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Immobilization and Characterization of Genistein Metabolizing and Glucose-tolerant β-Glucosidases from *Penicillium sp*. NRC24

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Abstract

Glucose tolerant β-glucosidases have attracted considerable attention due to their important roles in various biotechnological processes. Penicillium sp. NRC24, a new fungal isolate was used to produce soy glycosides-hydrolyzing β-glucosidase. The ability of β-glucosidase to convert soy glucosides into aglycone forms, as well as its activity on p-NPG, was investigated. Penicillium sp. NRC24 β-glucosidase was partially purified with 8.04-fold and final recovery of 45.7%. This enzyme exhibited a glucose-tolerant activity of 60.4% while the partially purified enzyme showed 85.6% activity at 0.5 M of glucose. Moreover, the produced β-glucosidase could tolerate ethanol up to 60%. β-glucosidase retained all its initial activity at 37 °C in presence of 15-60 % ethanol at pH 5.0. The immobilization and characterization of the produced β-glucosidase are described. The efficacy of β-glucosidase covalently immobilized on alginate/polysorbate complex was 70.1%. The impact of pH, and thermal stability, on the free and immobilized β-glucosidase activities and hydrolysis of soybean extract were evaluated. The optimum pH remained at 5.0 after immobilization, while the optimum temperature leveled up to 50 °C in comparison to the free enzyme, which remained at 37 °C. This is the first report to our knowledge on the use of Penicillium sp. NRC24 for the production, characterization, and immobilization of β-glucosidase, which can hydrolyze genistein and daidzein into their aglycone forms.

Keywords: β-glucosidase; Glucose and ethanol tolerance; Alginate/polysorbate complex; Covalent immobilization, Soy daidzein and genistein

1. Introduction

Soybean is an important source of isoflavones, a group of phytochemicals that have critical activity when used in aglycone form. Soybean-derived chemical compounds have a substantial impact on animal and human health. Daidzein (4′, 7-dihydroxyisoflavone) and Genistein (5, 7, 4′-trihydroxyisoflavone) are essential isoflavones found in products derived from soybeans, and their glycosides (genistin, and daidzin) are widely used **[1].**

Because isoflavones and estrogen have similar structural properties, the former can bind to estrogen receptors such as ER-α and ER-β and simulate estrogenic activities such as agonistic and antagonistic estrogenic actions, antioxidant, anti-inflammatory, and antimicrobial agents **[2]**. Acid, alkaline, and enzymatic hydrolysis methods are commonly used to create physiologically active glyconates from similar glycosides. Although acid hydrolysis has many advantages in terms of cost and process speed, it has a significant disadvantage in terms of target specificity and the formation of undesirable degradation products **[3]**.

From an industrial aspect, there is a need to investigate alternate selection processes for the

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production of aglycones. In this respect, using enzymes to convert isoflavone glycosides into aglycones is seen as a viable alternative. β-Glucosidase (β-D-glucoside glucohydrolase; EC 3.2.1.21) has become increasingly important for such purposes. β-glucosidase has a unique activity and produces a large amount of isoflavone aglycones as a result **[4]**.

Fungi capable of generating β-glucosidases were employed to establish industrial processes for making fermented soy products enriched with isoflavone aglycones. β-glucosidases hydrolyze carbohydrate or non-carbohydrate 1,4-glycosidic bonds to produce non-reducing glucosyl residues **[5]**.

β-glucosidases, an enzyme family found in bacteria, fungi, plants, and animals, can be used in a variety of biotechnological applications, including the enrichment of aromatic substances and flavor in the juice and beverage industries, as well as the textile, food, isoflavone hydrolysis, feed, and paper industries **[6]**.

Under profitable industrial conditions, there is an increasing demand for β-glucosidase synthesis from microbial sources. Because of the high amount of glucose produced by the hydrolysis of the enzymatic biomass, it is critical to use β-glucosidases that can withstand high glucose concentrations **[7]**.

When compared to conventional-based synthesis approaches, enzyme-mediated catalytic activities have demonstrated a wide range of capabilities and advantages in biotechnological and industrial fields **[8]**. Enzymes are frequently crippled and restricted due to limitations such as high cost, no recovery, non-recyclability, short catalytic lifetime, and poor operating stability against pH, temperature, and other factors **[9]**. Because immobilized enzymes on appropriate carrier supports have greater activity and stability than free enzymes, immobilization is a viable strategy for dealing with such problems. Furthermore, the goal of enzyme immobilization is to improve the properties of the enzymes as well as their reusability **[8]**.

Enzymes immobilization on a solid matrix may improve enzyme operational stability **[10]**. Furthermore, immobilization of an enzyme on an inert carrier provides significant cost savings by allowing the enzyme to be reused in multiple reactions and product separation cycles **[10, 11]**. Enzyme immobilization is commonly accomplished through three methods: enzyme entrapment, adsorption on a solid carrier, and ionic or covalent binding **[12, 13].**

Alginate, a natural hydrogel, is an excellent material for enzyme immobilization because it is inexpensive and safe for use in the food and pharmaceutical industries **[14]**. Alginate has been used in immobilization technology, either through entrapment **[15]** or through covalent techniques **[16].** This can be accomplished by activating with polyethyleneimine (PEI) and then glutaraldehyde (GA) **[17]**. Polysorbate was added to the alginate to increase its mechanical strength and immobilization efficiency, resulting in an effective complex of alginate/polysorbate beads.

Polysorbate is a type of emulsifier that is used in the food, pharmaceutical, and cosmetics industries. It is a type of oily liquid derived from oxysorbitan that has been esterified with fatty acids. It contains hydrophilic groups, which can enhance the properties of alginate. Polysorbate comes in several varieties, including polysorbate 20, 40, 60, and 80. Polysorbate 60 can be found in a variety of industries **[18].**

To attain our objective, *Penicillium* sp. was isolated and identified by 18S-RNA and tested for β-glucosidase production. The conditions for fungal isolation on soybean flour medium and production of β-glucosidase; purification of the enzyme, described, immobilized, and employed to produce aglycones from isoflavone glycoside conjugates obtained from soybeans. An effective complex of alginate/polysorbate gel beads for β-glucosidase immobilization has been designed. Catalytic activity maximization was assessed by studying the effect of temperature and pH values. Production and optimization of β-glucosidase were studied; the hydrolysis of soybeans was also studied and characterized using HPLC.

2. Materials and Methods

2.1. Materials:

2.1.1. Microorganisms

In the present work, the fungal isolate was previously isolated from a muddy soil sample in Egypt (El-Shazly et al. 2017). This isolate was purified and assessed for its production of β-glucosidase.

