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Assessment of Genistein and Daidzein Production By Some Local Fungal and Bacterial Isolates

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TWENTY-THREE different bacterial and fungal isolates were grown and screened for their capability to transform soy glycosides to their aglycone forms with higher titer of antioxidant activity compared to unfermented soy flour. Most of the bacterial isolates showed higher amounts of daidzein than of genistein, which are the aglycone products of daidzin and genistin. After fermentation of soybean flour using bacterial and fungal isolates, the content of isoflavone aglycones varied from 0.0 to 431.89 µg/g compared to unfermented autoclaved soybean flour. Extracellular β-glucosidase activity was ranged from 1.22 to 11.56 mU/mL and 0.3-534.3 U/mL for bacterial and fungal isolates, respectively, while, bacterial cell-bound β-glucosidase ranged from 44.72 to 128.89 mU/mL. Most of the bacterial isolates more efficiently transformed daidzin and genistin into the aglycones than fungal isolates. Among the tested bacterial isolates, the most potent one was selected, characterized according to the morphological and 16S rDNA sequence analysis and identified as *Bacillus licheniformis* NRC24.

Keywords: Daidzein, Genistein, β-glucosidase, free radical DPPH-scavenging activity, phylogentic tree, *B. licheniformis* NRC24.

Introduction

Elevated consumption of soybean products has been proposed to contribute to lowering the occurrence of chronic diseases, for example, atherosclerosis, cancer, osteoporosis and menopausal disorders. Different functional ingredients in soy-based food are of health benefits, especially isoflavones (Ewe et al., 2012). Soy isoflavones are mainly glucosides (malonyl, acetyl and β- glucosides) with a small amount of the more bioactive aglycones (Chun et al., 2008).The chemical structure of soy isoflavones influences their bioavailability and hence their biological and physiological efficiency. Highly polar and water-soluble glucosides are less absorbable by intestinal epithelium and thus have weaker biological activities than their respective counterpartswith the sugars removed (aglycones) (Ren et al., 2001). Fermenting soy flour by certain microorganisms has been shown to increase aglycone content conversion from their glucosides (Handa et al., 2014). Fermented soy products, like natto, tempeh and miso show higher antioxidant activity than unfermented soybeans (Murakami et al., 1984 and Berghofer et al., 1998).

Bacillus is one of the large genera of bacterial strains. It is a rod shaped, endospore bearing bacterium and belongs to the family Firmicutes. The genus *Bacillus* includes a great diversity of strains; some of them are strictly aerobic, while others are facultative anaerobic (Schallmey et al., 2004). *B. licheniformis* is a saprophytic bacterium found in nature and commonly found in soil and other natural environments. *B. licheniformis* is capable of growing on a large diversity of nutrient sources by means of synthesizing and secreting different hydrolytic enzymes and this quality makes it an industrially important microorganism (Raksha Rao et al., 2017 and Zhou et al., 2017).

In the current study, different bacteria and fungi were isolated from different soil samples and screened for daidzin and genistin biotransformation. The maximum daidzein and genistein transforming microbial isolate was identified using conventional and molecular techniques.

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Materials and Methods

Chemicals

Daidzein, genistein, 2, 2- diphenyl -1-picrylhydrazyl (DPPH) and *p*-nitrophenyl -β -D-glucoside (*p*-NPG) were obtained from Sigma Company (St. Louis, MO, USA). Soybean (Glycine max) flour was purchased from the soybean unit, Agricultural Research Centre, 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 and a Gram's staining kit from Biodiagnostic, Giza, Egypt. All other chemicals used were of analytical grade.

Media

Isolation media was composed of agar (25 g/L) supplemented and autoclaved separately with one of the following materials, 10 g/L soybean flour (Medium No.1) and 2 g/L cellobiose (Medium No.2). pH value was adjusted before autoclaving to 7 by adding 0.1 N NaOH for bacterial isolation and to 6.0 by 0.1 N HCl for fungal isolation. Screening medium was composed of defatted soybean flour, 10 g/L.

Microorganisms

Twenty-three local bacterial and fungal isolates from different soil samples were obtained from different locations in Egypt.

Isolation of bacterial and fungal isolates

Isolation of microorganisms was performed according to Stanbury et al. (1995). One gram of each soil sample was suspended in 10 mL of sterilized water, vortexed for 1 min and then sedimented. Each sample was serially diluted (till $X10^{-6}$) and 100 µL of each dilution sample was streaked on poured agar medium (Medium No. 1 and Medium No. 2) in sterilized petri dishes (Jensen, 1968). For bacterial isolation, the inoculated plates were incubated at 37^0C for 24 h while for fungal isolation; the plates were incubated at 28 ± 2^0C for 96 h. All morphologically contrasting colonies were purified using streak plate method. The pure bacterial and fungal cultures were preserved in 20% glycerol vials at -80^oC.

