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MEDIUM OPTIMIZATION FOR ISOFLAVONE AGLYCONES PRODUCTION FROM FERMENTED DEFATTED SOYBEAN FLOUR BY DISPLAY OF B-GLUCOSIDASE FROM *BACILLUS LICHENIFORMIS* NRC24 AND EVALUATION OF THEIR ANTIVIRAL AND ANTITUMOR ACTIVITIES

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ABSTRACT

The submerged fermentation parameters of defatted soybean flour with *Bacillus licheniformis* NRC24 were optimized using the one-factor at-a-time method and Box-Behnken design to maximize the production of β-glucosidase and transformation of glucosidic isoflavones (daidzin and genistin) into their aglycones. One-factor-at-a-time optimization studies revealed that 80 g/L of defatted soybean flour , fermentation time of 16 h, temperature at 37°C, initial pH value of 7, inoculum volume of 5% ($6x10^7$ CFU/mL), and medium volume of 50 mL in 250 mL Erlenmeyer flask with shaking conditions at 100 rpm were the most suitable conditions for aglycones production. The addition of cellobiose (1 percent w/v) to the soy flour medium resulted in a significantly increase in total aglycone. Further optimization using the Box-Behnken design resulted in a 1.11-fold increase in daidzein and genistein. Antiviral activity was observed against the (H5N1) influenza, herpes simplex type 1, and rota virus. The MTT cytotoxicity assay of the extract using MDCK, Vero, and MA104 cell lines revealed that fermented soybean flour reduced cytotoxicity more than the unfermented one. However, it showed higher cytotoxicity against HCT-116 colon cancer cells, MCF-7 breast and PC3 prostate cancer cells than the unfermented soy extract.

Keywords: *Bacillus licheniformis*, β-glucosidase, daidzein, genistein, optimization

INTRODUCTION

Isoflavones have drawn attention because of their ability to reduce the risk of cardiovascular diseases, protect against colon cancer and ageing skin, ease postmenopausal symptoms in women, and reduce the risk of osteoporosis. Also, they have antimutagenic, antioxidant, anti-obesity, and anti-inflammatory effects. Besides, they act as immunomodulators, preventing and treating various problems such as hypertension, hyperlipidemia, and neurological disorders (Yu-Hsuan *et al.*, 2020). To date, twelve chemical forms of isoflavone have been found in soybeans, including three glycosides (genistin, daidzin, and glycitin), their acetylglucoside (acetylgenistin, acetyldaidzin, and acetylglycitin) malonylglucoside (malonylgenistin, malonyldaidzin, and malonylglycitin) and aglycones (i.e. genistein, daidzein, and glycitein) (Wang *et al.*, 2013). Enzymatic digestion of isoflavone glucosides or fermentation of soy products results in the formation of isoflavone aglycone through the release of the sugar molecule from the isoflavone glycoside. Daidzein, genistein, and glycitein are the main aglycones of soy isoflavones, while daidzin, genistin, and glycitin are the isoflavone glycosides (Mizushina *et al.*, 2013). The combined effect of soybean isoflavones may be greater than the effect of a single isoflavone component under certain circumstances. Additionally, the biological activity of isoflavones in their aglycone counterparts are higher (Georgetti *et al.*, 2013), It also means that isoflavone aglycone-rich products are more useful in avoiding or preventing chronic diseases than glycoside-rich goods (Izumi *et al.*, 2000). Therefore, developing foods with high isoflavone aglycone content could also be a crucial strategy to enhance isoflavone bioavailability. In recent years, microbial fermentation has been widely applied within the biotransformation of glycosides to aglycones owing to several benefits such as nonpolluting, high activity of enzymes, and low cost (Doan *et al.*, 2019). Many studies have shown that during fermentation of cultures inoculated with lactic acid bacteria, *Bifidobacteria*, *Basidiomycetes*, *Bacillus subtilis* and *Saccharopolyspora erythraea*, isoflavone glycosides are deglycosylated to isoflavone aglycones by generating β-glucosidase enzyme, one of the cellulose-degrading enzymes (Ningtyas *et al.*, 2021). After selecting the most important factors affecting the production process, it is helpful to investigate and classify the most important parameters in deglycosylation of isoflavone glycosides and production of high concentrations of isoflavone aglycones during the fermentation process. Response surface methodology (RSM) is often used as a statistical method for the optimization of fermentation conditions (Jianming *et al.*, 2013). The antiviral and antitumor effects of soybean isoflavonoids have been investigated previously. Some reports have shown genistein has antiviral properties against several herpes viruses as well as non-herpes viruses (Andres *et al.*, 2009). In addition, the authors investigated the antiviral effects of daidzein. However, this isoflavone did not show anti-herpes activity (Argenta *et al.*, 2015). According to scientific studies, genistein has been described as an adjunct therapeutic drug in combinatorial cancer treatment and has been shown to inhibit tumor development also increased the antitumor effects of gemcitabine and docetaxel by inducing tumor cell apoptosis Zhang *et al.*, (2020). According to the above mentioned background, the main purpose of the present study was to optimize the culture conditions of *B. licheniformis* NRC24 to produce β-glucosidase enzyme for improving aglycones, genistein, and daidzein production through statistical experiments using RSM and assessment of their antiviral and antitumor activities.

MATERIALS AND METHODS

Materials

Daidzein, genistein and p-nitrophenyl-β-D-glucopyranoside (p-NPG) were obtained from Sigma Company (St. Louis, MO, USA). Soybean (Glycine max) flour was purchased from the soybean unit, Agricultural Research Center, Egypt. High-performance liquid chromatography (HPLC) grade methanol was purchased from Fisher Scientific (Hanover Park, IL). Nutrient agar medium was imported from Sisco Research Laboratories Pvt. Ltd, New Mumbai, India. Potato dextrose agar medium was imported from Laboratories Conda S.A., Madrid, Spain. Dulbecco's Modified Eagle's Medium (D-MEM) (Lonza, France) with 1% Penicillin-streptomycin (100U/ml penicillin; 100µg/ml streptomycin) and 10% Fetal bovine serum (FBS) (Lonza, France). Human colon carcinoma (HCT-116), human breast adenocarcinoma cells (MCF-7), and human prostate cancer cells (PC3) were purchased from the American Type Culture Collection (ATCC, NY, USA). All other used chemicals were of analytical grade.

Microorganism

Bacillus licheniformis NRC24 was isolated from Egyptian soil (El-Shazly *et al.*, 2017). The strain was stored in 20% (v/v) glycerol at -80°C. The strain was activated by two sequential transfers of *B. licheniformis* NRC24 in nutritional broth at 37°C for 15–18 hours.

Submerged fermentation

Unless in any other case indicated, submerged fermentation turned into carried out in 250 ml Erlenmeyer flasks with 50 ml of medium, autoclaved at 121°C for 20 minutes, inoculated with 10% (6x107 CFU / ml) of *B. licheniformis* NRC24 in nutrient broth obtained from 24 h old slant agar culture and incubated at 30°C with shaking conditions (200 rpm) for the proper time. Culture samples were centrifuged at 12000 rpm and the culture filtrate was used for the determination of aglycones and β-glucosidase. Experiments were done three times and the average value and standard deviations were provided.

One-factor-at-a-time optimization of daidzin and genistin transformation by *Bacillus licheniformis* NRC24

Optimization of the fermentation medium for the transformation of soy daidzin and genistin into their aglycone forms by *B. licheniformis* NRC24 in submerged cultures containing defatted soybean flour was performed using the one-factor at-a-time method, which is conducted by varying one factor/parameter at a time by keeping the rest of the parameters constant. The production medium in 250 mL Erlenmeyer flasks was optimized with respect to the investigations into the effects of the following parameters: different concentrations of defatted soybean flour (10, 20, 40, 50, 60, 80, and 100 g/L) according to Mukhtar and Haq (2013); incubation time for 4, 8, 12, 16, 20, 24, 28, 32, 36 and 48 h according to the method reported by Donkor and Shah (2008); incubation temperature at 28, 30, 33 and 37°C; medium volumes of 25, 50, 75, and 100 mL/250 mL Erlenmeyer flask according to Song *et al.* (2012); inoculum volume with 1.25, 2.5, 5, 10 and 15-mL from *B. licheniformis* NRC24 cell suspension; different initial pH values (4, 5, 6, 7 and 8) prepared by adjusting the fermentation medium pH with 0.1 N NaOH or 0.1 N HCl as described by Lakshmi *et al*. (2014), different agitation speeds (50,100, 150 and 200 rpm) according to Tian *et al.* (2016). To evaluate the effect of the addition of different inducers on daidzin and genistin transformation, fermentation medium was supplemented with different concentrations (0.02, 0.05 and 0.1 g/L) of inducers (glucose, cellulose, carboxymethyl cellulose (CMC) sodium salt, cellobiose, p-nitrophenyl-β-D-glucopyranoside (p-NPG), salicin) according to Hu *et al.* (2009). Both inoculated and uninoculated defatted soybean flour media were incubated under the same conditions and served as positive and negative controls, respectively.

Optimization of daidzin and genistin transformation using the Box-Behnken design

Optimization of daidzin and genistin transformation in soybean flour by *B. licheniformis* NRC24 was performed using Box-Behnken design (Box and Behnken, 1960) as a Response Surface Methodology (RSM). Factors including defatted soybean flour concentration (X1), incubation time (X2) and medium volume (X3) were studied at three levels coded as −1, 0 and +1 (low, basal and high), respectively. According to the design generated by Design-Expert software, seventeen experiments have been used together with twelve factorial points with five replicates at the center point for estimating the pure error sum of squares. The coded ranges and levels of the independent variables were shown in Table 1. A second-order polynomial function can be used to model the mathematical relationship between each response (Y) and the independent variables (Xi) as follows:

$$Y = \beta0 + \beta1X1 + \beta2X2 + \beta3X3 + \beta11X12 + \beta22X22 + \beta33X32 + \beta12\ X1X2 + \beta13\ X1X3 + \beta23\ X2X3 \quad (1)$$

Where Y is the predicted response; β0, intercept; β1, β2 and β3, linear coefficients; β11, β22 and β33, squared coefficients; β12, β13 and β23, the interaction coefficients of the equation; and X1, X2 and X3, the independent variables. The linear, quadratic, and interaction impacts of the independent variables on the response can all be calculated using this equation. For statistical data analysis, regression modeling, and drawing the response surface graphs, the Design-Expert programme (V. 8) was employed. ANOVA and Fisher's test were used to determine the significance of the independent variables' effect on the response, with a *p*-value less than 0.05 indicating significance. For the fit of the second-order polynomial model equation, the multiple determination coefficient (R^2) and adjusted R^2 were utilized as quality indicators. The remodeling and interactions between the coded parameters and the response were graphically illustrated using contour plots and three-dimensional surface plots. The optimal points were set by solving the equation arising from the final quadratic model and grid searching the RSM plot.