2.1.2. Chemicals

Sodium alginate (Alg) was purchased from Fluka. Polysorbate 60 was obtained from Acros Organics, Belgium. polyethyleneimine (PEI) (50%) and glutaraldehyde (GA) (50%), were purchased from Sigma-Aldrich. Defatted soybean flour was purchased from the soybean unit, Agricultural Research Centre, Egypt. Potato Dextrose Agar medium was imported from Laboratories Conda S.A., Madrid, Spain. Glucose, ethanol, daidzein, genistein, and *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG) were purchased from Sigma Aldrich Company (St. Louis, MO, USA). High-performance liquid chromatography (HPLC) grade methanol was obtained from Fisher Scientific (Hanover Park,

IL).Other chemicals were of Analar or equivalent quality. Inotech Encapsulator, model IE-50, was purchased from Inotech Encapsulator in Switzerland

2.2. Methods

2.2.1. Defatting of soybean flour

Soybean flour (6% fat) was mixed with n-hexane at a (1: 10 (w/v)) ratio for 2 h at 28 °C by incitement of constant rotary agitation and then it was decanted. A rotary evaporator was used to evaporate the remaining hexane (Hei-VAP Advantage, Heidolph Instruments GmbH, Germany) **[19]**.

2.2.2. Isolation and identification of the fungal isolate

The fungal isolate was previously isolated and screened for its ability to transform soy glucosides into their aglycone form by El-Shazly et al. (2017) **[20]**.

One gram of soil sample was vortexed for 1 min in 10 mL of sterilized water. Each sample was serially diluted (till $x10^{-6}$) and 100 µL of each dilution was streaked medium containing 25 g/L of agar was prepared and autoclaved separately with; 10 g/L defatted soybean flour as a selective substrate for screening of soy hydrolyzing glucosidase. pH was adjusted to 5 by using 0.1N HCl. Fungal colonies were then purified using PDA medium **[21, 22]** Then incubated for 96 h at 28 ± 2 °C. The pure fungal culture was preserved in 20% (v/v) glycerol vials at -80 °C.

2.2.3. Molecular Identification of fungal isolate

2.2.3.1. DNA extraction and PCR amplification of 18S rDNA

QIAamp mini kit (Qiagen) has been used for DNA extraction according to the manufacturer's instructions. After elution in 50 µl of AE buffer, DNA was stored at -80 °C until use. PCR was performed in a 50 µl reaction volume using 0.2 mM dNTPs (Invitrogen), 0.4 M primers, 5 µl 10x PCR buffer (Promega), 1.5 mM $MgCl_2$, and 1 U Taq DNA polymerase (Promega). NS1 (5'-GTAGTCATATGCTTGTCTC -3') and NS24 (5'-AAACCTTGTTACGACTTTTA -3') universal primer pairs were used to amplify bacterial 18S rDNA, and PCR amplification was carried out in 30 cycles as follows: denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min. UV light was used to separate and visualize amplification products using 1 percent agarose gel electrophoresis **[23]**.

2.2.3.2. Sequencing of 18S rDNA

The separation of PCR products was achieved using 1% (w/v) agarose gel electrophoresis. Following that, amplicons of various sizes were extracted from the gel and purified with a Qiagen gel extraction kit (Qiagen, Hilden, Germany). Products of purified PCR were used straightforwardly for cycle sequencing reactions using the BigDyeR Terminator (V.3.1) Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) as said by the manufacturer's instructions, and they were amplified for 26 cycles at 95 °C for 30 sec, 50 °C for 15 sec, and 60 °C for 4 min. Exclusion chromatography in CentriSep columns was used to purify the reaction product (Princeton Separations, Adelphia, NJ). Macrogen Inc. Korea sequenced the recovered materials using an ABI 3730XLDNA analyzer (Applied Biosystems, Foster City, CA). The SeqMan DNA Lasergene software (V.7) was used to generate the sequences (Dnastar, Madison, WI).

2.2.3.3. Phylogenetic analysis

The neighbor-joining algorithm and the Kimura 2-parameter model were used for phylogenetic analysis. The bootstrap method with 1000 replications was used to estimate the reliability of phylogenetic inference at each branch node, and evolutionary analysis was performed in MEGA (V.6).

2.2.4. Production of β-glucosidase by *Penicillium* sp. NRC24 through submerged fermentation

The fungal strain was grown in PDA slants at 28 ± 2 °C for 96 h then stored at 4 °C. The strain was grown in defatted soybean flour medium, pH 5.0, at 28 ± 2 °C for submerged fermentation.

To inoculate the fermentation medium, spore suspension (0.5%) was used. In 250 mL Erlenmeyer flasks, defatted soybean flour (0.5 g) was mixed with 50 mL distilled water and sterilized at 121 °C for 15 min. After mixing, the inoculated flasks were incubated under shaking culture conditions of 200 rpm at 28 ±2 °C for 96 h. An aliquot was analyzed for assessment of extracellular β-glucosidase activity and *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG) was used as synthetic substrate; all the experiments were done in triplicates**.**

2.2.5. Assessment of extracellular β-glucosidase activity

The β-glucosidase activity was determined by using *p*-nitrophenyl-β-D-glucopyranoside as a substrate. The reaction mixture consisted of 0.5 mL of 0.1% *p*-nitrophenyl-β-D-glucopyranoside in citrate phosphate buffer (0.05 M, pH 5), and 1 mL of the culture supernatant (as a source of β-glucosidase enzyme). To stop the reaction, 0.5 mL of sodium carbonate (1M) was added after 30 min of incubation at 37 °C. The aliquots were centrifuged at 15000 rpm for 5 min using an Eppendorf centrifuge (model 5415D; Eppendorf, Hamburg, Germany). These enzymatic conditions were used as a control in subsequent assays. The amount of released *p*-nitrophenol was measured using a spectrophotometer (SP-2000UV, Spectra, USA) at a wavelength of 401 nm. Enzyme activity unit was defined as the amount

of enzyme that liberates 1 mM *p*-nitrophenol per min under assay conditions **[24]**.

2.2.6. Partial purification of β-glucosidase from *Penicillium* sp. NRC24

In this experiment, the culture supernatant was subjected to centrifugation to get rid of the cells and residual wastes. A cold ethanol solution was added to the crude enzyme in an ice bath during stirring until the concentration of ethanol reached 20 % (v/v), and the precipitate was then collected by cooling centrifugation (4 °C) after 10 min at 4000 rpm. The precipitated protein was resuspended in 50 mM citrate phosphate buffer of pH 5. The procedure was repeated after another aliquot of fresh cold ethanol was gradually added to the supernatant fluid until the required ethanol concentration was reached. Thus, different fractions were obtained in ethanol concentrations of 40, 60, and 80 % (v/v). The enzymatic activity was assayed at pH 5 and 37°C as a control.

2.2.7. The effect of different glucose concentrations on the activity of β-glucosidase enzyme:

To determine the extent of glucose inhibition, 0.5 mL of different concentrations of glucose solution (0.1, 0.2, 0.3, 0.4, 0.5 M) were mixed with 0.5 mL of the substrate (*p*-NPG) and 0.5 mL of culture filtrate for the measurement of β-glucosidase activity in crude enzyme samples. The reaction conditions were maintained constant at pH 5 and a temperature of 37 °C.