Screening for biotransformation of soy isoflavone aglycones

Bacterial and fungal isolates were screened for daidzin and genistin biotransformation according to the method reported by Tsangalis et al. (2002). Bacterial isolates were previously activated by subcultured to nutrient agar slants and incubated at 37^0C for 24 h while fungal isolates were sub-cultured on potato dextrose agar medium and incubated at 28 ± 2^0C for 96 h. Defatted soybean flour (0.5 g) was dispersed in 250 mL Erlenmeyer flasks with 50 mL distilled water and autoclaved at 121^0C for 15 min. Submerged fermentation was performed by evenly inoculating the autoclaved soybean substrate with 5mL of cell suspension of the tested microbial isolates. The inoculated Erlenmeyer flasks were incubated under shaking culture at 200 rpm at 37^0C for 48 h and at 28 ± 2^0C for 96 h for bacterial and fungal isolates, respectively. 50 mL of uninoculated autoclaved defatted soybean flour medium was used as a negative control. At the end of incubation period, a 45 mL aliquot was taken from each flask and stored immediately at -80^0C for the analysis of isoflavones. The frozen aliquots were freeze dried using a freeze drier (Beta, Martin Christ Gefriertrocknungsanlagen GmbH, Germany) for isoflavone extraction and analysed using reverse phase high-performance liquid chromatography (HPLC). A sample of 5 mL was analyzed for assessment of β-glucosidase activity using *p*-nitrophenyl-β-D-glucoside (*p*-NPG) as the synthetic substrate.

Further screening with different concentrations of soybean flour was done according to Mukhtar & Haq (2013). Different concentrations of defatted soybean flour (10, 20 and 40 g/L) were tested for their efficiency in the biotransformation of daidzin and genistin to daidzein and genistein.

Measurement of pH

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

Assessment of β-glucosidase activity

Extracellular β-glucosidase activity

β-glucosidase activity was determined using *p*-nitrophenyl-β-D-glucopyranoside as a substrate according to the method reported by Otieno & 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*-nitrophenyl-β-D-glucopyranoside 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). For fungal samples 0.05 M citrate buffer with pH value 5 was used. After 30 min of incubation at 37^{0}C, 0.5 mL of (1 M) cold sodium carbonate was added to stop the reaction. The aliquots were then placed in 2-mL centrifuge tubes and subjected to centrifugation at 15000 rpm for 30 min using an Eppendorf centrifuge (model 5415D; Eppendorf, Hamburg, Germany). The amount of *p*-nitrophenol released was measured using a spectrophotometer (SP-2000UV, Spectra, USA) at a wavelength of 401 nm. One unit of enzyme activity was defined as the amount of enzyme that would liberate 1 mmol *p*-nitrophenol per minute under assay conditions.

Cell-bound β- glucosidase activity

The assay of cell-bound β-glucosidase activity was determined with the modified method of Kuo et al. (2006**).** 1 mL of culture broth was centrifuged at 15000 rpm for 5 min, then the supernatant was discarded and the cell pellets were washed with sodium phosphate buffer (pH 7.0). The cell pellets were re-suspended in 0.5 mL of sodium phosphate buffer (pH 7.0) containing 0.1% *p*-nitrophenyl-β-D-glucoside (*P*-NPG) and incubated at 37^{0}C for 30 min. The reaction was stopped by adding of 0.5 mL (1 M) Na_2CO_3 and centrifuged at 15000 rpm for 10 min. This reaction supernatant was measured using a spectrophotometer (SP-2000UV, Spectra, USA) at a wavelength of 401 nm. The amount of released *p*-nitrophenol was measured as described before.

Extraction of isoflavones

The extraction of isoflavones, including daidzein and genistein from fermented and unfermented soybean flour, was performed using a modified version of the method described by Fukutake et al. (1996). 1g of freeze-dried sample was added to 10 mL of 80% (v/v) aqueous methanol and extracted under agitation for 24 h at room temperature. The homogenates were centrifugated at 15000 rpm for 30 min and the obtained methanolic extracts were used to determine daidzein and genistein and antioxidant activity.

Assessment of the antioxidant activity DPPH free radical-scavenging assay

The ability of the isoflavone extracts to scavenge DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radicals was assessed spectrophotometrically (Pyo et al., 2005). A 20 µL aliquot of each isoflavone extract was mixed in a test tube with 1.0 mL methanol containing (0.1 mM) DPPH, which is a stable free radical, and has a typical absorbance at 517 nm. The decrease in absorbance at 517 nm was measured at 100 min. The decreased absorbance of DPPH was calculated by the following equation:

$$\text{Scavenging activity (\%)} = AA\% = 100 - [(Abs_{sample} - Abs_{blank}) \times 100 / Abs_{control}]$$

where $Abs_{control}$ is the absorbance of the DPPH solution without addition of the sample; Abs_{sample} is the absorbance of the mixture solution containing both the sample and DPPH, and Abs_{blank} is the absorbance of the blank solution without DPPH.

HPLC analysis of isoflavones

HPLC instrument Young Lin (Young Lin Cooperation, Seoul, South Korea) consists of a Reprosil-Pur Basic C18 5µm (dimension: 250 × 4.6 mm) column and a UV detector (λmax = 210 nm). Isocratic elution was used to isolate the isoflavones for detection. The mobile phase consisted of 100% methanol and 10 mmol/L of ammonium acetate buffer (60: 40) containing 1 mL of trifluoro-acetic acid per litre of solvent mixture. This was set at a flow rate of 1 mL/min according to the method by Otieno & Shah (2007) with some modifications. Injection volumes of isoflavone standards and of the samples were set at 100 µL throughout the run time of 30 min. Single standards were prepared for peak identification. Isoflavone concentrations were calculated back to dry basis (µg/g soybean flour).