Table 1 Variables used in the Box-Behnken design

Variable	Unit	Symbol	Level		
			−1	0	+1
Soybean flour concentration	g/L	X_1	60	80	100
Incubation time	h	X_2	8	16	24
Medium volume	mL	X_3	25	50	75

Measurement of pH

The pH value of the aliquots after the fermentation was monitored using a pH meter (Walk LAB Microprocessor pH meter HP9000) after calibrating with fresh standard buffers of pH 4.0 and 7.0.

Assessment of β-glucosidase activity
Extracellular β-glucosidase activity

The β-glucosidase activity was determined using p-NPG as a substrate according to the method reported by Otieno and Shah (2007) with some modifications. The reaction mixture in a total volume of 1.5 mL was composed of 0.5 mL of (0.1%) p-NPG in 0.05M sodium phosphate buffer with pH 7 (for bacterial samples) and 1 mL of the culture filtrate (as a source of extracellular β-glucosidase enzyme). After 30 minutes of incubation at 37°C, the reaction was stopped with 0.5 mL (1 M) cold sodium carbonate. Using an Eppendorf centrifuge (Model 5415D; Eppendorf, Hamburg, Germany), the samples were loaded in 2-mL centrifuge tubes and centrifuged for 30 minutes at 15000 rpm. A spectrophotometer (SP-2000UV, Spectra, USA) was used to measure the amount of emitted p-nitrophenol at a wavelength of 401 nm. The amount of enzyme that would liberate 1 mmol p-nitrophenol per minute under test conditions was defined as one unit of enzyme activity.

Activity of cell-bound β-glucosidase

The cell-bound β-glucosidase activity of *B. licheniformis* NRC24 was evaluated using the modified method of Kuo *et al*. (2006). The supernatant of the fermentation medium was discarded and the cell pellets were rinsed with sodium phosphate buffer after centrifuging 1 mL of the culture broth at 15000 rpm for 5 minutes (pH 7.0). The cell pellets were re-suspended in 0.5 mL of sodium phosphate buffer (pH 7.0) containing 0.1% P-NPG and then incubated at 37°C for 30 min. The reaction was stopped by adding of 0.5 mL (1 M) of Na_2CO_3 and the reaction mixture was centrifuged at 15000 rpm for 10 min. As previously stated, the amount of emitted p-nitrophenol was measured. One unit of enzyme activity was defined also as described before.

Extraction of isoflavones

A modified version of the method reported by Fukutake *et al.* (1996) was used to extract isoflavones, including daidzein and genistein, from fermented and unfermented soybean flour. 1g of freeze-dried sample was mixed with 10 mL of 80% (v/v) aqueous methanol and incubated for 24 hours at room temperature with agitation. The homogenates were centrifuged for 30 minutes at 15000 rpm to yield methanolic extracts, which were utilized to assess daidzein and genistein content as well as antioxidant activity.

HPLC analysis of isoflavones

HPLC instrument Young Lin (Young Lin Cooperation, Seoul, South Korea) consists of a Reprosil-Pur Basic C18, 5 µm (dimension: 250 × 4.6 mm) column and a UV detector (λmax = 210 nm). To isolate the isoflavones for detection, isocratic elution was utilized. The mobile phase was made up of 100% methanol and 10 mmol/L ammonium acetate buffer (60:40), with 1 mL trifluoroacetic acid per litre of solvent mixture. The flow rate was set at 1 mL/min according to the method of Otieno and Shah (2007) with some modifications. Throughout the 30-minute run, the injection volumes of isoflavone standards and samples were kept at 100 L. Single standards (daidzein and genistein) were prepared in the same solvent at 100 µg/mL for peak identification and isoflavone concentrations were calculated back to the dry basis (µg/g soybean flour).

Evaluation of cytotoxicity against mammalian cell lines

The cytotoxicity of fermented and unfermented soybean flour extracts was determined using 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT). Dulbecco's Modified Eagle's Medium was used to dilute the samples (DMEM). Stock solutions of the isolated chemical compounds were prepared in ddH2O containing 10% DMSO. The extracts were evaluated for cytotoxicity against Madin-Darby Canine kidney (MDCK) cells, African green monkey kidney cells (Vero), and Rhesus monkey kidney (MA104) cell lines using MTT, which was modified slightly from Mossman (1983). The cells were seeded in 96 well plates (100 µl/well at a density of 3105 cells/mL) and incubated overnight at 37°C in 5% CO_2. The cells were then treated in triplicates with varying doses of the

tested chemicals. The supernatant was discarded after another 24 hours, and the cell mono-layers were washed three times with sterilized 0.01 M phosphate buffer saline (PBS), and 20 µl of MTT solution (5 mg/mL) was added to each well and incubated at 37°C for 4 hours before medium aspiration. In each well, 200 µl of acidified isopropanol (0.04 M HCl in 100 % isopropanol = 0.073 mL HCl in 50 mL isopropanol) was used to dissolve the formazan crystals. A multi-well plate reader was used to measure the absorbance of formazan solutions at 570 nm. The following equation was used for the determination of the cytotoxicity percentage compared to the untreated cells.

Cytotoxicity % = [(Absorbance of cells without treatment - Absorbance of cells with treatment) X 100] / (Absorbance of cells without treatment) (2)

The concentration that displayed 50% cytotoxicity (TC50) was calculated using a plot of cytotoxicity (percentage) vs. sample concentration.

Assessment of antiviral activity

Using the plaque reduction assay, the antiviral activity of fermented and unfermented soybean flour extracts against herpes simplex virus type 1 (HSV-1) that causes cytopathic effect (CPE) in VERO cells, influenza virus H5N1 (A/CHICKEN/7217B/1/2013), and Rotavirus SA11 that causes CPE in MDCK cells according to the method of Hayden *et al*. (1980). The viral infection titer on host cells was measured using a tissue culture infectious dose endpoint of 50% (TCID50%) and a plaque formation unit (PFU). The assay was carried out in a six-well plate where MDCK cells (10^5cells/mL) were cultivated for 24 h at 37°C. Before being injected to the cells for 1 hour at 37°C, the A/chicken/M7217B/1/2013 (H5N1) virus was diluted to 104 PFU/well and combined with the safe quantities of the investigated drugs. The cells were inoculated with virus extract and virus-oseltamivir mixture (100 µl/well) after the growth media was withdrawn from the cell culture plates. After 1 hour of contact time for virus adsorption, 3 mL of DMEM supplemented with 2% agarose, as well as the assessed compounds, were applied to the cell monolayer. To allow viral plaques to develop, plates were allowed to harden before being incubated at 37°C for 3 to 4 days. After 2 hours, the plates were dyed with 0.1 percent crystal violet and formaldehyde (10%). The untreated virus was cultured with MDCK cells in control wells, and plaques were counted and the % reduction in plaque formation was estimated in relation to control wells as follows: Inhibition % = [(Viral count (untreated) -Viral count (treated)) /Viral count (untreated)] x100 (3)

Assessment of anti-cancer activity

McCoy's medium was used to culture human colon cancer (HCT-116) cells. MCF-7 human breast cancer cells and PC3 human prostate cancer cells were grown in DMEM. All media were supplemented with 10% fetal bovine serum, 2mol/mL L-glutamine, 250 mg/mL, 100 units/mL penicillin G sodium salt, and 100 units/mL streptomycin sulphate at 37°C in a humidified CO_2 (5%) incubator (Model: HF 1600, and Shanghi Lishen Scientific Co., Shanghai, China). Every four days, the cultures were trypsinized for five minutes at 37°C using a 1 mL trypsin/EDTA solution. When confluence had reached 75%, cells were utilized. 0.25 mM trypsin and 1 mM EDTA are dissolved in phosphate buffer to make the trypsin/EDTA solution.

Assessment of cytotoxicity against tumor cells

The active mitochondrial dehydrogenase enzyme's ability to cleave the tetrazolium rings of yellow MTT to form a dark blue insoluble formazan crystal that is largely impermeable to cell membranes, resulting in its accumulation within healthy cells, was used to assess the cytotoxic effects of the samples. Cells are dissolved once they are solubilized, resulting in the liberation of crystals. The amount of soluble formazan dark blue colour is directly linked to the number of viable cells. The absorbance at 570 nm was used to determine the extent of MTT decrease (Hansen *et al.*, 1989).In a flat bottom 96-well microplate, cells ($0.5X10^5$ cells/well) were introduced in a serum-free medium and treated with 20 µl of varying concentrations of each investigated substance for 20 h at 37°C in a humidified 5% CO_2 atmosphere. After incubation, the media was withdrawn and a 40 µl MTT solution was added to each well, which was then incubated for another 4 hours. MTT crystals were solubilized by shaking the plate at room temperature with 180 µl of acidified isopropanol per well. A microplate ELISA reader was used to do the photometric determination at 570 nm. For each concentration, triplicate repeats were done and the average was computed. Data were expressed as a percentage of relative viability compared to untreated cells (control), with 100% relative viability indicating cytotoxicity.
The percentage of relative viability was calculated using the following equation:

Relative viability % = [(Absorbance of treated cells/Absorbance of control cells)] X100 (4)

The IC50 value is the half-maximal inhibitory concentration. It is calculated from the linear equation of the curve of concentration/viability percentage of each sample in each cell line.