After 30 min, the reaction was stopped with 0.5 mL of sodium carbonate (1M), and the concentration of *p*-nitrophenol produced was estimated at 401 nm. The relative activity is defined as the enzymatic activity at each glucose concentration in comparison to the activity without glucose as a control **[25]**.

2.2.8. Ethanol tolerance

To test the extent of ethanol tolerance, different ethanol concentrations (15, 30, and 60%) were mixed with 0.5 mL of culture supernatant and 0.5 mL of substrate (*p*-NPG) and used to measure the activity of the β-glucosidase enzyme. The pH and temperature of the reaction were kept constant at 5 and 37 °C, respectively. After 30 min, the reaction was stopped with 0.5 mL of sodium carbonate (1M), and the concentration of *p*-nitrophenol produced was estimated at 401 nm. The relative activity is defined as the enzymatic activity at each ethanol concentration in comparison to the activity without ethanol as a control.

2.2.9. Carriers' preparation for covalent immobilization

2.2.9.1. Alginate gel beads

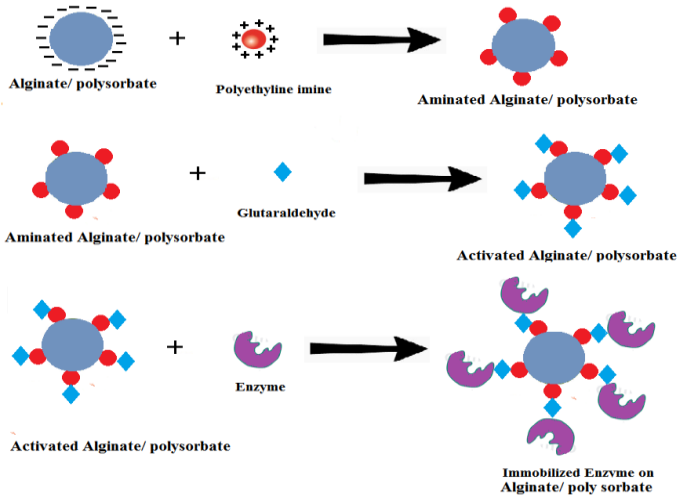
Using an overhead mechanical stirrer, sodium alginate (Alg) (2%) was dissolved in distilled water until complete dissolution. Innotech Encapsulator (Innotech Co. in Switzerland) was used to drop alginate polymer solution through a 300 mm nozzle in 3% $CaCl_2$ as a hardening solution and soaked in it for 3 h **[26]**.

2.2.9.2. Alginate/polysorbate gel beads

Alginate/polysorbate gel beads can be prepared by dissolving alginate (Alg) and polysorbate (PS) in distilled water in a 1:1 ratio with a final concentration of 2% using an overhead mechanical stirrer. The complex was then dropped in 3% $CaCl_2$ through a 300 mm nozzle by using Innotech Encapsulator.

2.2.9.3. Activation of gel beads

The activation of gel beads for covalent immobilization was done by using polyethyleneimine (PEI) and glutaraldehyde (GA). First, gel beads were soaked in 4% PEI at 9.5 pH for 3 h; then distilled water was used for washing it well to remove unreacted PEI. The aminated gel beads were then immersed for 3 h in a 2.5% GA solution. The gel beads were then washed three times to remove the unreacted GA, as shown in Scheme 1. Finally, the activated gel beads were ready to be used in subsequent enzyme immobilization steps **[27, 28]**.



Scheme 1 activation scheme of alginate/polysorbate complex and immobilization processes.

2.2.9.4. Immobilization of β-glucosidase

The partially purified β-glucosidase was immobilized covalently with both carriers (alginate and alginate/Polysorbate) beads. An appropriate amount of the enzyme (236 mU/ml) was added to pre-activated gel beads. The gel beads and enzyme were incubated for 16 h at room temperature with gentle stirring using a roller mixer. Finally, the

immobilized gel beads were washed several times with buffer solution and stored until they were needed again **[12].** The enzymatic activity was assayed at pH 5 and 37°C. The yield of immobilization (Y_e) was measured through equation (1)

$Y_e = E_i/E_f * 100\%$ equation (1)

E_i and E_f are the activity of the immobilized enzyme and the total activity of free enzyme added respectively.

2.2.10. Elucidation of the modified gel beads using FT-IR

Infra-red transmission spectra of carriers (alginate/polysorbate), (alginate/polysorbate-PEI), (alginate/polysorbate - PEI – GA), and (alginate/polysorbate – PEI – GA – Enzyme) were described by using FT-IR spectrophotometer (FT-IR-800, Shimadzu, Japan). This test is used to prove the modification of gel beads by the presence of new functional groups. These four formulas were prepared by air drying and mixing with KBr, separately. It was then ground into fine powder using a mortar and compressed into discs using a hydraulic press at 10,000 psi. Scan it in the wavelength range 400 – 4000 cm^{-1} at ambient temperature **[27].**

2.2.11. Reusability studies

The gel beads were assayed as described in the section "enzyme activity assay" to investigate the reusability of β-glucosidase enzyme covalently immobilized onto activated alginate/polysorbate gel beads. The same gel beads were then thoroughly washed with a buffer solution and reused. This procedure was carried out 13 times. The relative activities were expressed as a percentage of the initial activity, which was assumed to be 100% **[29].** The enzymatic activity was assayed at pH 5 and 37°C.

2.2.12. Factors affecting the activity β-glucosidase enzyme in both forms of the enzyme (the free and the immobilized forms)

2.2.12.1. Effect of different temperatures and thermal stability

The purpose of this experiment was to investigate the effect of temperature on both free and immobilized β-glucosidase enzyme activity. The enzyme assay was performed at five different temperatures (25, 37, 50, 65, and 75 °C). At each of the five temperatures, 1 mL of crude enzyme or an equivalent amount of immobilized enzyme was incubated with 0.5 mL of substrate (*p*-NPG) (30 min). To stop the reaction, 0.5 mL of sodium carbonate (1M) was added. At 401 nm, the absorbance was measured. For the thermal stability test, 1 mL of crude enzyme or an equivalent amount of immobilized enzyme was incubated at 3 different temperatures (50, 60, and 70 °C) for 1h., followed by the addition of *p*-NPG (0.5 mL), and incubated for 30 min at 37 °C. 0.5 mL of 1M sodium carbonate has been added to stop the reaction. The absorbance was measured at 401 nm **[30].** The enzymatic activity was measured at pH 5 and 37°C as a 100% control.

2.2.12.2. Effect of initial pH value

The experiment was carried out to determine the most optimum pH value for enzyme activity in both forms (free and immobilized). The enzyme assay pH was adjusted to 3, 5, 7, and 9. After 30 min at 37 °C, 0.5 mL of cold sodium carbonate (1M) was added to stop the reaction, and the absorbance at 401 nm was measured **[31]**. The enzymatic activity was measured at pH 5 and 37°C as a 100% control.