Calibration curves of Isoflavone standard

Soy isoflavone aglycones were quantified by analysing each methanolic standard of dadzein and genistein by HPLC using a diode array detector (HPLC-DAD), following the procedure described by da Silva et al. (2011). Standard solutions of known concentrations of daidzein (0.25, 0.5, 0.75, 1.25, 2.5 and 5 µg/mL) and genistein (0.5, 1, 2, 3, 4 and 5 µg/mL) were used. The areas obtained were matched to their respective concentrations. The concentrations of daidzein and genistein were calculated by interpolation of the areas and expressed in µg of each isoflavone aglycone per gram of soybean flour, on a dry weight basis. The isoflavone peaks were identified by a comparison of the retention times (RT) and confirmed by a comparison of the UV spectra with those of the reference materials.

Identification of the most potent bacterial isolate

The identification of the most potent bacterial isolate was performed on the basis of morphological and molecular characteristics.

Morphological characterization

Cultural characteristics such as colony morphology (color, shape, margin and surface) and cell morphology (shape, Gram reaction, and arrangement) of the selected bacterial isolate were carried out according to Bergey & Holt (1994) by using a bright field microscope (Olympus CX41RF, Olympus cooperation, Tokyo, Japan).The selected bacterial isolate was characterized by Gram's stainingand results of the staining procedure were observed under oil immersion lens (100x).

Molecular approach

DNA extraction and PCR amplification of 16S rDNA

QIAamp mini kit (QIAGEN) was used for DNA extraction according to the procedure of manufacturer. The total DNA was eluted in 50 µL of AE buffer and stored at -80 ^{0}C until used. PCR was performed in a reaction volume of 50 µL, consisting of 0.2 mM each dNTP (Invitrogen), 0.4 M each primer, 5 µL 10X PCR buffer (Promega), 1.5 mM $MgCl_2$ and 1 U Taq DNA polymerase (Promega). The universal primer pairs used to amplify bacterial 16S rDNA were 27f (59-AGAGTTTGATCCTGGCT CAG-39) and 1492r (59-GGTTACCTTGTTACGACTT-39) (Lane et al., 1985). PCR amplification was performed with 30 cycles as follows: denaturation at 94 ^{0}C for 1 min, annealing at 55 ^{0}C for 1 min and extension at 72 ^{0}C for 2 min. Amplification products were separated by 1·0 % agarose gel electrophoresis and visualized under UV light.

Sequencing of 16S rDNA

The PCR products were separated by (1%) agarose gel electrophoresis. Amplicons of the appropriate sizes were subsequently excised from the gel and purified using QIAGEN gel extraction kit (Qiagen, Hilden, Germany). The purified PCR products were directly used for cycle sequencing reactions using BigDyeR Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions, and were further amplified for 26 cycles at 95^{0}C, 30 s; 50^{0}C, 15 s; 60^{0}C, 4 min. The reaction product was purified by exclusion chromatography in CentriSep columns (Princeton Separations, Adelphia, NJ). The recovered materials were sequenced using an ABI 3730XLDNA analyzer (Applied Biosystems, Foster City, CA). Sequences were generated using SeqMan DNA Lasergene 7 software (DNASTAR, Madison, WI).

Phylogenetic analysis of the selected bacterial isolate

Phylogenetic analysis was done using the neighbor-joining algorithm with the Kimura 2-parameter model. *Vibrio rotiferianus* was used as an out-group and used as the root for the tree. The reliability of phylogenetic inference at each branch node was estimated by the bootstrap method with 1000 replications, evolutionary analysis was conducted in MEGA6.

Results

Isolation and screening of some Egyptian bacterial and fungal isolates for the biotransformation of soy glucosides into soy aglycones

Screening of the twenty-three microbial isolates was conducted to detect their ability to deglycosylate daidzin and genistin into daidzein and genistein using submerged fermentation.

The data recorded in Table 1 demonstrated that the bacterial isolate No. 3 was the most potent daidzein and genistein producer (176.05, 255.84 µg/g, respectively), while the other microbial isolates produced daidzein and genistein in a range from 0.0 to 162.59 and from 1.32 to 149.67µg/g, respectively. The HPLC analysis of the fermented and unfermented samples revealed an increase of the aglycones forms for some tested microbial isolates (Fig. 1 a-d).

For bacterial isolates, it was noticed that the amount of daidzein was almost greater than that of genistein except for isolates No.3, 4 and 8.

Few fungal isolates could transform isoflavone glycosides under the conditions used. Meanwhile, some fungal isolates were more potent producers of β-glucosidase than bacterial isolates. Bacterial isolates produced extracellular β-glucosidase with activity of 11.56 mU/mL and a cell-bound form with activity ranging from 44.72 to 128.89 mU/mL, while fungal isolates produced extracellular β-glucosidase with activity ranged from 0.30 to 534.32 U/ml. The highest extracellular β-glucosidase activity was produced by fungal isolate No. 9.

Concerning the DPPH scavenging activity, all the tested microbial isolates exhibited enhanced antioxidant activity in all fermented soybean extracts compared to unfermented controls (Fig. 2). In case of bacterial isolates, the scavenging DPPH free radical activity ranged from 32.56% to 93.26%, while, fungal isolates showed scavenging DPPH free radical activity ranging from 36.44% to 96.59%. The highest values of DPPH free radical scavenging activity were obtained with bacterial isolates No. 1 and 5 and fungal isolates No. 6, 7 and 8, while bacterial isolate No. 6 and fungal isolate No. 11 showed the lowest DPPH scavenging activity. On the other hand, the unfermented soybean flour

extract showed a scavenging activity of 25.04% for DPPH free radicals.