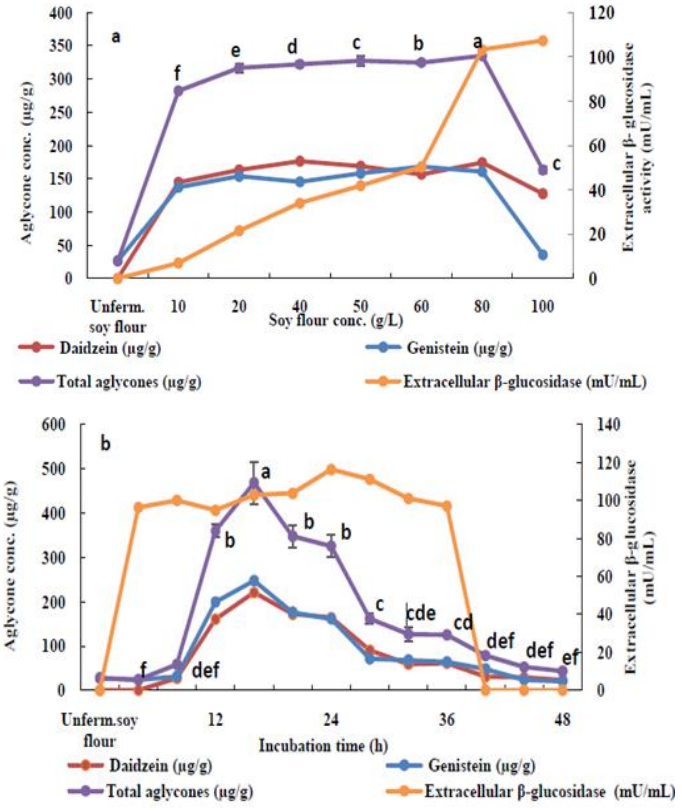
Statistical analysis

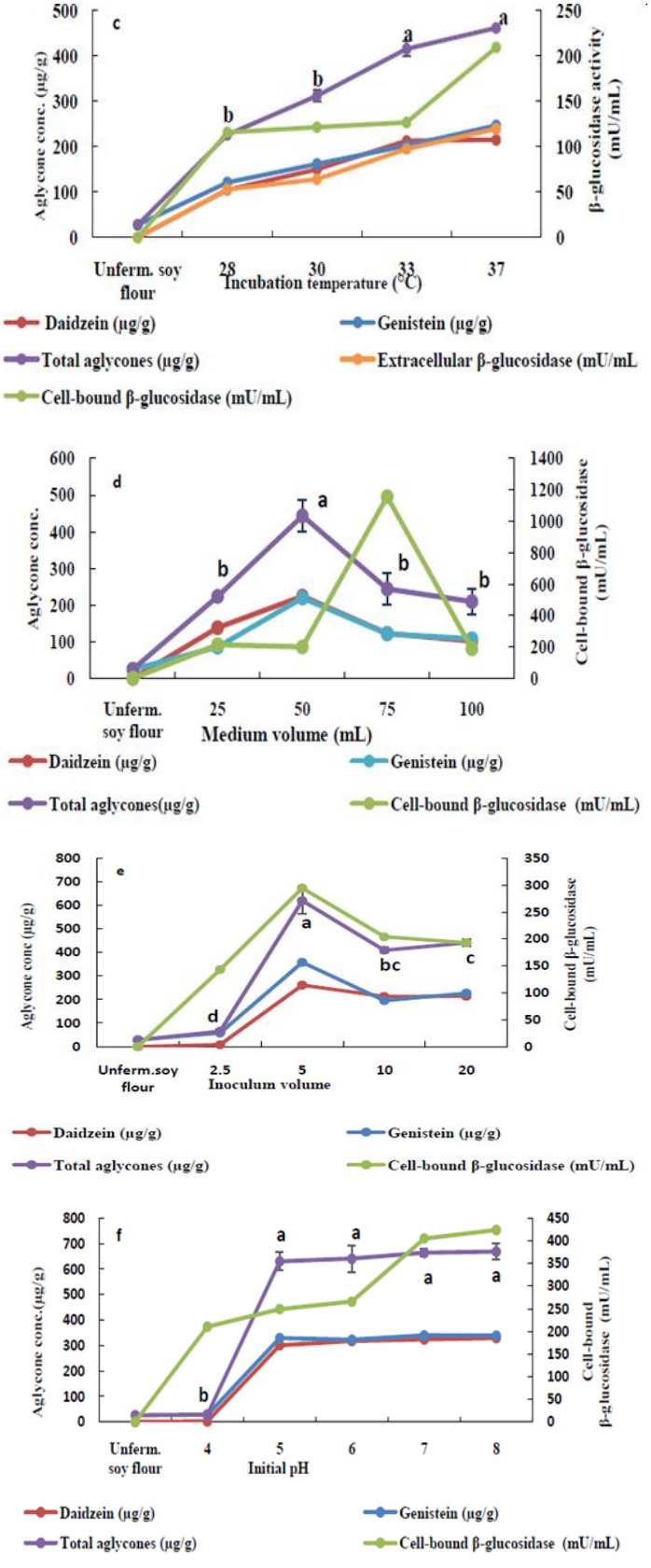
Statistical analysis was performed using Minitab (V.17.3.1.). Comparisons were evaluated using one-way ANOVA followed by Tukey post hoc comparisons. Differences were considered statistically significant in the case of $P<0.05$. 3.

RESULTS AND DISCUSSION

Optimization of culture and environmental conditions for daidzein and genistein production

The one-factor-at-a-time methodology was used to determine the optimized conditions of soy daidzin and genistin transformation using *B. licheniformis* NRC24. The effect of different soy flour concentrations on aglycones production was shown in Fig. 1a. Defatted soybean flour of concentration 80g/L was the optimum concentration that played an effective role in significantly enhancing the transformation ($p < 0.05$) of daidzin and genistin after 24 h in 50 mL of the medium. It has been found that soy flour provided the desired nutrients for the growth of *B. licheniformis* NRC24 and was accountable for the transformation of glucosides, daidzin and genistin, into aglycones daidzein and genistein. The soybean kind may be performed a role in the formation of aglycones. Kuo *et al.* (2006) found that the isoflavones, daidzin, and genistin were highly deglycosylated in flask cultures at a concentration of 50g/L black soymilk after 24 hours. The changes in isoflavone concentrations in relation to increasing fermentation time during 48 h are shown in Fig. 1b. Daidzein and genistein became detectable 8 hours after inoculation and increased significantly ($P< 0.05$) to their maximum (228.57 and 247.513, respectively) at 16 hours, then decreased dramatically until 48 hours. Optimum fermentation time may vary among different microorganisms for the biotransformation process. In flask cultures, deglycosylation of isoflavone glycosides of black soymilk was highly deglycosylated and the maximum level of both daidzein and genistein was obtained after 16 h of incubation as mentioned by Kuo *et al.* (2006). Alternatively, it was reported that the aglycone isoflavones concentration in soymilk fermented with lactic acid bacteria strains was found to be significantly higher during the first 24 hours of the fermentation process. *Lactobacillus rhamnosus* CRL981 could flourish in soymilk and also have high β-glucosidase activity, attaining 100% hydrolysis of glucosides and isoflavones after 12 hours of fermentation, according to Marazza *et al.* (2009). Otieno and Shah (2007), Tsangalis *et al.* (2004) and Sumarna (2010) additionally declared that once fifteen hours of fermentation at 37°C, the concentration of aglycone in soybean milk fermented with *L. acidophilus* ATCC 4461, *L. casei* 2607, and *Bifidobacterium animalis* ssp. *lactis* Bb12 was more accumulated.





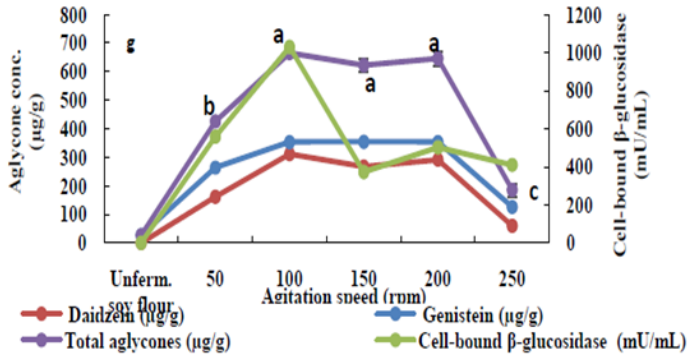


Figure 1 Effect of nutritional and environmental parameters affecting daidzin and genistin transformation and β-glucosidase production by *B. licheniformis* NRC24. a) Soy flour concentration, b) Incubation time, c) Incubation temperature, d) Medium volume, e) Inoculum volume. f) Initial pH value and g) Agitation speed.

Concerning the effect of fermentation temperature on deglycosylation of daidzin and genistin, results showed that *B. licheniformis* NRC24 could transform daidzin and genistein at temperatures ranging from 28 to 37°C with no obvious difference in daidzein concentration (Fig. 1c) with the maximum transformation ($p<0.05$) at 37°C. Additionally, there was no observed significant difference between total aglycone content at 33 and 37°C. As stated in several reports, the incubation temperature influenced the growth of microorganisms, product formation and other responses (Fadahunsi *et al.*, 2020). In agreement with that finding, the optimal fermentation temperature of soy flour fermented by *L. paracasei* subsp. *paracasei* and *B. longum* was 37°C (Rodríguez de Olmos *et al.*, 2015). The activity of β-glucosidase produced by lactic acid bacteria (LAB) was defined mainly in the cell-free extract via cell-bound β-glucosidase activity and was not detected in culture supernatant (El-shazly *et al.*, 2017).Therefore, the cell-bound β-glucosidase activity will be considered as a toll for the biotransformation of soy glucosides.

Experiments with varied volumes of fermentation medium were done to explore the influence of aeration on the biotransformation of daidzin and genistin. Fig. 1d shows the influence of different medium volumes of defatted soybean flour (25, 50, 75, and 100 mL) on the production of daidzein and daidzein by *B. licheniformis* NRC24. The results showed that as the medium volume was raised, the concentrations of daidzein and genistein, as well as the overall aglycone content, steadily increased until they reached their maximum levels at 50 mL/250 mL flask. At 75 mL/flask, the enzyme β-glucosidase was steadily raised until it reached its maximum value (1157.106 mU/mL). The amount of β-glucosidase, daidzein, and genistein was considerably reduced when the medium volume was increased to 100mL. High medium volume lowered oxygen transport during the fermentation process, whereas low medium volume may inhibit microbial development due to lower nutrient solubility and quick water loss by evaporation (Lonsane *et al.*, 1985).

The variation of *B. licheniformis* NRC24 inocula concentration was investigated, and no significant changes were found among the tested inocula (10-20%). However, by *B. licheniformis* NRC24, a 5% (v/v) inoculation volume has a substantial effect on daidzein, genistein, total aglycone, and cell-bound β-glucosidase. The findings also show that the size of the inoculum has a substantial relationship with the transformation of soy glucoside to its aglycone and cell-bound β-glucosidase (Fig. 1e). Unfortunately, increasing the inoculum dose did not result in a significant improvement in daidzein, genistein total aglycones or β-glucosidase biotransformation after 24 hours. Our findings for *B. licheniformis* NRC24 coincide with those for *L. plantarum* 128/2 reported by Izaguirre *et al.* (2021). Due to differences in cell growth rates, medium composition, ability to metabolise medium nutrients, mass transfers, and medium volume, the optimal inoculum size varies from producer strain to other strain (Jawan *et al.*, 2020). Mudgetti (1986) stated that a lower inoculum density results in insufficient biomass, which leads to poor product production, but a larger inoculum density may result in too much biomass, which also leads to poor product creation. Throughout the biotransformation and manufacture of various products, microbial species have shown a wide range of pH values that are optimal. The results showed no significant difference in the transformed daidzein, genistein and total aglycone concentrations by adjusting the initial pH value of soybean flour medium in the range of 5–8 (Fig. 1f.). It was also observed that the maximum activity of β-glucosidase was recorded at an initial pH value of 7-8. It has been reported that *B. licheniformis* KIBGE-IB4 produced maximum α-1,4-glucosidase activity at an initial medium pH value of 7.0. However, *Bacillus* sp. NPST-AK15 secreted maximum protease enzyme as the growth medium pH was adjusted at 11 (Nawaz *et al.*, 2016; Ibrahim *et al.*, 2015).

