obtained when cellobiose was the substrate. This is very high compared with other purified β-glucosidases.

2.2. Optimisation of genistein production

Plackett–Burman experimental design was applied to reflect the relative importance of various enzymatic biotransformation factors. The data shown in (Table S8) revealed a wide variation in genistein conc. (0.681–5.4) mg/g, thereby reflecting the significant effect of the studied factors for attaining a higher productivity. Sorted parameter estimates of the eight tested variables revealed that time had a significant positive effect on genistein aglycone production, whereas pH had a significant negative effect (Table S9). To identify the optimum response region for genistein aglycone, the significant independent variables [reaction time and pH] were further explored at three levels using Box–Behnken design. (Table S10) presented the design matrix for the variables, given in both coded and natural units, plus the experimental genistein aglycone production results. The optimal levels for the factors were found to be time (5 h), and pH (4), where genistein concentration reaches up to (9.38 mg/g).

There was 11.73 folds increase in the concentrations of genistein aglycones compared to the non-biotransformed suspension. Similar results were obtained where the content of daidzein had increased by 34 folds in soybean flour suspension biotransformed by a thermostable β-glucosidase enzyme from Paecilomyces thermophile (Yang et al. 2009). Maitan-Alfenas et al. 2014 found that β-glucosidase increased genistein content from 1.28 mg/g in the non-biotransformed to 6.37 mg/g in the biotransformed soy molasses.

The value of the determination coefficient (RSq) was 0.99 (Figure S5). This indicates a high degree of correlation between the experimental and predicted values, and confirms the high accuracy of the model.

2.3. Antioxidant activity

The antioxidant properties of the bio-transformed soy flour extracts were evaluated by DPPH method. (Figure 1) shows that both non-biotransformed and biotransformed soy flour extracts have a scavenging activity towards the DPPH radical in a dose-dependent manner (2.5–15 mg/mL). However, there was four folds increase in the antioxidant activity of the non-biotransformed soy flour extract and biotransformed soy flour extract (1) Non-biotransformed soy flour extract (2) Biotransformed soy flour extract using the produced β-glucosidase.
biotransformed extract compared to the non-biotransformed one. The increase in the antioxidant activity is explained by that the hydrolysis of β-glucosidic linkage leads to liberation of hydroxyl groups in free aglycone which might be important to the antioxidant activity of the extract (Okawa et al. 2001). EC50 was (10 and 2.5) mg/mL for non-biotransformed and biotransformed soy flour extracts using the produced β-glucosidase enzyme, respectively.

3. Experimental

3.1. Micro-organisms

A. niger NRRL 3122, obtained from Agricultural Research Service (ARS) Culture Collection (Peoria, Illinois USA), was used in this study.

3.2. β-glucosidase production

The stock culture was maintained on potato-dextrose-agar (PDA) slants at 4 °C and sub-cultured every 4 weeks. Spores were harvested by scraping from PDA slants (7 days incubated at 30 °C) with 10-mL distilled water to give a spore suspension of approximately 2 × 10^7 spores/mL. Each 250-mL flask containing 50 mL of the fermentation medium (g/L): 5 glycerol (analytical grade), 3.5 wheat bran, 7.5 corn steep liquor (CSL, 50% dry matters), 1 NaNO₃, 0.3 K₂HPO₄, 0.1 KCl, MgSO₄·7H₂O and 0.01 FeSO₄·7H₂O was inoculated with 0.25 mL of spore suspension (2 × 10^7 spores/mL) and incubated at 30 °C with agitation at 200 rpm. Broth samples were taken on the 8th day and assayed for their β-glucosidase activities after removing the mycelia by centrifugation.

3.3. Purification of β-glucosidase enzyme

β-glucosidase enzyme was partially purified by ultrafiltration using Amicon Ultra centrifugal filters MWCO 100 kDa. Then by slow addition of ammonium sulphate with stirring to the crude enzyme gives 60% saturation at 4 °C, and then allowed to stand for 24 h at 4 °C. After centrifugation at 10,000 rpm for 20 min, the supernatant was decanted and the precipitate was discarded. Ammonium sulphate was added to bring the supernatant to 80% saturation under the same conditions. After centrifugation at 10,000 rpm for 20 min, the supernatant was decanted and the precipitate was dissolved in 10 mL, 0.05 M citrate phosphate buffer (pH 4.8), and then dialysed against the same buffer for 48 h.