The pH values increased from 7.0 to 8.97 after soybean flour fermentation time (48 h) using bacterial isolates and increased from 5.0 to 9.26 after soybean flour fermentation time (96 h) using fungal isolates (Table 1) except for fungal isolates No. 6 and 8, where the final pH shifted to acidic range 3.58 and 5.94.

Among the candidates, bacterial isolates No. 3 and 8 had the ability to effectively transform daidzin and genistin into their aglycone forms, so, screening with different concentrations of soybean flour was investigated for both isolates. The changes in total isoflavone aglycones content after defatted soybean flour fermentation are summarized in Fig. 3. The isoflavone aglycones content (daidzein and genistein) of the fermented soybean flour using bacterial isolate No. 3 reached 289.03, 316.37 and 323.55 µg/g after 24 h of fermentation of different soybean flour concentrations; 10, 20 and 40 g/L, respectively. Meanwhile, the total isoflavone content reached to 422.56, 233.35 and 207.21 µg/g, respectively after 48 h. Relating to bacterial isolate No. 8, the isoflavone aglycones content reached 198.57, 302.36 and 328.98 and 276.45, 209.41 and 257.76 µg/g after 24 h and 48 h at soybean flour concentration of 10, 20 & 40 g/L, respectively.

TABLE 1. Quantitative determination of daidzein and genistein production by selected bacterial and fungal isolates.

	Isolate No.	Final pH	Extracellularβ-glucosidase activity		Cell-bound-β-glucosidase (mU/ml)	Daidzein (µg/g)	Genistein (µg/g)	Total aglycones (µg/g)
			24h	48h	48h			
			(mU/ml)					
	Unfermented soybean flour medium	7.0	0.0	0.0	0.0	0.0	26.98	26.98
Bacteria	#1	8.8	1.22	0.0	86.11	162.59	71.02	232.21
	#2	8.8	11.56	0.02	83.79	108.81	85.29	194.10
	#3	8.3	5.33	0.02	73.98	176.05	255.84	431.89
	#4	8.6	5.22	0.03	123.39	70.02	97.98	168.0
	#5	8.9	0.0	0.0	44.72	108.94	54.64	163.57
	#6	7.3	0.0	0.0	128.89	0.0	0.0	0.0
	#7	8.9	6.33	0.0	67.96	65.57	38.41	103.98
	#8	9.1	6.76	0.02	109.54	139.79	149.67	289.41
	#9	8.9	4.57	0.0	57.702	115.17	41.79	156.98
	#10	8.8	4.33	0.02	110.0	161.62	28.08	189.70
			(U/ml)					
Fungi	#1	7.4	428.19			0.0	2.34	2.34
	#2	8.3	197.28			0.0	1.57	1.57
	#3	8.8	408.22			0.0	3.75	3.75
	#4	8.7	130.64			0.0	1.32	1.32
	#5	8.8	108.33			10.85	4.31	15.16
	#6	3.5	262.71			0.0	1.77	1.77
	#7	8.8	0.30			0.0	0.0	0.0
	#8	5.9	136.52			0.0	0.0	0.0
	#9	8.1	534.32			0.0	0.0	0.0
	#10	7.7	122.23			34.79	0.0	34.79
	#11	9.1	0.56			19.42	0.0	19.42
	#12	9.1	0.62			0.0	0.0	0.0
	#13	9.2	0.34			13.0	0.0	13.0