2.2.13. Hydrolysis of soybean flour using free and immobilized β-glucosidase enzyme

This experiment was carried out to investigate the effect of different forms of the enzyme on the hydrolysis of soybean flour. 40 mg of defatted soybean flour suspended in 400 µl citrate phosphate buffer (pH 5), was hydrolyzed with 183 mU/ml of β-glucosidase enzyme or an equal amount of immobilized one and incubated at 50 °C for 10 min before being stopped by boiling for 5 min. Lyophilization was performed on the hydrolyzed samples.

Isoflavones were extracted from the lyophilized samples using methanol 80% in a ratio (1:5 (w/v)) and stirring at room temperature for 2 h. The mixtures were centrifuged for 5 min at 1500 rpm. The supernatants were filtered with a 0.45 µm filter before being subjected to additional isoflavone analysis via HPLC. The enzyme-free reaction mixture was used to express the controlled samples **[32].**

2.2.14. HPLC analysis of isoflavones

HPLC isocratic elution was used to estimate concentrations of daidzein and genistein. The mobile phase contained 100% methanol, 10 mM/L ammonium acetate (60:40), and 1 mL trifluoroacetic acid per liter of solvent mixture. The flow rate was set to 1 mL/min. During the 30-minute run time, injection volumes of isoflavone standards (daidzein and genistein) and samples were kept constant at 100 µl. Single standards were prepared for peak identification. Isoflavone concentrations were calculated on a dry basis (g/g soybean flour) **[24]**.

2.2.15. Calibration curves of isoflavone standard

Soy isoflavone aglycones were quantified by HPLC using a diode array detector (HPLC-DAD) to analyze each methanolic standard of daidzein and genistein. Standard solutions of daidzein (0.25, 0.5, 0.75, 1.25, 2.5, and 5 g/mL) and genistein (0.5, 1, 2, 3, 4, and 5 g/mL) were used. The areas obtained were

matched with their corresponding concentrations. The concentrations of daidzein and genistein in soybean flour were calculated using area interpolation and expressed in g of each isoflavone aglycone per gram of dry-weight soybean flour. Isoflavone peaks were detected by comparing retention times (RT) and confirmed by comparing UV spectra to those of reference materials **[33]**.

3. Results and discussion

3.1. Isolation and identification of β-glucosidase-producing strain

A wild fungal strain was previously isolated on a medium containing defatted soybean flour only as a substrate for β-glucosidase. On PDA media, the strain colonies formed green and dense felt surface colonies, and also the morphological characteristics were similar to those of *Penicillium* sp. **(Figure 1).**

Figure 1 *Penicillium* sp. NRC-24 culture cultivated for 4 days on potato dextrose agar medium.

The 18S rRNA region was amplified by PCR, yielding a 1,678-bp sequence that was submitted to GenBank with accession number MZ913018.1. The partial DNA sequence matched the 18S rRNA sequences of other *Penicillium* sp. 99.7% of the time in BLAST search results. The phylogenetic analyses were determined using neighbor-joining methods and displayed as a phylogenetic tree **(Figure 2)**.

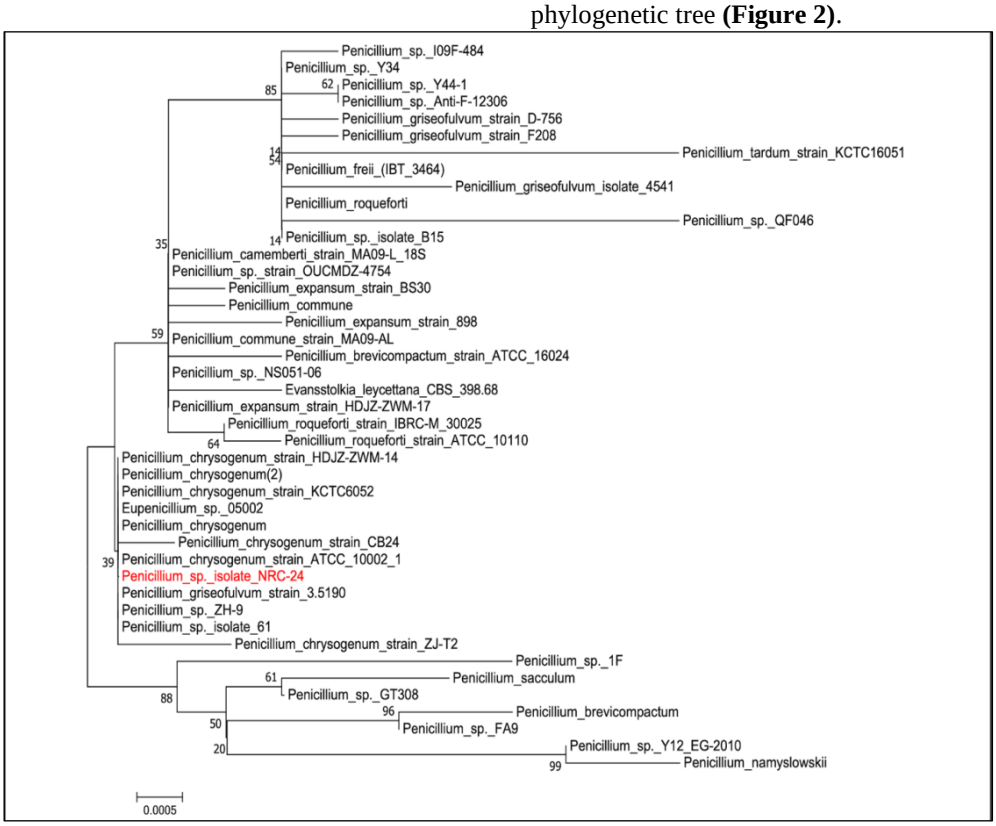


Figure 2 Phylogenetic tree of *Penicillium* sp. NRC24 with other species.

3.2. Production of β-glucosidase:

β-Glucosidase is a worldwide enzyme that is found in all living things, including bacteria, fungi, plants, as well as animals. In flasks containing defatted soybean flour, the fungus *Penicillium* sp. NRC24 was assessed for producing extracellular β-glucosidase. This activity under submerged fermentation was 227.5±10 mU/ml. **Handa *et al.*, (2014)** grew *Aspergillus oryzae* IOC 3999/1998 or *Monascus purpureus* NRRL 1992 on defatted soybean flour (DSF) to improve β-glucosidase production and convert glycosidic isoflavones into aglycones **[19]**. **El-Shazly *et al.*, (2017)** reported that twenty-three various bacterial and fungal isolates were grown on defatted soybean flour and tested for their ability to convert soy glycosides to aglycone forms. The activity of extracellular β-glucosidase was found to range from 1.2 to 11.5 mU/mL. While it was 0.3-534.3 mU/mL for bacterial and fungal isolates, respectively, whereas, β-glucosidase bound to bacterial cell- activity varying from 44.7 to 128.9 mU/mL **[20]**. **Sun *et al.*, (2021)** found that the production of a β-glucosidase enzyme (113 mU/mL) by fermentation of *Trichoderma reesei* S12 from mangrove soil on cellulose as a substrate **[34]**.