Agitation speed is one of the culture parameters that regulates the amount of dissolved oxygen in the fermentation media. Therefore, different agitation speeds were evaluated for isoflavone glucoside transformation, and the results demonstrated that *B. licheniformis* NRC24 yielded the highest of both isoflavones when the agitation was set at 100 rpm (Fig. 1g). Similarly, β-glucosidase activity

reached its peak (1031.02 mU/mL) at 100 rpm. While agitation speeds of 250 rpm reduced both β-glucosidase activity and aglycone formation. Cheng *et al.* (2010) reported a similar outcome for black soybean fermented by *Rhizopus oligosporus* NTU-5. In fermentation processes, agitation is necessary for mixing and shearing. It not only enhances mass and oxygen transport between phases, but it also keeps the medium's chemical homogeneity and physical conditions consistent. Agitation, on the other hand, can produce morphological alterations, growth and metabolite formation variations, and even damage to cell structures due to shear stresses (Zhou *et al.*, 2018).

In comparison to the normal fermented soybean flour, supplementing defatted soybean flour with 1% (w/v) cellobiose resulted in a significant increase in β-glucosidase activity (1.32-fold) and aglycones (1.34%) as indicated in Table 2. However, adding other sugars to the soybean flour process, such as glucose, cellulose, p-NPG, and salicin, had no effect on the transformation of glycosidic daidzein and genistein in soybean flour into their respective aglycones. Adding glucose (0.05 g/L) or additional inducers [CMC (0.1 g/L), cellobiose (0.02 g/L), p-NPG, and salicin (0.05 g/L)] to the soybean flour medium increased β-glucosidase production to nearly the same level as the other inducers. Adding cellulose to soybean flour at concentrations of 0.2, 0.5, and 1% reduced the synthesis of β-glucosidase by 29.06, 59.44, and 69.62 %, respectively.

Several investigations have demonstrated that LAB with β-glucosidase activity can improve the aglycone concentration during soya milk fermentation. The β-glucosidase enzyme is in charge of breaking down the β-1-6 glycosidic linkage, which connects the isoflavone's pyran ring to the sugar moieties. β-glucosidase is an inducible enzyme generated by microorganisms in response to carbon sources in the fermentation medium. The optimal carbon supply for β-glucosidase synthesis depends on the microorganism, fermentation methods, alternative fermentation parameters, and their interactions. When cultured in soymilk, the majority of LAB demonstrated detectable levels of β-glucosidase activity. With the exclusion of *Bifidobacterium animalis* BB12, *L. acidophilus* 33200, *L. acidophilus* 4962, *L. casei* 2607, and *L. casei* ASCC 290 showed an increase in enzyme activity up to 24 h followed by a drop as the fermentation process go forward (Otieno *et al.*, 2007). Any decrease or increase in the enzyme activity was strongly linked to the formation of isoflavone aglycone forms. On the other hand, a quantitative relationship between β-glucosidase activity and daidzein aglycone yield could not be demonstrated, but the lack of β-glucosidase in *Bifidobacterium longum* MB 220 and *Bifidobacterium longum* MB 224 was linked to the strains' inability to produce daidzein (Raimondi *et al.*, 2009).

Table 2 Effect of different inducers on the production of cell bound β-glucosidase activity daidzein and genistein by *B. licheniformis* NRC24.

Inducer	Inducer's conc. (g/L)	Final pH	Cell-bound β-glucosidase activity (mU/mL)	Daidzein (µg/g)	Genistein (µg/g)	Total Aglycones (µg/g)
UFSF	0.0	7.0	0.0	0.0	26.32	26.32
Control (without inducer)	0.0	6.4	1054.63	307.49	354.77	662.27
Glucose	0.02	5.8	1379.63	304.59	346.22	650.82
	0.05	5.7	1596.29	308.03	348.99	657.02
	0.1	5.7	1350.93	285.52	322.57	608.09
Cellulose	0.02	5.9	748.15	301.78	308.32	609.85
	0.05	5.8	427.78	296.59	308.07	604.66
	0.1	5.7	320.37	302.93	308.70	611.64
CMC	0.01	5.6	1199.07	325.85	334.25	660.09
	0.02	5.6	1376.85	415.06	391.33	806.39
	0.05	5.9	1505.56	316.88	349.27	666.15
	0.1	5.9	1638.88	292.37	328.67	621.03
Cellobiose	0.02	5.9	1674.07	303.41	332.87	636.27
	0.05	5.9	1452.78	328.58	362.09	690.68
	0.1	5.8	1392.59	418.91	468.09	887.00
	0.2	5.8	1103.70	298.38	266.09	564.47
	0.5	5.7	1032.41	271.37	261.83	533.19
p-NPG	0.02	5.8	1393.52	325.04	359.61	684.65
	0.05	5.9	1438.89	276.59	341.08	617.67
	0.1	5.9	1081.48	255.09	313.87	568.96
Salicin	0.02	5.6	1381.48	385.62	347.94	733.55
	0.05	5.6	1650.0	322.19	307.43	629.62
	0.1	5.7	1379.63	297.66	277.44	575.09

Optimization based on Response Surface Methodology (RSM)

Based on the 'One factor at a time' experimental approach, three variables (defatted soybean flour concentration, incubation duration, and medium volume), which significantly influenced the transformation of daidzin and genistin in soybean flour, were chosen to determine their optimum response region for soy glucosides transformation. The box-Behnken design was used as a quadratic model and RSM in this study to better optimize the transformation of daidzin and genistin in soybean flour. Table 1 shows the three components as well as their coded values.

The experimental results of cell bound β-glucosidase activity, daidzein, genistein and the total aglycones are shown in Table 3. The β-*glucosidase* activity, daidzein, and genistein concentrations in soybean flour media fermented by *B. licheniformis* NRC24 ranged from 653.31 to 2001.71 mU/mL, 23.77 to 515.09 g/g, and 23.96–470.49 g/g, respectively. The minimal values for daidzein, genistein, total aglycones, and β-glucosidase activity were found in run number 10, while the greatest values were found in runs 16 and 7, respectively.

Table 3 Box-Behnken design of factors affecting daidzein and genistein transformation using *B. licheniformis* NRC24

Run	X1 Conc.of soy flour	X2 Incubation time	X3 Medium volume	R1 Cell bound β-glucosidase activity (mU/mL)	R2 Daidzein (µg/g)	R3 Genistein (µg/g)	Total aglycones (µg/g)
Unfermented soy flour			0.0	0.0	26.488	26.488	
Fermented soy flour							
1	1	1	0	1391.13±124.8	339.42±18.1	337.72±11	677.13
2	0	1	1	1252.49±149	425.69±9.4	364.95±67.4	790.64
3	1	0	1	1514.58±28.1	308.24±12.5	325.30±8.7	633.54
4	-1	-1	0	1637.07±212.1	219.90±37.7	338.581±49.4	558.48
5	0	0	0	1747.22±115.4	361.76±12.6	390.09±5.5	751.85
6	-1	0	-1	1941.89±6.7	455.49±15.5	429.885±2.1	885.37
7	0	-1	-1	2001.71±126.2	351.02±21.4	374.96±25.1	725.98
8	0	0	0	1747.22±115.4	361.75±12.6	390.09±5.5	751.85
9	1	0	-1	1710.19±119.5	361.80±17.5	379.16±21.8	740.96
10	0	-1	1	653.309±18.8	23.769±1.1	36.99±18.7	60.76
11	0	0	0	1747.22±115.4	361.76±12.6	390.09±5.5	751.85
12	-1	0	1	1412.24±92.3	391.79±6	425.46±2.5	817.24
13	1	-1	0	1616.18±174.5	26.71±1.1	23.96±1.8	50.67
14	0	0	0	1747.22±115.4	361.757±12.6	390.09±5.5	751.847
15	0	0	0	1747.22±115.4	361.76±12.6	390.09±5.5	751.85
16	-1	1	0	1610.48±72.1	515.09±22.7	470.49±25.7	985.58
17	0	1	-1	1633.27±99.3	368.44±25.8	388.48±39.7	756.92

All responses R1, R2, and R3 (β-glucosidase activity, daidzein, and genistein) have Model F-values of 6.69, 14.40, and 7.13, respectively, indicating that the model is relevant, as indicated by Fisher's F-test with a low probability value [(Model>F) = 0.0101, 0.0010, and 0.0084, respectively] (Table 4, 5 and 6). For both daidzein and

genistein, values of ''Prob> F" less than 0.05 indicated that model variables X1, X2, X3, X2X3, and X22 are significant. For β-glucosidase activity, model terms X3, X2X3, and X22 are also significant. R^2 values ranged from 0.8959 to 0.9488, suggesting a fair correlation between expected and actual outcomes for β-glucosidase activity, daidzein and genistein and indicating that the model accurately reflected the real relationship between the three variables. This indicates more than 89-95 percent of response variability can be explained by the selected model and indicating that the model fit well in the planned experiments. The values of adjusted R^2 for the three responses (0.7620, 0.8829, and 0.7752, respectively) were also high, which indicated the significance of the model

Table 4 Analysis of variance of the Box-Behnken experimental design for assessment of β-glucosidase production by *B. licheniformis* NRC24.

Source	Sum of squares	DF	Mean square	*F*-value	*p*-value Prob>*F*
Model	1.348E+006	9	1.497E+005	6.69	0.0101
X1	17075.89	1	17075.89	0.76	0.4113
X2	54.58	1	54.58	2.439E-003	0.9620
X3	7.530E+005	1	7.530E+005	33.66	0.0007
$X_1 X_2$	9847.09	1	9847.09	0.44	0.5283
$X_1 X_3$	27893.84	1	27893.84	1.25	0.3010
$X_2 X_3$	2.341E+005	1	2.341E+005	10.46	0.0144
X_1^2	6083.52	1	6083.52	0.27	0.6181
X_2^2	2.066E+005	1	2.066E+005	9.23	0.0189
X_3^2	83128.38	1	83128.38	3.72	0.0953
Residual	1.566E+005	7	22372.51		
Lack of Fit	1.566E+005	3	52202.52		
Pure Error	0.000	4	0.000		
Cor. Total	1.504E+006	16			
R^2	0.8959				
Adj. R^2	0.7620				
Adequate Precision	10.828				
Coefficient of variation	9.38%				

Note: X1; defatted soybean flour (g/L), X2; incubation time (h) , X3 and medium volume (mL). Cor.; Corrected, * Values of "probability >*F*" less than 0.05 were indicated that model terms were significant.

Table 5 Analysis of variance of the Box-Behnken experimental design for assessment of daidzin transformation by *B. licheniformis* NRC24.