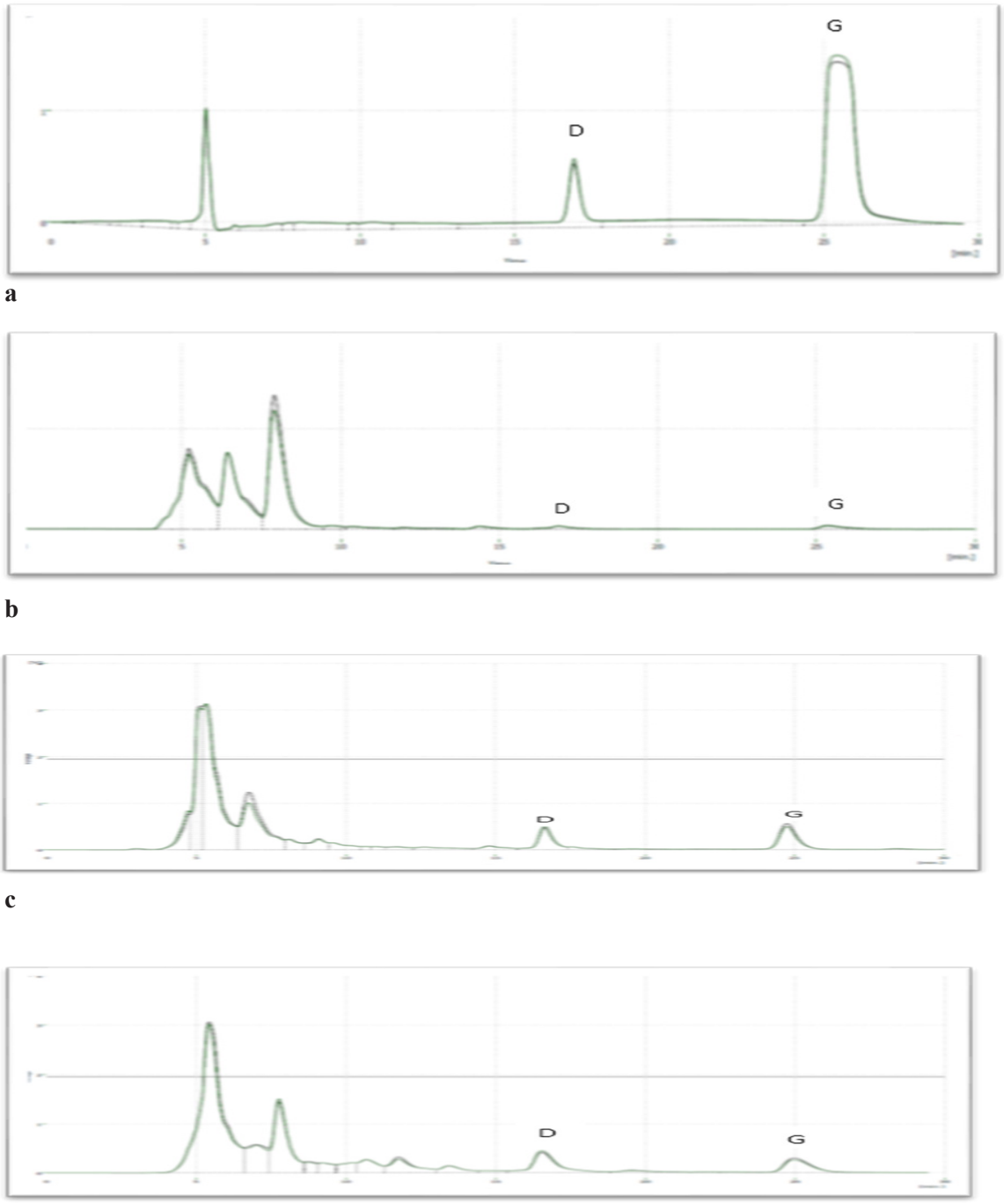

Fig. 1. HPLC chromatogram showing the retention times of (a) standard daidzein (D) followed by genistein (G), (b) unfermented soybean flour, (c) bacterial isolate No. 3 (d) bacterial isolate No. 8.

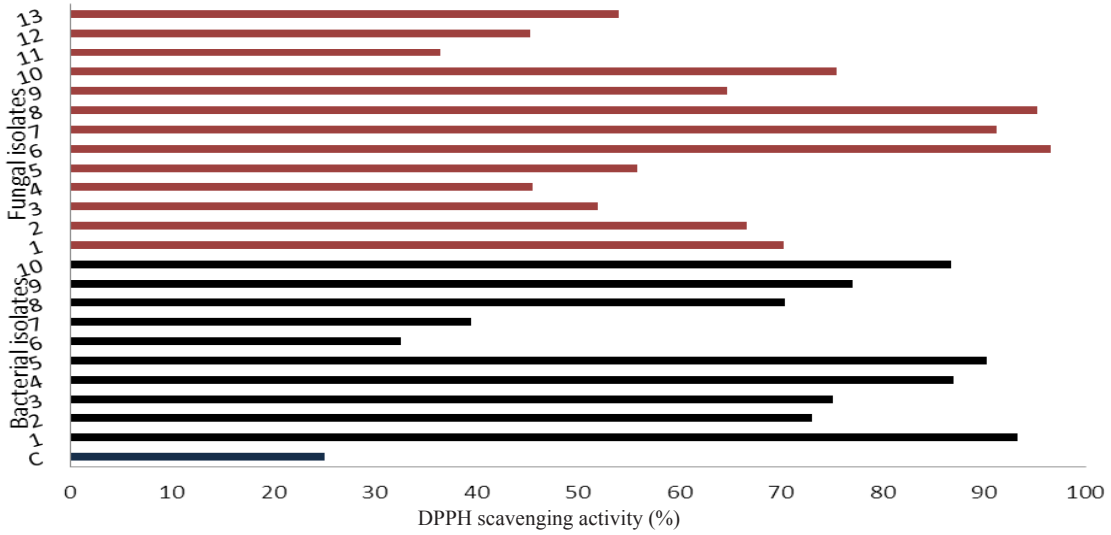

Fig. 2. Free radical DPPH scavenging activity (%) of unfermented and fermented soybean by bacterial and fungal isolates.