3.3. Partial purification of β-glucosidase from *Penicillium* sp. NRC24:

To investigate the characteristics of the glucose-tolerant β-glucosidase, the lyophilized culture filtrate of *Penicillium* sp. NRC-24 grown on soybean flour medium (1000 ml) was precipitated using chilled ethanol. The effect of ethanol concentration from 20 to 80% (v/v) indicated 40–60% to give a precipitate that showed 8.04-fold purity of β-glucosidase with 6.58% recovery of the enzyme (Table1). The precipitate was redissolved in 5 ml of 0.05 M citrate phosphate buffer (pH 5.0). The concentrated sample, which contained 6.58% of the initial activity, was immobilized. Kim *et al.* (2021) partially purified β-glucosidase using ammonium sulphate (10-80%) with 1.86-purification fold **[35]**. Asati *et al.* (2019) used ammonium sulphate to partially purify β-glucosidase with a purification fold of 0.99 **[36]. Singhvi and Zinjardeam (2020)** used ion exchange and gel filtration chromatography to purify β-glucosidase, resulting in a 44.4-fold purification with a 39.7% recovery **[1]. Das *et al.* (2013)** stated that the enzyme purification by saturation with 80% ammonium sulphate yielded a 12.8-fold purification of extracellular β-glucosidase with a recovery of 21.0 % **[12]. Maitan-Alfenas *et al.* (2014)** also purified the enzyme using two methods: anion exchange with DEAE-Sepharose and gel filtration with Sephacryl S-300 **[32]**. Also, **Mahapatra & Manian (2022)** purified the enzyme by two methods, ammonium sulfate precipitation and gel filtration by Sephadex G-100 **[37].** In addition, **Neculai *et al.*, (2018)** purified the enzyme with 50% acetone which resulted in 13–fold purification **[38].**

Table1 Partial purification of *Penicillium* sp. NRC24 β-glucosidase. All measurements were accomplished in triplicate (n 3), and data obtained were expressed as mean standard deviation (± SD).

Ethanol conc. %	Total protein content of fraction (mg)	Recovered protein (%)	Total activity of fraction (mU)	Recovered activity (%)	Specific activity (mU/mg protein)	Purification fold
Culture filtrate	21.98	100	27777.78 ±916	100	1263.78	1
0-20	12.21	55.55	6438.27 ±166	23.18	527.29	0.42
20-40	2.65	12.06	3421.296 ±32	12.32	1291.06	1.02
40-60	0.18	0.82	1828.70 ±24	6.58	10159.44	8.04
60-80	2.18	9.92	1018.52 ±8	4.49	467.21	0.37
Total	17.22	78.34	12706.79	45.74	12445.01	9.85

3.4. Glucose and ethanol tolerance

The inhibitory effect of glucose and ethanol on the produced extracellular β-glucosidase activity was evaluated. As shown in Fig. 3, the enzyme could tolerate different concentrations of glucose ranging from 0.1 to 0.5 M. At the highest concentration

tested, 0.5 M glucose, the enzyme-maintained 60.4 percent of its initial activity. The glucose-tolerance character of the partial-purified enzyme, fractions 20%, 40%, and 60% were also tested in the presence of 0.1–0.5 M glucose **(Figure 3).** Fraction 60 showed the maximum purification fold (8.04) so this fraction retained the highest concentration of the purified enzyme demonstrated the highest glucose tolerance characteristic. At the maximum concentration tested (0.5 M glucose) the enzyme retained 28.3, 38.29, and 83.2 % activity, for fractions, 20, 40, and 60 % respectively. The results **(Figure 4)** also indicated an increase in enzyme activity of approximately 25–45% was observed after incubation in the presence of different ethanol concentrations 0, 15, 30, and 60 at 37 °C and pH 5.0. It also was found that no inhibition of enzyme activity (it retained 100% of its initial activity) was observed after the enzyme had been incubated with 15% ethanol at 65 °C for 30 min.

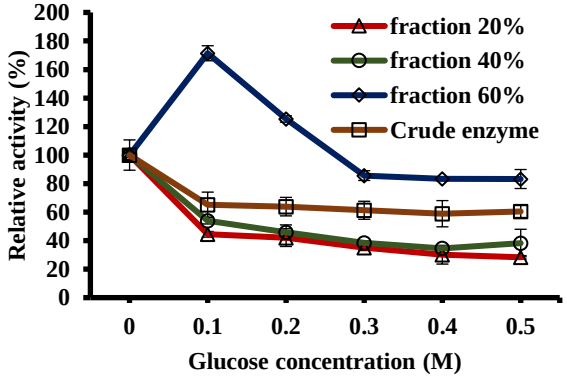


Figure 3 The glucose concentration effect on extracellular β-glucosidase activity, both crude and partially purified forms. All measurements were accomplished in triplicate (n 3), and data obtained were expressed as mean standard deviation (± SD).

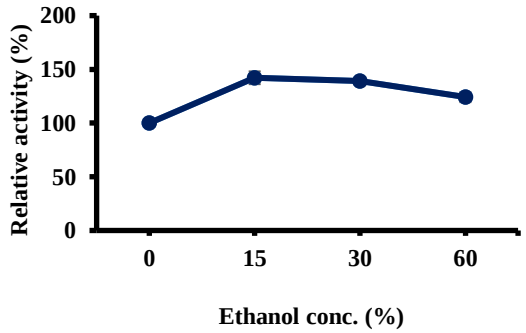


Figure 4 The effect of ethanol concentration on the crude extracellular β-glucosidase activity at 37 °C and pH 5.0. All measurements were accomplished in triplicate (n 3), and data obtained were expressed as mean standard deviation (± SD).

3.5. Immobilization of β-glucosidase on different carriers

The partially purified β-glucosidase was immobilized onto Alginate and alginate/polysorbate complex via the covalent immobilization method. As shown in Fig. 5, the immobilization efficacy increased in the case of alginate/polysorbate complex as it was about 70.1% while it was 68.2 % in the case of alginate. This may have occurred as this complex contains more hydroxyl groups than alginate alone. These groups increase the amount of PEI reacted with the gel beads which leads to more enzyme particles immobilized on the surface of the alginate/polysorbate complex. Previously, many studies recorded the enzyme immobilization with different methods due to this process being cheap and enable the enzyme to be reused numerous times. The enzyme was immobilized on glutaraldehyde cross-linked commercial gelatin by **Nishida *et al.*, (2018) [39]**. **Figueira *et al.*, (2011)** investigated the immobilization of a partially purified *Aspergillus* sp. β-glucosidase on a variety of substrates**.** To improve the biocatalytic performance of *Fusarium solani* β-glucosidase (FBgl1) **[40]**, **Boudabbousa *et al.*, (2020)** immobilized it on chitosan **[41]**. *Aspergillus fumigatus* ABK9 β-glucosidase was purified from a pre-optimized solid-state fermentation medium and then entrapped in 4% alginate beads, according to **Das et al., (2015) [12]**. According to **Nishida *et al.*, (2018)** the immobilized enzyme was more thermally stable and less susceptible to glucose inhibition than the free form, and this result supports our findings **[39]**. We suggest that the cause of these beneficial characteristics was due to the immobilization process resulted in covalent binding between both the carrier and the enzyme which was difficult to break so the immobilized enzyme tolerates more severe conditions as high temperature, alkaline and acidic pH, and thermal stability.