Source	Sum of squares	Df	Mean square	*F*-value	*p*-value Prob>*F*
Model	2.716E+005	9	30172.36	14.40	0.0010
X1	41013.34	1	41013.34	19.57	0.0031
X2	1.389E+005	1	1.389E+005	66.27	< 0.0001
X3	18745.90	1	18745.90	8.95	0.0202
$X_1 X_2$	488.92	1	488.92	0.23	0.6438
$X_1 X_3$	25.75	1	25.75	0.012	0.9148
$X_2 X_3$	36959.87	1	36959.87	17.64	0.0040
X_1^2	38.60	1	38.60	0.018	0.8959
X_2^2	34201.87	1	34201.87	16.32	0.0049
X_3^2	1786.80	1	1786.80	0.85	0.3865
Residual	14667.74	7	2095.39		
Lack of Fit	14667.74	3	4889.25		
Pure Error	0.000	4	0.000		
Cor. Total	2.862E+005	16			
R^2	0.9488				
Adj. R^2	0.8829				
Adequate Precision	13.841				
Coefficient of variation	13.97%				

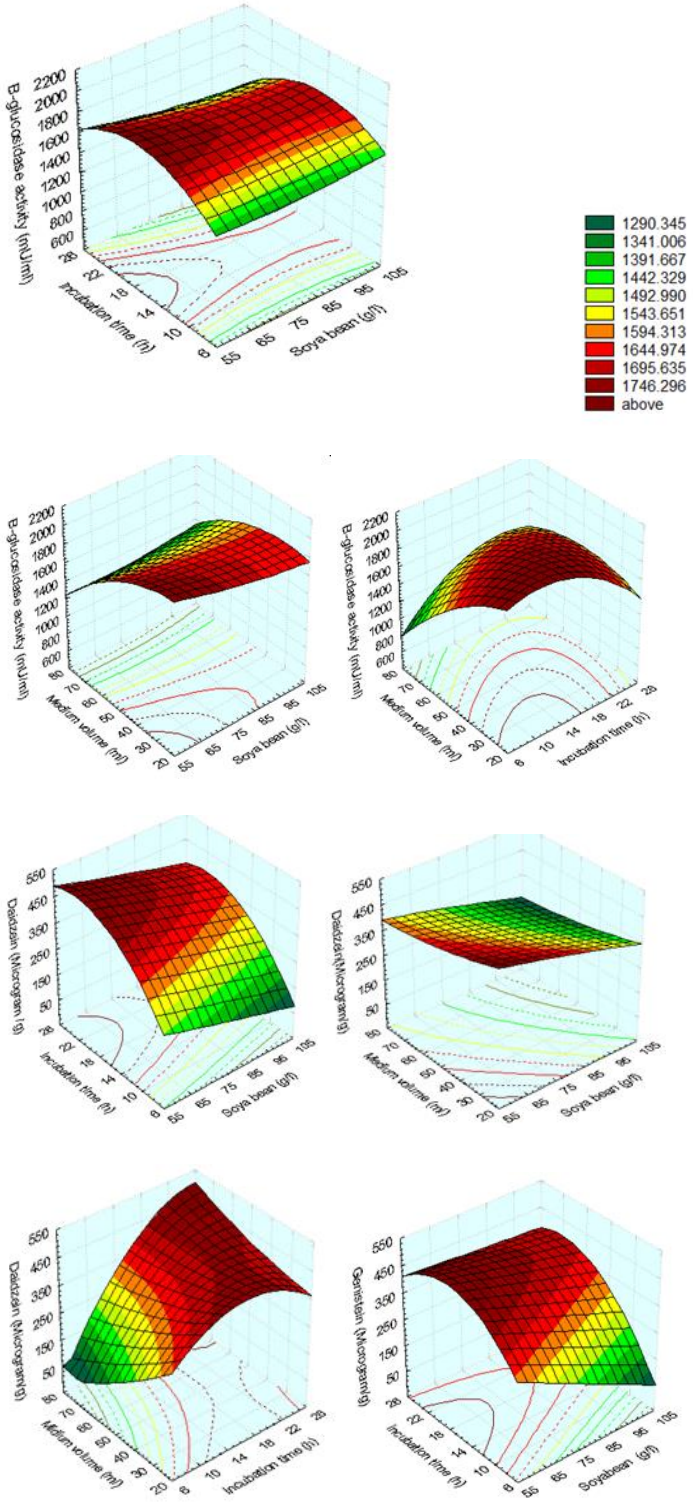
Note: X1; defatted soybean flour (g/L), X2; incubation time (h) and X3; medium volume (mL). Cor.; Corrected, * Values of "probability >*F*" less than 0.05 indicated that the model terms were significant.

Table 6 Analysis of variance of the Box-Behnken experimental design for assessment of genistin transformation by *B. licheniformis* NRC24.

Source	Sum of squares	Df	Mean square	*F*-value	*p*-value Prob>*F*
Model	2.185E+005	9	24278.06	7.13	0.0084
X1	44741.17	1	44741.17	13.14	0.0085
X2	77446.11	1	77446.11	22.74	0.0020
X3	22028.48	1	22028.48	6.47	0.0385
$X_1 X_2$	8266.90	1	8266.90	2.43	0.1632
$X_1 X_3$	611.05	1	611.05	0.18	0.6846
$X_2 X_3$	24719.07	1	24719.07	7.26	0.0309
X_1^2	1.53	1	1.53	4.481E-004	0.9837
X_2^2	40441.81	1	40441.81	11.88	0.0107
X_3^2	2.31	1	2.31	6.775E-004	0.9800
Residual	23839.11	7	2.31		
Lack of Fit	23839.11	3	3405.59		
Pure Error	0.000	4	7946.37		
Cor. Total	2.423E+005	16	0.000		
R^2	0.9016				
Adj. R^2	0.7752				
Adequate Precision	9.897				
Coefficient of variation	16.97%				

Note: X1; defatted soybean flour (g/L), X2; incubation time (h) and X3; medium volume (mL). Cor.; Corrected, * Values of "probability >*F*" less than 0.05 indicated that the model terms were significant.

Two crucial measurements for graphically visualizing the pattern of interactions are three-dimensional response surface plots (Fig. 2) and correlation plots (Fig. 3). The response surface plots showed a curvature along the incubation time axis due to the statistical significance of quadratic coefficients of incubation time in the model for the three responses (R1, R2 and R3). The contour plots for R1, R2, and R3 demonstrate the importance of the link between incubation time and medium volume. The ideal values of independent variables in uncoded (real) units for maximizing the response were 60 g/L defatted soybean flour, a 24 h incubation duration, and a medium volume of 50 mL, as determined by solving and analyzing the response surface plots generated with Design-Expert software. Under the estimated conditions, the highest levels of daidzein and genistein of *B. licheniformis* NRC24 predicted by the model were computed to be 515.092 and 470.489 µg/g, respectively.



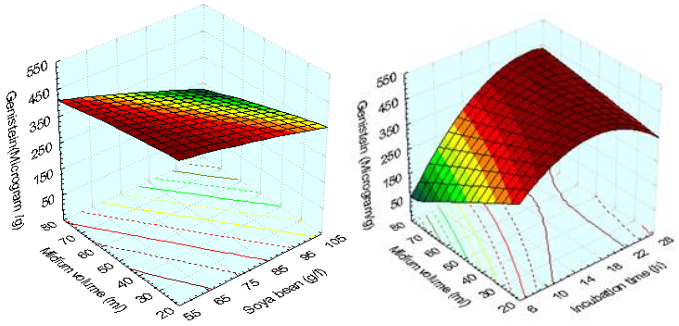


Figure 2 Response surface plot of the interaction effect between defatted soybean flour concentration, incubation time, and medium volume on β-glucosidase (a,b,c), daidzein (d,e,f) and genistein (g,h,i) production by *B. licheniformis NRC 24*.

The correlation plot (Fig. 3a) of daidzin and genistin biotransformation was found to be very close, showing a high correlation between their biotransformations. The correlation coefficient was 0.995, indicating a good connection between their biotransformations. Correlation plots (Fig. 3 a, b and c) revealed a reasonable relationship between β-glucosidase production and daidzin and genistin biotransformation (r=0.498 and r=0.576, respectively). The predicted and experimental findings were extremely close, demonstrating the RSM's accuracy and utility in optimizing the process for daidzein, genistein, and β-glucosidase synthesis. The regression significance of the model for β-glucosidase, daidzein and genistein was demonstrated by adequate *F*-values in conjunction with the *p*-values (less than 0.05). The more meaningful the related coefficient is, the bigger the magnitude of the *F*-value and the smaller the *p*-value (Liu *et al.*, 2003).

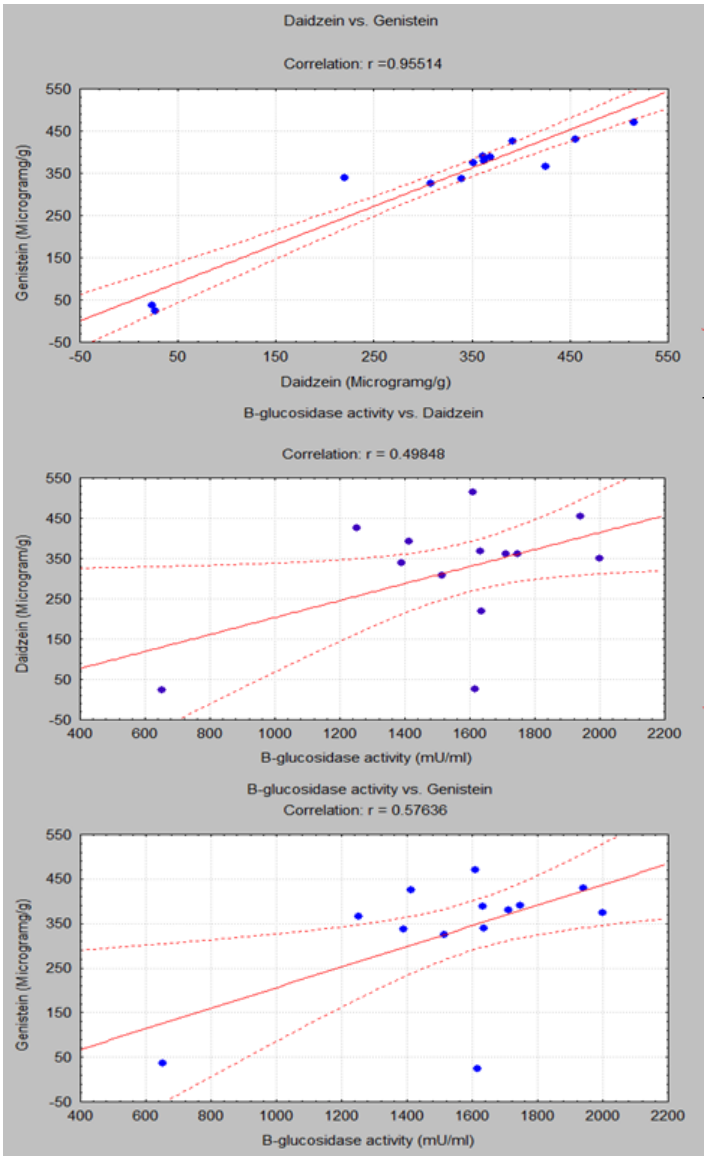


Figure 3 (a, b, c): Correlation plots of daidzein vs genistein, β-glucosidase vs daidzein,