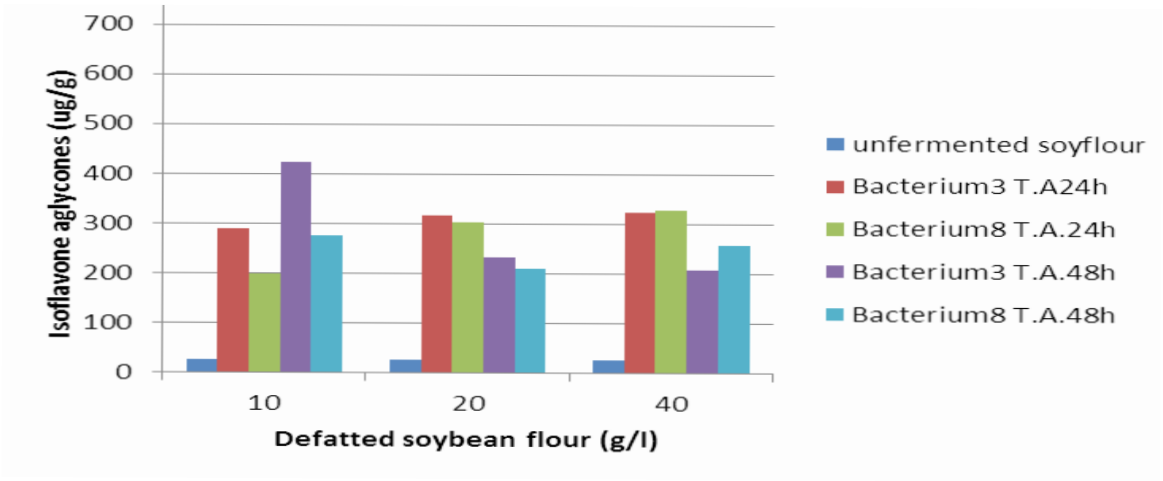

Fig. 3. Changes in isoflavone aglycones content at 24 h and 48 h submerged fermentation of defatted soybean flour by bacterial isolate No.3 and 8.

Identification of the most potent bacterial isolate

The identification of the selected bacterial isolate was done on the basis of morphological and 16S rDNA sequence analysis.

Morphological characterization

On the basis of morphological characteristics, the selected isolate was Gram stain positive (Fig. 4). Additionally, under microscopic examination, the cells appeared rods with rounded ends and the spores were lateral and cylindrical in shape. Cells appeared adherent, finely wrinkled, dull and opaque when cultivated on nutrient agar medium.

Molecular characterization

The genomic DNA of bacteria isolate No. 3 was extracted and 16S rDNA was amplified by PCR. The PCR product was examined by agarose gel electrophoresis. The PCR product was purified and sequencing was done in triplicate in order to get the correct sequence. The nucleotide sequence of 16S rDNA was submitted in GenBank database under the accession number KX712228. The 16S rDNA sequence of the bacterial isolate was aligned with all available 16S rDNA sequences in GenBank database and finally the phylogenetic tree was constructed (Fig.5). The phylogenetic tree indicated that this bacterial isolate belonged to the genus *Bacillus* and the pattern of the tree determined that the strain is closely related to other *B. licheniformis* strains with 99% 16S rDNA similarity.

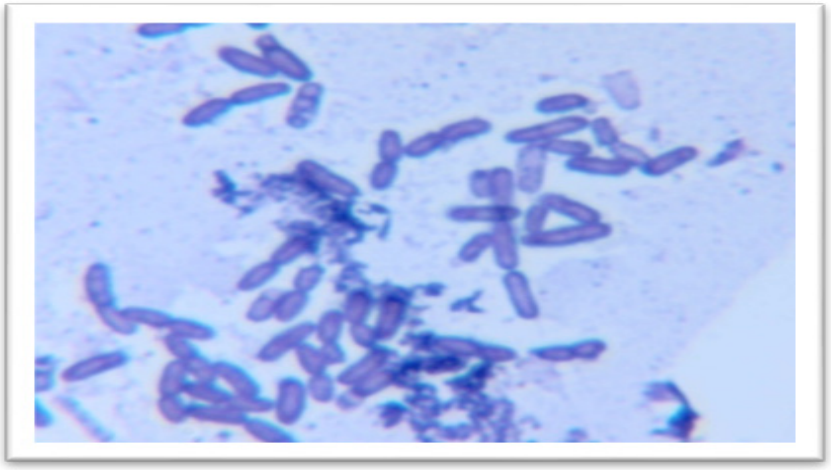

Fig. 4. Gram staining of bacterial isolate No. 3.