3.4. Native polyacrylamide gel electrophoresis (Native-PAGE) and zymogram analysis

Standard protocols for SDS and Native PAGE were employed to prepare gels with 10% strength and were used throughout the study. Samples were concentrated using Amicon ultra centrifugal filter 100 KDa before loading on to the gels. Protein was estimated by Bradford method and samples were normalised to contain equal protein concentrations before loading the gel in duplicates. Gels were loaded as two halves with each half containing the same samples exactly in the same order and concentration. After completion of the electrophoresis, the gels were washed once in distilled water and were divided into two
parts, each corresponding to a half containing all the samples as the other one. One of the halves was incubated with 10 mM MUG (Methyl umbeliferyl glucopyranoside) solution in citrate buffer (0.05 M, pH 4.8) for 10 min at room temperature (28 °C). The second half was stained with Coomassie Brilliant Blue staining. BGL activity was visualised as blue-green fluorescence under long wavelength UV trans-illumination. Then it was photographed using an imaging system (Red Cell Bioscience, USA).

3.5. Characterisation of the β-glucosidase

3.5.1. Determination of the molecular weight
The partially purified β-glucosidase enzyme was run on native PAGE (Laemmli 1970) along with standard protein markers (Blue stain protein ladder, (20-245) KDa, Gold Biotechnology, USA). β-glucosidase activity band was visualised by Methylumbeliferyl glucopyranoside (MUG) (Bioworld, USA) staining and the zymogram was photographed. The position of β-glucosidase enzyme was confirmed by comparison with the standard protein markers of known molecular weights.

3.5.2. Determination of the optimal temperature and pH
0.5 mL of the substrate (0.4% cellobiose in citrate phosphate buffer) was added to 0.5 mL of appropriately diluted enzyme solution (fermentation broth filtrate) and incubated under the conditions indicated in the experimental designs for 30 min (Table S2 and S4). Then, the enzymatic activity was measured.

3.5.3. Temperature and pH stability of the β-glucosidase enzyme
The thermal stability of enzyme was evaluated by incubating the enzyme in citrate phosphate buffer (0.05 M, pH 4.8) at (30 to 90) °C for 30 min. Then, the residual enzymatic activity was measured. The pH stability of enzyme was evaluated by incubating the enzyme in a series of buffers at pH range of (3 to 9) at 4 °C for 24 h. Then, the enzyme solution was adjusted to pH 4.8. Then, the residual enzymatic activity was measured.

3.5.4. Substrate specificity of the β-glucosidase
0.5 mL of appropriately diluted enzyme solution was incubated with 0.5 mL of each substrate in citrate phosphate buffer (0.05 M, pH 4.8) at 50 °C for 30 min. The total amount of reducing sugars from 1% polysaccharides (dextran, soluble starch, maltodextrin and microcrystalline cellulose, pullulan) was determined by DNS. The glucose released from 10 mM (sucrose, maltose, and cellobiose) was determined. Then, the enzymatic activity was measured.

3.5.5. Determination of Kinetic Parameters of β-glucosidase
The effect of cellobiose (1–20 mM) on the reaction rate was determined at 50 °C and pH 4.8. The values of the Michaelis constant (Km) and the maximum velocity (Vmax) were determined from Lineweaver–Burk plots.

3.6. Assay of the β-glucosidase enzyme
0.5 mL of the substrate (0.4% cellobiose in 0.05 M citrate phosphate buffer, pH 4.8) was added to 0.5 mL of appropriately diluted enzyme solution and incubated at 50 °C for 30 min. The
reaction mixture was placed in boiling water for 5 min to stop the reaction and then immediately cooled in an ice bath. The glucose concentration in the mixture was determined using high-performance liquid chromatography (HPLC) with an Aminex HPX-87H column (300 × 7.8 mm) at 45 °C and 0.005 M H₂SO₄ as the mobile phase at 0.6 mL/min. (Zaldívar et al. 2001) One unit of β-glucosidase activity was defined as the amount of enzyme that produced 1 µmol of glucose per min from cellobiose.

3.7. Enzymatic hydrolysis of the soy flour

The defatted soy flour was suspended in citrate phosphate buffer and was hydrolysed by its incubation with β-glucosidase enzyme under the conditions indicated in the experimental design (Table S8 and S10). The reactions were terminated by boiling the suspension for 5 min, and then the hydrolysed solutions were centrifuged at 11,000 rpm for 15 min to collect supernatants containing isoflavones.