Soybean concentration (X1), incubation period (X2), and medium volume (X3) were significantly affected by the formation of daidzein and genistein. The linear effect (X1, X2, X3), squared effect (X22), as well as interactive effect (X2X3), were highly significant (p-value < 0.005). Among the three variables, it was also concluded that the incubation time is the key factor influencing daidzein and genistein production, due to its largest F-value (66.27 and 22.74, respectively), followed by soybean flour concentration and medium volume. Furthermore, the interaction terms (X1X2 and X1X3) were not significant, indicating that there is no significant correlation between the two variables and that they did not significantly aid in increasing the production of daidzein, genistein, and β-glucosidase, but they still act as limiting factors in the fermentation medium.
The application of RSM for β-glucosidase activity, daidzein and genistein production yielded the following regression equations:

$$Y_{\text{Cell bound β-glucosidase}} = 1747.22 - 46.20X1 - 2.61X2 - 306.80X3 + 38.01X12 - 221.52X22 + 140.51X32 - 49.62X1X2 + 83.51X1X3 + 241.9X2X3 \quad (5)$$

$$Y_{\text{Daidzein}} = 361.76 - 71.60X1 + 131.74X2 - 48.41X3 - 3.03X12 - 90.13X22 + 20.60X32 + 11.06X1X2 + 2.54X1X3 + 96.12X2X3 \quad (6)$$

$$Y_{\text{Genistein}} = 390.09 - 74.78\ X1 + 98.39\ X2 - 52.47\ X3 + 0.6X12 - 98.00X22 - 0.74X32 + 45.46\ X1X2 + 12.36X1X3 + 78.61\ X2X3 \quad (7)$$

The negative coefficients X1 and X3 suggest that when defatted soybean flour content and medium volume increased, daidzein and genistein production by *B. licheniformis* NRC24 declined. The positive coefficient X2 revealed that the generation of daidzein and genistein by *B. licheniformis* NRC24 increased when the incubation period was increased under those culture conditions. Simultaneously, as all of the variables were increased, β-glucosidase activity declined. The Box-Behnken design model was found to be successful in determining the optimum yield of daidzein and genistein from fermented soybean flour by *B. licheniformis* NRC24 in this investigation. Thai soybean type TG145 fermented with *B. subtilis* TN51 had a lower total isoflavone compound content than Chungkukjang, which was prepared from Korean and Chinese soybean but had a higher number of aglycone kinds than other fermented soybean products (Lee *et al.*, 2007). The influence of incubation time as an environmental factor on the transformation of daidzin and genistin in soybean flour has been previously reported by Silva *et al.* (2011). In accordance with our study, we found that there was a significant effect of incubation time on daidzin and genistin transformation in soybean flour. Each isoflavone aglycone concentration was increased with increasing fermentation time as a result of the hydrolytic action on relevant glucosides. Additionally, the daidzein to genistein ratio appeared to be equal. These results are crucial for optimizing soybean flour fermentation, which was designed with the goal of achieving high aglycone concentrations and ideal aglycone equivalent ratios at a specified fermentation duration.

The cytotoxicity of fermented and unfermented soybean flour extracts against mammalian cell lines

In comparison to the unfermented soybean flour extract, the cytotoxicity of the fermented soybean flour extract was investigated in mammalian cell lines (MDCK cells, Vero cells, and MA104 cells). As demonstrated in Table (7), there was no cytotoxicity against MDCK and Vero cell lines at a concentration of 100 µg/L of fermented soybean flour extracts, but increasing the concentration resulted in cytotoxicity against all cell lines examined. The unfermented and fermented soybean flour extracts have TC50 values of 1315.73 and 1508.38 µg/L on the MDCK cell line, 560 and 516.8 µg/L on the Vero cell line, and 537.03 and 1186.78 µg/L on the MA104 cell line, respectively. However, there was no difference observed with TC50 for fermented and unfermented soybean extracts on the Vero cell line. Using the MTT assay, the maximum concentrations of both daidzein and genistein (50 and 100 µM) were found to be cytotoxic to HTC cells (Lepri *et al.*, 2013). On Vero cells, 100µM genistein caused morphological alterations such as enhanced roundness and cell fusion, but 50 M caused no morphological changes (Yura *et al.*, 1993).

Antiviral activity of the fermented and unfermented soybean flour extracts against the Avian influenza A virus, HSV-1 and Rotavirus SA11

The obtained data (Fig. 4) showed that the extract of fermented soybean flour by *B. licheniformis* NRC24 was significantly reduced 40% of the plaque number of avian influenza virus (A/chicken/ Egypt/ M7217B/1/2013 (H5N1), 42% of HSV-1 and 80% RV plaque formation in comparing to the unfermented soybean flour extract and untreated control in a dose-dependent manner. A previous study showed that genistein has a neuraminidase inhibitory effect against each of A/NWS/33 (H1N1) and A /chicken/Korea/MS96/96 (H9N2) (Wei *et al.*, 2015). Genistein, the main component of fermented soy flour was reported that have an impact on the replication of herpes HSV-1 through inhibition of protein-tyrosine kinas that play a role within the phosphorylation of tyrosine residues in viral polypeptides (LeCher *et al.*, 2019). Infections with the rotavirus are a leading cause of acute gastroenteritis in newborns and young children, as well as a variety of domestic animals (Huang *et al.*, 2015). Andres *et al.* (2007) discovered that combining the soy aglycones genistein, genistin, and acetylgenistin reduced RV infection to the same extent via modifying virion attachment to host cells and a post-binding phase in RV-infected MA104 cells.

Table 7 Evaluation of cytotoxicity of fermented soybean flour extract by *B. licheniformis* NRC24.

Sample	Cell line	Sample concentration (µg/µl)	Cytotoxicity (%)
UFSF	MDCK	100	0
		200	5.69656
		400	6.27669
		800	30.28765
		TC_{50}	1315.727µg/µl
FSF1		100	0
		200	0.217549
		400	2.409153
		800	25.61437
		TC_{50}	1508.38 µg/µl
UFSF	Vero	50	0
		100	0
		200	14.36
		TC_{50}	560 µg/µl
FSF1		50	0
		100	0
		200	15.76
		TC_{50}	516.8 µg/µl
UFSF	MA104	50	29.8452469
		100	20.8302628
		200	22.672562
		TC_{50}	537.03µg/µl
FSF1		50	16.90002456
		100	21.5917465
		200	21.83738639
		TC_{50}	1186.78 µg/µl
FSF2		50	16.23679686
		100	22.99189388
		200	30.41021862
		TC_{50}	411.86

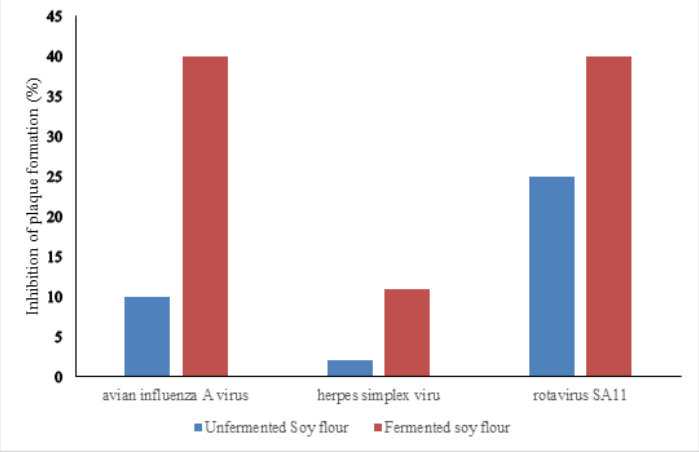


Figure 4 Assessment of antiviral activity of the fermented and unfermented soy flour extracts (50 µg/µl) against avian influenza A virus (M7217B) 2013(H5N1), herpes simplex virus (HSV-1) and rotavirus SA11.

Antitumor activity of fermented and unfermented soybean flour extracts against colon cancer, breast cancer and prostate cancer

After 48 hours of incubation, both unfermented and fermented soy flour extracts decreased cell viability on HCT-116 human colon cancer cell lines, breast cancer cell lines (MCF-7 and MDA-MB-231 breast cancer cell lines), and inhibited prostate cancer cell proliferation and induced apoptosis using PC3 prostate cancer cell lines in a concentration-dependent manner. As can be shown in Figs. 5 and 6, their computed IC50 values are 98.27g/mL and 48.14g/mL, respectively. Furthermore, both extracts had a negligible cytotoxic effect on breast cancer (IC50 values of 173.94g/mL and 137.93g/mL, respectively). Generally, the fermented

soy flour extract was more effective at inducement necrobiosis and inhibiting proliferation altogether neoplastic cell lines mentioned above than unfermented soy flour extract at an equivalent concentration (50 µg/mL) due to the increase in aglycones (genistein and daidzein) than fresh soybean meal.