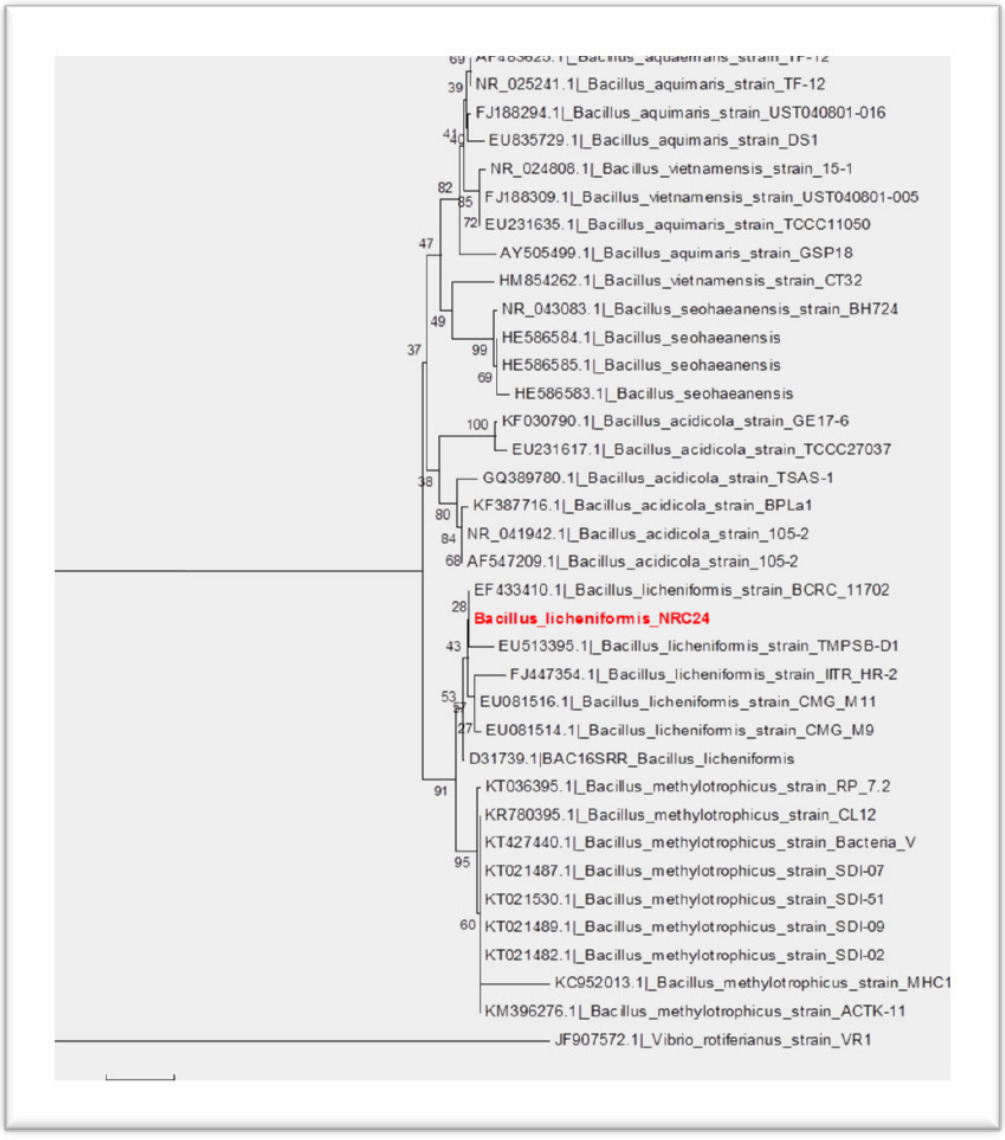

Fig. 5. Phylogentic tree showing the relation of B. licheniformis NRC24 with other species.

Discussion

β-glucosidases are prevalent enzymes that hydrolyze broad glycosides including aryl and alkyl-β-d-glycosides. These enzymes, produced by many microorganisms, may also hydrolyse β-glucosidic linkages of soybean polyphenol β-glucosides mobilizing the polyphenol aglycones (Vong et al., 2016; Berrin et al., 2003 and Wolosowska & Synowiecki, 2004).

Some microbial species were isolated based on the selection of the desired character (Stanbury et al., 1995). The growth of the bacterial and fungal isolates on cellobiose agar or defatted soybean flour agar showed that they were able to utilize cellobiose and soybean flour.

Most of the bacterial isolates efficiently hydrolyzed daidzin and genistin. Even if wide differences in the biotransformation of both daidzin and genistin were found, only one bacterial isolate (isolate No. 6) was incapable to perform the biotransformation of both daidzin and genistin. Variation in individual isoflavone contents as affected by different bacterial strains, *Lactobacillus plantarum B1–6, Streptococcus thermophilus* S10, *Bacillus subtilis* CSY191 were reported by Xiao et al. (2015), Lee et al. (2015) and Shin et al. (2014).

In fermented soybean flour, the isoflavone aglycone, daidzein, was found in larger amounts than that of genistein for almost all bacterial isolates, except isolates No. 3, 4 and 8 compared to unfermented soybean flour. Our results are in agreement with previous work for *Bacillus spp*. (Wei et al., 2008). The selective action of the β-glucosidase synthesized by most of the tested bacterial isolates on daidzin, could explain higher increase in the concentration of the product, daidzein, than that of genistein. That finding was also reported by Shin et al. (2014) and Kuo et al. (2012).

Some tested bacterial isolates did not produce extracellular β-glucosidase as their supernatants did not hydrolyze *p*-PNG, but all bacterial isolates showed positive cell-bound enzyme, which indicates that hydrolytic activity of the tested bacterial isolates mainly functions via cell-bound β-glucosidase activity. These results are similar to those reported by Raimondi et al. (2009), Tsangalis et al. (2002), Kuo et al. (2006) and Otieno & Shah (2007). β-glucosidases produced intracellularly by many microorganisms usually show broad substrate specificity (Wolosowska & Synowiecki, 2004) and their physiological functions vary greatly depending upon their origin and substrate specificity (Berrin et al., 2003).

Among the screened bacterial isolates, the level of DPPH radical-scavenging activity increased from 1.3 to 3.47 fold compared to unfermented soybean flour. The changes in isoflavone aglycones had a great effect on improving DPPH radical-scavenging activities of fermented soybean flour using bacterial isolates. The observed variations in scavenging activities may depend on various factors, including differences in the population of bacteria, strain type and the ratio of aglycones to glucosides. Genistein and daidzein have direct free radical scavenging ability (Lee et al., 2015 and Shon et al., 2007).