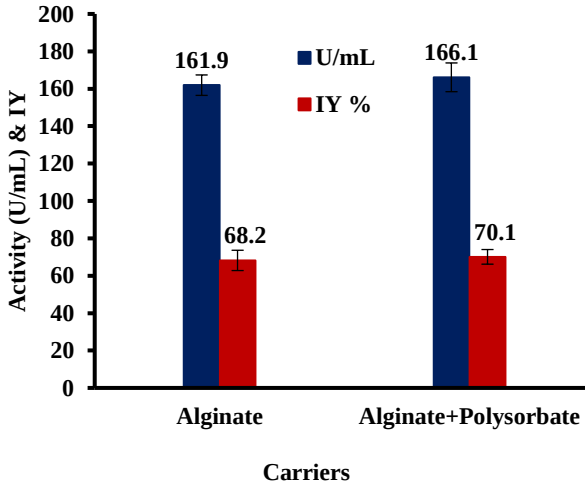


Figure 5 Activity (U/mL) and Immobilization yield (IY) of β-glucosidase immobilized on alginate and alginate/polysorbate complex. All measurements were accomplished in triplicate (n 3), and data obtained were expressed as mean standard deviation (± SD).

3.6. Elucidation of the modified gel beads using Fourier Transform Infra-Red (FT-IR)

FT-IR spectroscopic analysis of the four formulas, (alginate/polysorbate), (alginate/polysorbate - PEI), (alginate/polysorbate - PEI – GA), (alginate/polysorbate – PEI – GA – Enzyme), were demonstrated in figure (6). In this figure, as in curve (Alg/Ps) that is related to alginate/polysorbate mixture, it shows the characteristic band at 3454 cm^{-1} that refers to OH groups, and two bands at 2928 cm^{-1} and 2854 cm^{-1} that refer to C-H groups of alginate and polysorbate, also band at 1741 cm^{-1} that corresponding to C=O group of polysorbate. Curve (Alg/Ps/PE) that is related to aminated gel beads shows a broad band at 3272 – 3570 cm^{-1} that refers to the amine group (NH_2) that is present on the surface of gel beads and also there is a new band at 1560 cm^{-1} that refers to CN groups resulting from the interaction of the carbonyl groups present in the mixture with the amine groups present in polyethylene amine. While in the curve (Alg/Ps/PE/GA) that is related to activated gel beads, it shows the band of amine at 3197- 3544 cm^{-1} and a band at 1590 $cm^{-1}$for CN groups resulting from the interaction of the carbonyl groups in glutaraldehyde and the amine groups on the surface of gel beads, and a band at a wavelength of 1771 cm^{-1} which is specific to the terminal carbonyl groups present in the glutaraldehyde present on the surface of the gel beads. On the other hand, when the activated gel beads reacted with the enzyme, the amine range 3195-3655 cm^{-1} became wider due to numerous amine groups present in the enzyme as shown in the curve (Alg/Ps/PE/GA/En) **[27, 42]**.

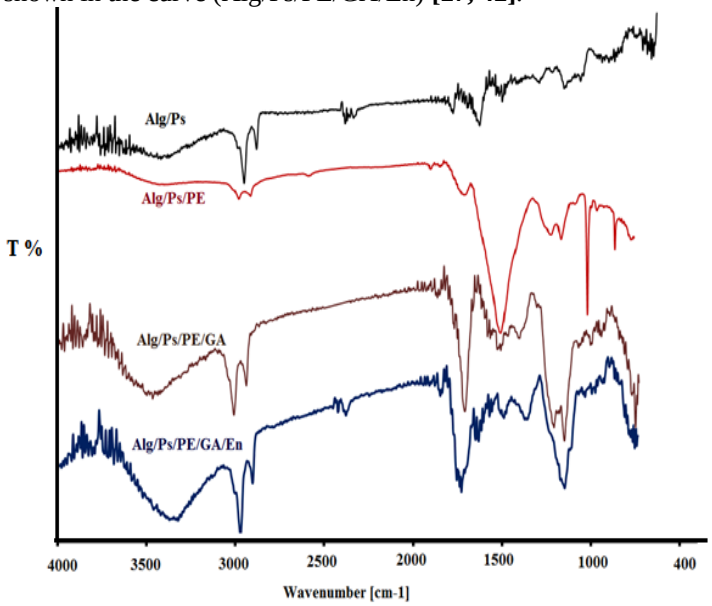


Figure 6 FT-IR of Alg/polysorbate beads (Alg/Ps). Alg/polysorbate beads treated with polyethylenimine (Alg/Ps/PE). Alg/polysorbate beads treated with polyethylenimine followed by glutaraldehyde (Alg/Ps/PE/GA). Treated Alg/polysorbate beads with the β-glucosidase (Alg/Ps/PE/GA/En).

3.7. Reusability studies

The reusability of immobilized enzymes is very important because of the economics of better processes. After thirteen consecutive cycles, β-glucosidase that immobilized on alginate/polysorbate complex show an increase in activity to more than 164 % of its initial activity while in case of alginate it was only 56% (Fig. 7). The reason for that increase in case of the alginate/polysorbate complex could be regarded to: first, the enzyme was immobilized via a covalent bond, so there is no expectation of activity leakage; alternatively, the immobilized β-glucosidase may require a relaxation period in contact with the substrate before reaching its maximum activity. On the other hand, in case of alginate beads (control beads), the activity decreased by time due to the weak interaction between β-glucosidase and the gel beads **[43]**. Many studies investigated the reusability due to it is a very important process for commercial applications. Immobilized β-glucosidase was reused for 10 cycles **[39]**; 12 cycles **[41]**; 9 cycles **[12]**.

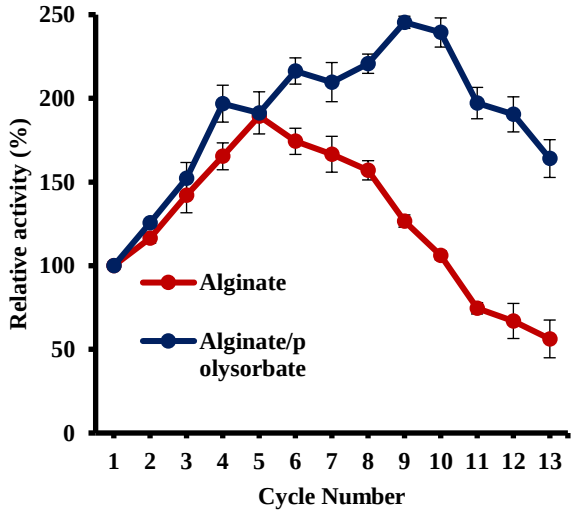


Figure 7 The reusability of immobilized β-glucosidase on alginate and alginate/polysorbate complex under assay conditions of pH 5 and 37°C. All measurements were accomplished in triplicate (n 3), and data obtained were expressed as mean standard deviation (± SD).