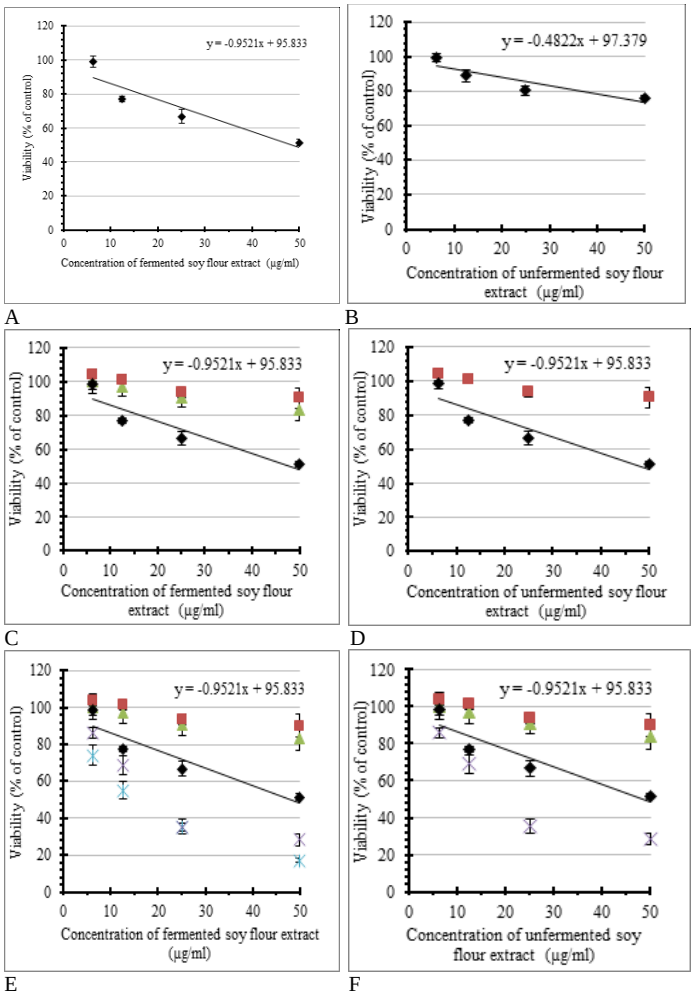


Figure 5 Cytotoxicity of fermented soybean flour extract and unfermented soybean flour extract on colon cancer HCT-116 cells (a, b), breast cancer MCF-7 cells (c, d) and prostate cancer PC3 cells (e, f), respectively, as estimated by MTT assay. Data are represented as viability

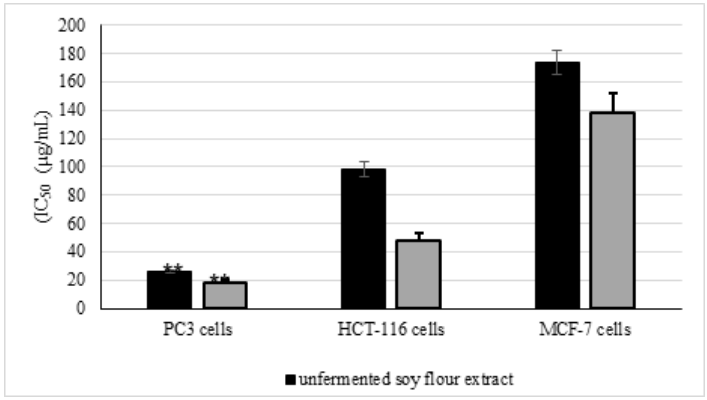


Figure 6 Comparative cytotoxicity (IC_{50}) of samples of unfermented and fermented soy flour extracts on different human cancer cell lines as estimated by MTT assay. Data are represented as µg/mL [mean ± SE]. ** represent P<0.01. (%) of control [mean ± SE].

Carcinogenesis, specifically the initiation, development and advancement of cancer, has been shown to be modulated by the fundamental components of soy isoflavones. Soybean aglycones such as genistein and daidzein, in particular, have piqued interest due to a rising variety of biological effects as well as chemo-preventive properties against breast and prostate cancer (Taylor *et al.*, 2009). Our results are in agreement with Dong *et al.* (2013) who mentioned that the combination of genistein and daidzein showed significant apoptotic effects on both LNCaP cells and C4-2B cells. At lower doses (30 µM), Yuan-jing *et al.* (2009) found that genistein inhibited the proliferation of cancer cells (Hela and DU-145) rather than normal cells (LO-2). Breast cancer is the second most common diagnosed cancer and also the top cause for mortality among women in both developed and developing countries. The actual fact that the bulk of breast cancers are hormone-receptor-positive is well-known. Phytoestrogens found in soy, like genistein and daidzein, are almost like human 17 β-estradiol but have a lower estrogenic effect (Messina and Hilakivi-Clarke, 2009).

Furthermore, genistein, a key component of fermented soy flour, has been shown to block pathways that control metastatic transformation in human prostate tissue. At concentrations existing in a human dietary intake, genistein repressed cell detachment, proteinase activity, cell invasion, and human adenocarcinoma prostate cancer metastasis, in line with Pavese *et al.* (2014). Tempeh is a fermented soybean food product that looks like a solid cake and is produced with *Rhizopus oligosporus* (Haron *et al.*, 2009). Tempeh isoflavones (a combination of genistein, daidzein, glycitein, genistin, and daidzin) suppress metastasis in 3 neoplastic cell lines: MCF-7 (breast neoplastic cell line), Hel (immortal cervical neoplastic cell line), and HO-8910 (ovarian cancer cell). Therefore, fermented soybean meal and or soy isoflavones containing genistein are food components that have the potential for the prevention of cancer and other health care applications.

CONCLUSION

The study looked at how different culture and environmental factors, as well as inducers and their levels in the production medium, affected the production of the soy aglycones genistein and daidzein by the local strain *B. licheniformis* NRC24. The findings support the use of a non-statistical one-factor-at-a-time approach for identifying and mobilizing appropriate factors. RSM was also found to be successful in optimizing the chosen factors in a manageable number of experimental trials, resulting in a 1.11 fold increase in aglycone output overall. The optimal medium and, as a result, the culture conditions described in this study are useful for the further creation of a large-scale fermentation process for the economical production of toper aglycones from the local strain *B. licheniformis* NRC24. Furthermore, the biological activities of the fermented defatted soy flour against viral and tumor cells were investigated. As a result, the fermented soybean meal produced by the local strain *B. licheniformis* NRC24 could be a promising option for further research, development, and commercial and industrial uses. We intend to continue to expand and improve both the logical information base and provide better living aid in the future.

Conflict of Interests: The authors declare that they have no conflicts of interest.

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REFERENCES

Andres, A., Donovan, S. M. & Kuhlenschmidt, M. S. (2009). Soy isoflavones and virus infections. *Journal of Nutritional Biochemistry, 20*(8), 563-569. https://doi.org/10.1016/j.jnutbio.2009.04.004

Andres, A., Donovan, S. M., Kuhlenschmidt, T. B. & Kuhlenschmidt, M. S. (2007). Isoflavones at concentrations present in soy infant formula inhibit rotavirus infection in vitro. *Journal of Nutrition*, 137 (9), 2068- 2073. https://doi.org/10.1093/jn/137.9.2068 Argenta, D. F., Silva, I. T., Bassani, V. L., Koester, L. S., Teixeira, H. F., and Simoes, C. M.O. (2015). Antiherpes evaluation of soybean isoflavonoids. Archives of *Virology*,160, 2335-2342. https://doi.org/10.1007/s00705-015-2514-z

Box, G. E. P., and Behnken, D. W. (1960). Some new three level designs for the study of quantitative variables. *Technometrics* 2(4), 455-475. https://doi.org/10.1080/00401706.1960.10489912

Cheng, K. C., Lin, J.T., Wu, J.Y. & Liu, W.H. (2010). Isoflavone conversion of black soybean by immobilized *Rhizopus* spp. *Food Biotechnology* ,24(4), 312-331. https://doi.org/10.1080/08905436.2010.524459

Doan, D. T.; Luu, D. P.; Nguyen, T. D.; Thi H. B.; Thi H. M. P.; Do, H. N.; Luu, V. H.; Pham, T. D.; Than, V. T.; Thi, H. H. P.; Pham, M. Q. & Tran, Q. T. (2019). Isolation of *Penicillium citrinum* from roots of *Clerodendron cyrtophyllum* and application in biosynthesis of aglycone isoflavones from soybean waste fermentation. *Foods*, 8(11), 554. https://doi.org/10.3390/foods8110554

Dong, X., Xu, W., Sikes, R. A. & Wu, C. (2013). Combination of low dose of genistein and daidzein has synergistic preventive effects on isogenic human prostate cancer cells when compared with individual soy isoflavone. *Food Chemistry*, 141(3), 1923-1933. https://doi.org/10.1016/j.foodchem.2013.04.109

Donkor, O. N. & Shah, N. P. (2008). Production of *β-glucosidase* and hydrolysis of isoflavone phytoestrogens by *Lactobacillus acidophilus*, *Bifidobacterium lactis*, and *Lactobacillus casei* in soymilk. *Journal of Food Science*, 73(1), 15-20. https://doi.org/10.1111/j.1750-3841.2007.00547.x

El-Shazly, A. I., El-Deen, A. M. N., Ibrahim, N. A., Abdelwahed, N. A. M., El-Beih, A. A., Shetaia, Y. M. H. & Farid, M. A. (2017). Assessment of genistein and daidzein production by some local fungal and bacterial isolates. *Egyptian Journal of Microbiology*, 52, 49-61. https://doi.org/10.21608/ejm.2017.1134.1023

Fadahunsi, I.F., Busari, N.K. & Fadahunsi, O.S. (2020). Effect of cultural conditions on the growth and linamarase production by a local species of *Lactobacillus fermentum* isolated from cassava effluent. *Bulletin of the National Research Centre*, 44, 185. https://doi.org/10.1186/s42269-020-00436-3

Fukutake, M., Takahashi, M., Ishida, K., Kawamura, H., Sugimura, T. & Wakabayashi, K. (1996). Quantification of genistein and genistin in soybeans and soybean products. *Food and Chemical Toxicology*, 34(5), 457-461. https://doi.org/10.1016/0278-6915(96)87355-8

Georgetti, S. R., Casagrande, R., Vicentini, F. T. M. C., Baracat, M. M., Verri, Jr. W. A. & Fonseca, M. J. V. (2013). Protective effect of fermented soybean dried extracts against TPA-induced oxidative stress in hairless mice skin. *BioMed Research International*, 2013, 1-8. https://doi.org/10.1155/2013/340626

Hansen, M.B., Nielsen, S.E. & Berg, K. (1989). Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. Journal of *Immunological Methods*, 119, 203-210 . https://doi.org/10.1016/0022-1759(89)90397-9 Haron, H., Ismail, A., Azlan, A., Shahar, S. & Peng, L.S. (2009). Daidzein and genistein contents in tempeh and selected soy products. *Food Chemistry*, 115, 1350–1356. https://doi.org/10.1016/j.foodchem.2009.01.053

Hayden, F. G., Cote, K. & Douglas, R. G. (1980). Plaque inhibition assay for drug susceptibility testing of influenza viruses. *Antimicrobial Agents* and *Chemotherapy*, 17(5), 865-870. https://doi.org/10.1128/AAC.17.5.865

Huang, H., Liao, D., Liang, L. Song, L. & Zhao, W. (2015). Genistein inhibits rotavirus replication and upregulates AQP4 expression in rotavirus-infected Caco-2 cells. *Archives of Virology* , 160(6), 1421-1433. https://doi.org/10.1007/s00705-015-2404-4.