3.8. Optimisation of soy isoflavone aglycone production based on multifactorial experiments

3.8.1. Evaluation of the factors affecting soy isoflavone aglycone production

Eight assigned variables (pH, reaction time, temperature, substrate conc., agitation rate, CaCl₂, MnCl₂ and buffer strength) were screened in 12 experimental trials using Plackett–Burman experimental design (Liu et al. 2010). Table (S7) illustrates the examined factors, as well as the levels of each factor used in the experimental design. The magnitude and the ranking of each variable in Plackett–Burman design were estimated by statistical analyses of the data. The main effect of each variable was calculated as the difference between the average of measurements made at the high value (+) and at the low value (−). All experiments were carried out in triplicate and the concentration of genistein aglycone (mg/g) was taken as the response (dependent variable).

3.8.2. Optimisation of the factors affecting enzymatic deglycosylation of genistin

The variables with significant effects on enzyme production, as identified by the Plackett–Burman design, were further optimised using a response surface Box–Behnken design (Ferreira et al. 2007). The design comprised 15 experiments where pH and reaction time were tested at three levels and in multiple combinations with other parameters. The whole set of experiments was performed in triplicate and the concentration of genistein aglycone (mg/g) was taken as the response (dependent variable).

3.9. HPLC determination of genistein

After centrifugation of the hydrolysed soy flour suspension, the supernatant was freeze dried. The freeze-dried samples were stirred in 15 mL of 80% aqueous methanol solvent for 3 h at room temperature, filtered through Whatman No. 42 filter paper. A rotatory evaporator was used to dry the filtrate under vacuum at a temperature below 37 °C. The residue was re-dissolved in 10-mL 80% MeOH solution and filtered through a 0.45 Fm filter unit. The genistein content was determined by Shimadzu HPLC (Kyoto, Japan) system model LC-10AD VP controlled by CLASS-VP System Software, using pump (Model LC-10 ADvp), UV/ VIS
detector (Model SPD 10A VP). Chromatographic separation was carried out on Dikma Diamonsil C18 column (4.6–250 mm Dima Co., Ltd., Orlando, FL). The mobile phase had the following composition: 0.1% (v/v) acetic acid in filtered MilliQ water (solvent A), and 0.1% (v/v) acetic acid in acetonitrile (solvent B). The following gradients for solvent B were applied: 15–25% over 35 min, 25–26.5% over 12 min and 26.5–50% over 30 s, followed by isocratic elution for 14.5 min with a flow rate of 1.0 mL/min and column temperature setting at 40 °C and absorbance was measured at 254 nm (Wang and Kurzer1997).

### 3.10. Statistical analysis

The optimal value for production of soy isoflavone aglycone was estimated using SAS JMP 8 NULL program tools for a regression analysis of the obtained experimental data. The quality of the fit of the polynomial model was expressed by the coefficient of determination ‘RSq’. The experiments were performed in triplicate and the mean values were given.

### 3.11. Antioxidant assay

Antioxidant activity of soy flour methanolic extract was determined by the ability to scavenge 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radicals. One-mL DPPH (0.1 mM) solution in methanol was mixed with 100 µL of the crude soy extract, vortexed well and then incubated for 30 min in the dark at room temperature and the absorbance was measured at 517 nm. For the control sample, the crude soy extract was replaced with 100 µL methanol. This antioxidant activity was given as percent (%) DPPH scavenging and EC 50 was obtained by extrapolation of the data and calculated on the basis of the total solid contents of each methanolic extract (McCue et al. 2004).

### 4. Conclusion

The β-glucosidase of A. niger NRRL 3122 shows a potential as an industrial source of β-glucosidase. It has a high affinity for cellobiose and one of the highest specific activities ever reported for cellobiase activity. The enzyme is extremely stable over a wide range of temperatures and pH. One of its most important attributes is that its pH and temperature optima match those reported for *Trichoderma* cellulase, indicating a potential for supplementing *Trichoderma* cellulase with β-glucosidase from *A. niger*. The biotransformation of soy flour by β-glucosidase enzyme using these optimal factors increased genistein concentration by 11.723 folds. There was four folds increase in the antioxidant activities of the biotransformed soy flour suspension comparing to the non-biotransformed one.

### Supplementary material

Supplementary material relating to this article is available online, alongside with Figures S1–S5 and Tables S1–S11.

### Disclosure statement

No potential conflict of interest was reported by the authors.
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