3.8. Optimal temperature and thermal stability of free and immobilized β-glucosidase

One of the most important goals of enzyme immobilization is to improve enzyme resistance to various tempering forces. Temperature optimization of free and immobilized β-glucosidase on the alginate/polysorbate complex was accomplished by performing enzyme activity at various temperatures ranging from 25 to 75 °C (Fig. 8a). The thermal properties of the free and immobilized enzymes differed. After immobilization, there was a major shift in enzyme optimized reaction temperature from 37 to 50 °C, as well as an increase in enzyme relative activity at high temperatures. At 75 °C, free β-glucosidase retained approximately 24.2 % of its relative activity, while immobilized β-glucosidase retained 38.6%. Thermal stability character for free and immobilized β-glucosidase was investigated after incubation at temperatures ranging from 50 to 70 °C for 1 h. As demonstrated in (Fig. 8b). In accordance, **Das *et al.*, (2015)** immobilized β-glucosidase and discovered that the immobilization process, after 30 days, increased the stability of storage by 80% and also glucose tolerance **[12]**. According to **Hu *et al.*, (2018)** after immobilization, there is an increase in the optimum temperature for β-glucosidase by 5 °C **[31]**. **Das *et al.*, (2015)** indicated increased thermal stability of the immobilized enzyme **[12].** Also, table 2 summarizes the comparison of optimum temperature of the isolated enzyme with previously characterized enzymes, other reported studies of optimal temperature and thermal stability of free and immobilized β-glucosidase are summarized in Table (2).Table 2 comparison of optimum temperature of *Penicillium* sp. NRC24 β-glucosidase with previously characterized enzymes**.**

Optimum temperature of Free enzyme	Optimum temperature of Immobilized enzyme	Microorganism	Reference
37	50	*Penicillium* sp. NRC24	**This study**
30	40	*Aspergillus awamori*	**[39]**
60	65	*Aspergillus sp*	**[40]**
65	75	*Thermoascus aurantiacus*	**[31]**
55	70	*Agrocybe aegirit*	**[9]**
50	60	*Aspergillus fumigatus*	**[12]**

3.9. Optimal pH of free and immobilized β-glucosidase

The optimal pH was conserved after immobilization on the alginate/polysorbate complex. It is worth noting that the free and immobilized enzyme tolerates a broader range of pH values ranging from 3 to 9 (Figure **8c)**. The free and immobilized enzyme retained 61.8 % and 62 % of its original activity in pH 3, respectively. Moreover, the free and immobilized enzyme was still active in extreme pH value 9, showing 60.6 % and 53.6 % of its original activity.

The pH/activity profile was unaffected by immobilization, however the temperature/activity profile was enhanced after immobilization. Because of the immobilization, thermal stability was improved. When compared to free-glucosidase, the immobilized enzyme demonstrated a shift in optimal temperature, preservation of the optimum pH with strong residual activity at severe acidic pH, and

improved thermal stability **[9, 42].** This may be due to the immobilization process (covalent binding) done between the carrier and the enzyme which was difficulty broken, so the immobilized enzyme tolerates more severe conditions as high temperature, alkaline and acidic pH, and thermal stability, and also the carrier protects the active site of the enzyme.

Table 3 summarizes the comparison of optimum pH of the isolated enzyme with previously characterized enzymes**.** Other reported studies of Optimal temperature and thermal stability of free and immobilized β-glucosidase are summarized in Table (3).

Table 3 comparison of optimum pH of *Penicillium* sp. NRC24 β-glucosidase with previously characterized enzymes**.**

Optimum pH of Free enzyme	Optimum pH of Immobilized enzyme	Microorganism	Reference
5	5	*Penicillium* sp. NRC24	**This study**
4.5	4.5	*Aspergillus sp*	**[40]**
5	5	*Thermoascus aurantiacus*	**[31]**
6	6	*Agrocybe aegirit*	**[9]**
6	5	*Aspergillus fumigatus*	**[12]**