Ibrahim, A. S., Al-Salamah, A. A., Elbadawi, Y. B., El-Tayeb, M. A. & Ibrahim, S. S. S. (2015).Production of extracellular alkaline protease by new halotolerant alkaliphilic *Bacillus* sp. NPST-AK15 isolated from hyper saline soda lakes. *Electronic Journal of Biotechnology*, 18(3), 236-243. https://doi.org/10.1016/j.ejbt.2015.04.001

Izaguirre J. K., Barañano L., Castañón S., Alkorta I. , Quirós L.M. & Garbisu C. (2021). Optimization of the bioactivation of isoflavones in soymilk by lactic acid bacteria. *Processes* 9 (6), 963. https://doi.org/10.3390/pr9060963

Izumi, T., Piskula, M. K., Osawa, S., Obata, A., Tobe, K., Saito, M., Kataoka, S., Kubota, Y. & Kikuchi, M. (2000). Soy isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans. *Journal of Nutrition* 130 (7), 1695-1699. https://doi.org/10.1093/jn/130.7.1695

Jianming, W., Yuan, G., Ping, L., Feng, H. & Liying, L. (2013). Optimization of ultrasound-assisted extraction procedure to determine total isoflavones in Chinese soybean cheese by Box–Behnken design. *Food Analytical Methods*, 6(1), 221-226. https://doi.org/10.1007/s12161-012-9434-2

Kuo, L. C., Cheng, W. Y., Wu, R. Y., Huang, C. J. & Lee, K. T. (2006). Hydrolysis of black soybean isoflavone glycosides by *Bacillus subtilis* natto. *Applied Microbiology and Biotechnology*, 73(2), 314-320. https://doi.org/10.1007/s00253-006-0474-7

Jawan, R., Abbasiliasi, S., Joo Shun, T., Shuhaimi, M., Halim, M. & Ariff, A. (2020). Influence of culture conditions and medium compositions on the production of bacteriocin-like inhibitory substances by *Lactococcus lactis* Gh1. *Microorganisms*, 8 (10), 1454, 1-14. https://doi.org/10.3390/microorganisms8101454

Lakshmi, B. K. M, Ratna Sri, P. V. Ambika Devi, K. and Hemalatha, KP. J. (2014). Media optimization of protease production by *Bacillus licheniformis* and partial characterization of alkaline protease. *International Journal* of *Current Microbiology* and *Applied* Sciences, 3(5), 650-659.

LeCher, J. C., Diep, N., Krug, P. W., and Hilliard, J. K. (2019). Genistein has antiviral activity against Herpes B virus and acts synergistically with antiviral treatments to reduce effective dose. *Viruses* 11(6), 499, 1-13. https://doi.org/10.3390/v11060499

Lee, J. Y., Kim, H. S., & Song, Y. S. (2012). Genistein as a Potential Anticancer Agent against Ovarian Cancer. *Journal of traditional and complementary medicine*, *2*(2), 96–104. https://doi.org/10.1016/S2225-4110(16)30082-7

Lee, Youn-Woo ., Kim, Jae-Duck ., Zheng, Jinzhu . & Row, Kyung. (2007). Comparisons of isoflavones from Korean and Chinese soybean and processed product. *Biochemical Engineering Journal* ,36, 49-53. https://doi.org/10.1016/j.bej.2006.06.009 Lepri, S. R., Luiz, R. C., Zanelatto, L. C., Da Silva, P. B. G., Sartori, D., Ribeiro, L. R. & Mantovani, M. S. (2013). Chemoprotective activity of the isoflavones, genistein and daidzein on mutagenicity induced by direct and indirect mutagens in cultured HTC cells. *Cytotechnology*, 65(2), 213-222. https://doi.org/10.1007/s10616-012-9476-8

Liu, J. Z., Weng, L.P., Zhang, Q.L., Xu, H. & Ji, L.N. (2003). Optimization of glucose oxidase production by *Aspergillus niger* in a benchtop bioreactor using response surface methodology. *World Journal of Microbiology and Biotechnology*, 19, 317–23. https://doi.org/10.1023/A:1023622925933

Lonsane, B., Ghildyal, N., Budiatman, S. & Ramakrishna, S. (1985). Engineering aspects of solid-state fermentation. *Enzyme* and *Microbial Technology*, 7(6), 258-265. https://doi.org/10.1016/0141-0229(85)90083-3

Marazza, J. A., Garro, M. S. & de Giori, G. S. (2009). Aglycone production by *Lactobacillus rhamnosus* CRL981 during soymilk fermentation. *Food Microbiology*, 26(3), 333-339. https://doi.org/10.1016/j.fm.2008.11.004

Messina, M. & Hilakivi-Clarke, L. (2009). Early intake appears to be the key to the proposed protective effects of soy intake against breast cancer. *Nutrition and Cancer*, 61(6), 792-798. https://doi.org/10.1080/01635580903285015

Mizushina, Y., Shiomi, K., Kuriyama, I., Takahashi, Y. & Yoshida, H. (2013). Inhibitory effects of a major soy isoflavone, genistein, on human DNA topoisomerase II activity and cancer cell proliferation. *International Journal of Clinical Oncology* , 43, 1117-1124. https://doi.org/10.3892/ijo.2013.2032

Mossman, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, 65(1-2), 55-63. https://doi.org/10.1016/0022-1759(83)90303-4

Mukhtar, H., & Haq, I. (2013). Comparative evaluation of agroindustrial byproducts for the production of alkaline protease by wild and mutant strains of *Bacillus subtilis* in submerged and solid state fermentation. *Scientific World Journal*, 2013, 1-6. https://doi.org/10.1155/2013/538067

Mudgetti, R.E. (1986). Solid-state fermentations. In Manual of Industrial Biotechnology; Demain, A. L., Solmen, N.A. (eds). American Society for Microbiology, Washington DC, USA. pp. 66–83.

Nawaz, M. A., Bibi, Z., Karim, A., Rehman, H. U., Jamal, M., Jan, T., Aman, A. & Qader, S. A. U. (2016). Production of α-1, 4 *β- glucosidase* from *Bacillus licheniformis* KIBGE-IB4 by utilizing sweet potato peel. *Environmental Science and Pollution Research*, 24, 4058–4066. https://doi.org/10.1007/s11356-016-8168-x

Ningtyas, D.W., Hati, S. & Prakash, S. (2021). *Bioconversion and bioaccessibility of isoflavones from sogurt during in vitro digestion. Food Chemistry*, 343, 128553. https://doi.org/10.1016/j.foodchem.2020.128553

Otieno, D. & Shah, N. (2007). Endogenous *β-glucosidase* and β-galactosidase activities from selected probiotic micro-organisms and their role in isoflavone biotransformation in soymilk. *Journal of Applied Microbiology* , 103(4), 910-917. https://doi.org/10.1111/j.1365-2672.2007.03438.x

Pavese, J. M., Krishna, S. N. & Bergan, R. C. (2014). Genistein inhibits human prostate cancer cell detachment, invasion, and metastasis. *American Journal* of *Clinical Nutrition*, 100 (suppl-1), 431-436. https://doi.org/10.3945/ajcn.113.071290

Raimondi, S., Roncaglia, L., De Lucia, M., Amaretti, A., Leonardi, A., Pagnoni, U. M., and Rossi, M. (2009). Bioconversion of soy isoflavones daidzin and daidzein by *Bifidobacterium* strains. *Applied Microbiology and Biotechnology* 81(5), 943. https://doi.org/10.1007/s00253-008-1719-4

Silva, L. H., Celeghini, R. M. S., and Chang, Y. K. (2011). Effect of the fermentation of whole soybean flour on the conversion of isoflavones from glycosides to aglycones. *Food Chemistry* 128(3), 640-644. https://doi.org/10.1016/j.foodchem.2011.03.079

Song, Q., Huang, Y., and Yang, H. (2012). Optimization of fermentation conditions for antibiotic production by *Actinomycetes* YJ1 strain against *Sclerotinia sclerotiorum*. *Journal of Agricultural Science* 4, 95-102. https://doi.org/10.5539/jas.v4n7p95

Sumarna, (2010). Hydrolysis of bioactive isoflavone in soymilk fermented with *β-glucosidase* producing lactic acid bacteria from local fermented foods of Indonesian. *Malaysian Journal of Microbiology* 6(1), 30-40. https://doi.org/10.21161/mjm.17709

Taylor, C. K., Levy, R. M., Elliott, J. C., and Burnett, B. P. (2009). The effect of genistein aglycone on cancer and cancer risk: a review of in vitro, preclinical, and clinical studies. *Nutrition Reviews* 67(7), 398-415. https://doi.org/10.1111/j.1753-4887.2009.00213.x

Tian, Y., Fan, Y., Liu, J., Zhao, X., & Chen, W. (2016). Effect of nitrogen, carbon sources and agitation speed on acetoin production of *Bacillus subtilis* SF4-3. *Electronic Journal of Biotechnology* 19, 41-49. https://doi.org/10.1016/j.ejbt.2015.11.005

Tsangalis, D., Ashton, J. F., Stojanovska, L., Wilcox, G., and Shah, N. P. (2004). Development of an isoflavone aglycone-enriched soymilk using soy germ, soy protein isolate and bifidobacteria. *Food Research International* 37(4), 301-312. https://doi.org/10.1016/j.foodres.2004.01.003

Wang, Q., Ge, X., Tian, X., Zhang, Y., Zhang, J. & Zhang, P. (2013). Soy isoflavone: The multipurpose phytochemical (Review). *Biomedical Reports*, 1(5), 697-701. https://doi.org/10.3892/br.2013.129

Wei, B., Cha, S.-Y., Kang, M., Kim, Y. J., Cho, C.-W., Rhee, Y. K., Hong, H.-D. & Jang, H.-K. (2015). Antiviral activity of Chongkukjang extracts against influenza A virus in vitro and in vivo. *Journal of Ethnic Foods*, 2(2), 47-51. https://doi.org/10.1016/j.jef.2015.04.001

Yuan-jing, F., Nan-shan, H. & Lian, X. (2009). Genistein synergizes with RNA interference inhibiting survivin for inducing DU-145 of prostate cancer cells to apoptosis. *Cancer Letters*, 284(2), 189-197. https://doi.org/10.1016/j.canlet.2009.04.024

Yu-Hsuan, H., Chi-Tang, H. & Min-Hsiung, P. (2020).Bioavailability and health benefits of major isoflavone aglycones and their metabolites. *Journal of Functional Foods*, 74, 104164. https://doi.org/10.1016/j.jff.2020.104164

Yura, Y., Yoshida, H., & Sato, M. (1993). Inhibition of herpes simplex virus replication by genistein an inhibitor of protein- tyrosine kinase. *Archives of Virology*, 132(3-4), 451-461. https://doi.org/10.1007/BF01309554

Zhang, Z., Zhou, L., Xie, N. *et al*. (2020). Overcoming cancer therapeutic bottleneck by drug repurposing. *Signal Transduction* and *Targeted Therapy*,5,113. https://doi.org/10.1038/s41392-020-00213-8

Zhou Y., Han L., He H., Sang B., Yu D., Feng J. & Zhang X. (2018). Effects of agitation, aeration and temperature on production of a novel glycoprotein GP-1 by *Streptomyces kanasenisi* ZX01 and scale-up based on volumetric oxygen transfer coefficient. *Molecules*, 23(1), 125. https://doi.org/10.3390/molecules23010125