The fungal isolates produced more extracellular β-glucosidases than those obtained by bacterial isolates during soybean flour fermentation. These results are in accordance with that reported by Georgetti et al. (2009). That difference could be related to the cellular localization of the synthesized β-glucosidase or to the culture conditions. Bacterial synthesized β-glucosidases can be bound to the cell wall or secreted into the periplasmic space (Pyo et al., 2005). Soybean flour fermented by the tested bacterial isolates resulted in a greater biotransformation of daidzin and genistin into daidzein and genistein than that fermented by the tested fungal isolates. Fungal isolates No. 5, 10, 11 and 13 only had the capability to transfom daidzin into daidzein. Yang et al. (2009) repoted that there are differences in hydrolysis of soybean isoflavones by microbial β-glucosides, which depend on their specificity towards various chemical forms of isoflavones. These results were related to substrate specificity of the produced enzyme that influencing the biotransformation of daidzin and genistin. Similarly, β-glucosidases produced by most our fungal isolates were still not able to hydrolyse β-glycosidic linkages of the isoflavonoids daidzin and genistin.

Although the methanolic extracts prepared from fermented soybean flour using fungal isolates presented higher scavenging activity (increased from 1.39 to 3.69 fold) than that of unfermented extract, they showed poor daidzein and genistein content. Thus, other phenolic compounds may be mobilized by fermentation and probably they are good hydrogen donor to the DPPH radicals as described by Georgetti et al. (2009). Additionally, Fan et al. (2009) mentioned that peptides and

amino acids were liberated during douchi (a traditional fermented soybean product in China) fermentation, and that the antioxidant activity of the douchi depended on the increase in peptides with fermentation, rather than on the increase in isoflavone aglycons. Besides isoflavones and low-molecular-weight peptides, soybean fermentation also produced other materials, such as amino acids and melanoidin, which can also cause changes in the antioxidant activities of fermented foods. Amino acids obtained during soybean fermentation can increase the antioxidant activity of tempeh, another fermented soybean food (Hoppe et al., 1997).

The pH values increased during soybean flour fermentation using bacterial and fungal isolates except for two fungal (*Aspergillus niger*) isolates No. 6 and 8. That rise in pH values was presumably a result of proteolysis and the release of ammonia following the use of amino acids by the fermenting microorganisms. The release of ammonia is responsible for the ammonia-like odor characteristic of most vegetable protein fermentations (Cho et al., 2009; Cho et al., 2011; Kim et al., 2012 and Shin et al., 2014).

Combined results suggested that bacterial isolates No. 3 and 8 synthesized β-glucosidases with higher specificity to hydrolyse daidzin and genistin β-glycosidic bonds than those produced by the other tested isolates. Additionally, their fermented methanolic extracts showed an increase in antioxidant activities. However, bacterial isolate No. 3 showed an efficient potency at different concentrations of soybean flour in transforming daidzin and genistin into their aglycone forms other than the tested bacterial isolate No. 8. As a result, bacterial isolate No. 3 was adopted as the working isolate for soy isoflavone glycosides biotransformation.

On the basis of morphological characteristics and 16S rDNA sequence analysis, the bacterial isolate No.3 was identified as *B. licheniformis NRC24*. The sequences of the current isolate were found to be 99% similar to 16S rDNA sequences of *B. licheniformis* from various sources in GenBank database. It is not only *B. licheniformis* NRC24 that can produce β-glucosidase during soybean fermentation; other microorganisms used for fermented soybeans such as lactic acid bacteria and Bifidobacteria with soymilk (Chun et al., 2007 and 2008), *Actinomucor elegans* with sufu (Yin et al., 2004 and 2005), *Rhizopus* with tempeh (Miura et al., 2002), *Aspergillus* with miso (Yamabe et al., 2007), and *B. subtilis* with natto (Dajanta et al., 2009).

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تقييم إنتاج الجينيستين والدايدزين بواسطة بعض العزلات البكترية والفطرية المحلية

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تم عزل وتنقية ثلاثة وعشرون سلالة بكتيرية و فطريه من بعض الترب الطينية من اماكن مصرية مختلفة وذلك لاختبار قدرتها على تحويل جليكوسيدات الصويا إلى الاجليكونات و النشاط المضاد للأكسدة مقارنة بدقيق الصويا غير المخمر. أظهرت معظم العزلات البكتيرية كميات أعلى من الديدزين منها للجينيستين، وهي منتجات أجليكونية من جليكوسيدات الصويا. بعد تخمير دقيق فول الصويا باستخدام العزلات البكتيرية والفطرية، تباين محتوى الاجليكونات من 0.0 إلى 431.89 ميكروغرام / جم مقارنة بدقيق فول الصويا المعقم غير المخمر. تراوح نشاط إنزيم البيتا-جلوكوزيداز المنتج خارج الخلية من 1.22 إلى 11.56 مللى وحدة / مل و 0.3-534.3 وحدة / مل للعزلات البكتيرية والفطرية، على التوالي، في حين تراوح نشاط إنزيم البيتا-جلوكوزيداز المرتبط بالخلايا البكتيرية من 44.72 إلى 128.89 مللى وحدة / مل. معظم العزلات البكتيرية كانت أكثر كفاءة فى تحويل جلكوسيدات الصويا إلى اجليكونات من العزلات الفطرية. ومن بين العزلات البكتيرية التي تم اختبارها، تم اختيارالعزلة البكترية الأكثر فعالية فى تحويل جلكوسيدات الصويا، وتم تعريفها وفقا للتحليل المورفولوجي وبالإضافة إلى تحليل 16S rDNAوعرفت بأنها *Bacillus licheniformis* NRC24