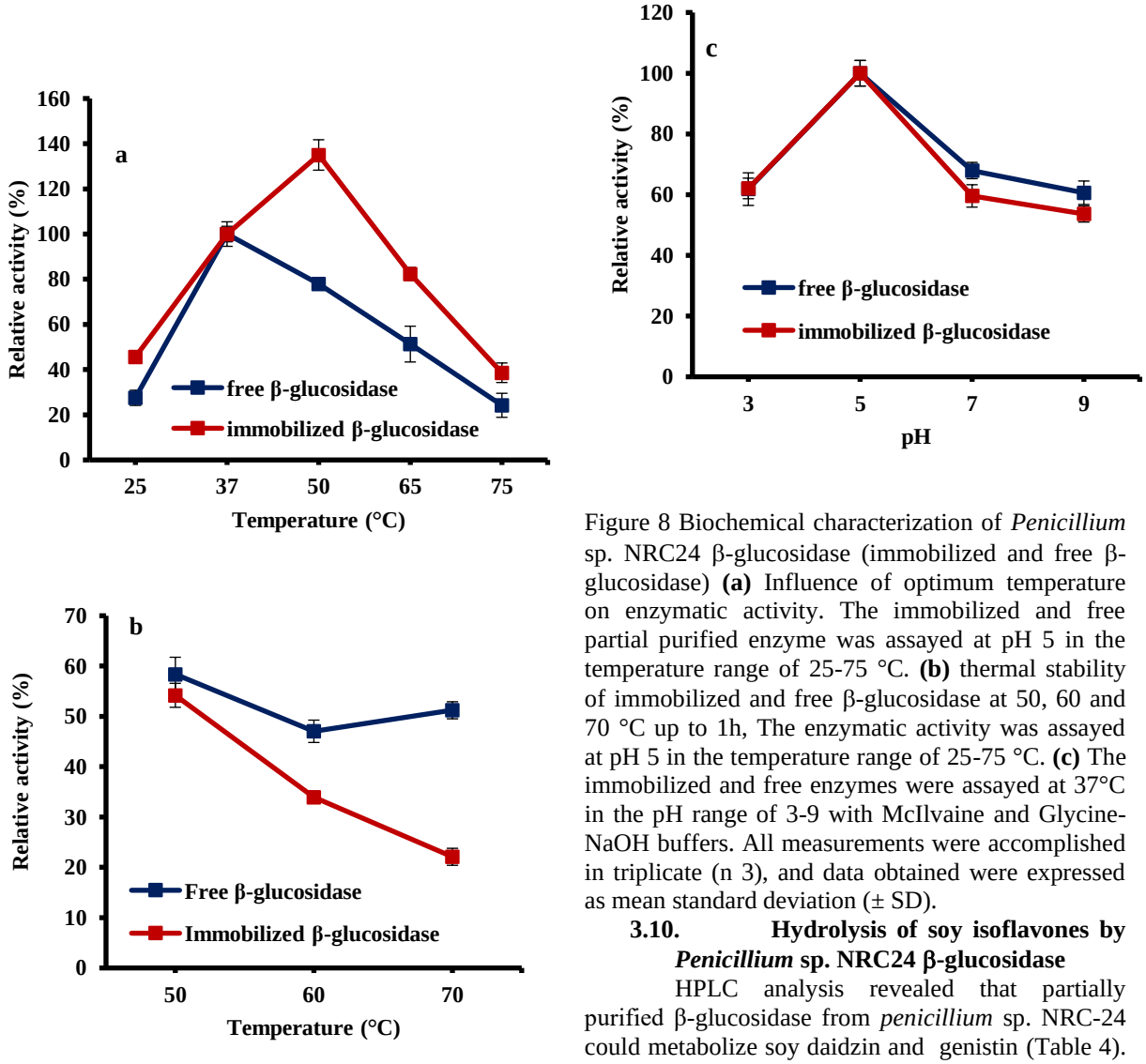


Figure 8 Biochemical characterization of *Penicillium* sp. NRC24 β-glucosidase (immobilized and free β-glucosidase) **(a)** Influence of optimum temperature on enzymatic activity. The immobilized and free partial purified enzyme was assayed at pH 5 in the temperature range of 25-75 °C. **(b)** thermal stability of immobilized and free β-glucosidase at 50, 60 and 70 °C up to 1h, The enzymatic activity was assayed at pH 5 in the temperature range of 25-75 °C. **(c)** The immobilized and free enzymes were assayed at 37°C in the pH range of 3-9 with McIlvaine and Glycine-NaOH buffers. All measurements were accomplished in triplicate (n 3), and data obtained were expressed as mean standard deviation (± SD).

3.10. Hydrolysis of soy isoflavones by *Penicillium* sp. NRC24 β-glucosidase

HPLC analysis revealed that partially purified β-glucosidase from *penicillium* sp. NRC-24 could metabolize soy daidzin and genistin (Table 4). After treating defatted soy flour with free partially

purified β-glucosidase, genistein concentration increased 2.41-fold from 25.25 µg/g to 61 µg/g. It was 1.62-fold for daidzein. Immobilized β-glucosidase, on the other hand, was unable to metabolize soy genistin and daidzin. This could be attributed to the low incubation reaction time (10 minutes), as the immobilized enzymes may need more incubation time than free enzymes. This result could be attributed to the steric hindrance of the immobilized enzyme and the polymer which slows the chemical reactions due to steric bulk.

Table 4 Metabolizing of soy daidzin and genistin by the free and immobilized enzyme (183 mU/ml β-glucosidase)

β-glucosidase	**Daidzein (µg/g)**	**Genistein (µg/g)**
Free	78.63±0.93	61±1.06
Immobilized	48.0±0.0	22.75±0.0
Soy flour extract	48.5±2.83	25.25±0.0

The production of isoflavone aglycones by β-glucosidase has grown in popularity **[44]**. Isoflavone glycosides were adapted to a 2.0 mM concentration (1.17 mM genistin, 0.75 mM daidzin) and converted to aglycones using 0.1 U/mL immobilized β-glucosidases **[31].** After decomposing soy glycosides extracted with purified β-glucosidase, they were converted to daidzein (95%) and genistein (92%), showing productivity of 17.1 g/L/h and 19.3 g/L/h, respectively **[1].** Free and immobilized β-glucosidases showed a promising trend for industrial applications as they both could hydrolyze soy molasses isoflavone glucosides. The results indicate that glucosidases produced by *D. hansenii* UFV-1 – were applied to increase the aglycon content of soy products raising their nutritional value **[32]**.

4. Conclusion

For the first time, this work evaluates the fungal isolate *Penicillium* sp. NRC-24 as a new source for glucose and ethanol-tolerant extracellular β-glucosidase enzyme. The β-glucosidase enzyme was glucose tolerant and retained 60.4 % of initial activity at 0.5 M glucose. Moreover, the β-glucosidase could tolerate ethanol up to 60%.In this work, the partially purified *Penicillium* sp. NRC-24 β-glucosidase was successfully immobilized onto the alginate/polysorbate complex. Under optimal conditions, the immobilization yield was reported to be 70%. Properties of the enzyme, such as optimum temperature have been improved via the immobilization process. The initial activity of the immobilized enzyme was the same for 13 cycles which indicates a successful immobilization process. The produced β-glucosidase from the new fungal isolate, purified *Penicillium* sp. NRC-24, could hydrolyze the glycosidic bond of isoflavones of soybean to their active aglycones forms: Genistein, and daidzein. We recommend using the *Penicillium* sp. NRC-24 β-glucosidase in pharmaceutical and industrial applications.

5. Data Availability

All data generated or analyzed during this study are included in this published article. The 18S rRNA region was amplified by PCR, yielding a 1,678-bp sequence that was submitted to GenBank with accession number MZ913018.1.

6. Author contributions

A.E., D.A., A.G., and M.H. contributed to conceptualizing and planning experiments. M.H. designed the carrier. A.E., D.A., and A.G. carried out enzymatic experiments. A.E., D.A., A.G., and M.H. contributed equally to the interpretation of the results. All authors contributed to the writing of the manuscript.

7. Conflict of interest

The authors declare that there is no conflict of interest.

8. Acknowledgments

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9. References

[1] Singhvi MS & Zinjarde SS (2020) Production of pharmaceutically important genistein and daidzein from soybean flour extract by using β-glucosidase derived from *Penicillium janthinellum* NCIM 1171, *Process Biochem*, **97:** 183–190. https://doi.org/10.1016/j.procbio.2020.07.014.

[2] Paterni I, Granchi C, Katzenellenbogen JA, Minutolo F (2014) Estrogen receptors alpha (ERα) and beta (ERβ): subtype-selective ligands and clinical potential, Steroids **90:** 13–29. https://doi.org/10.1016/j.steroids.2014.06.012.

[3] Chen PX, Tang Y, Zhang B, Liu R, Marcone MF, Li X, Tsao R (2014) 5-hydroxymethyl-2-

furfural and derivatives formed during acid hydrolysis of conjugated and bound phenolics in plant foods and the effects on phenolic content and antioxidant capacity, *J. Agric. Food Chem.* **62(20):** 4754-61. https://doi.org/10.1021/jf500518r.

[4] Pei X, Zhao J, Cai P, Sun W, Ren J, Wu Q, Zhang S, Tian C (2016) Heterologous expression of a GH3 β-glucosidase from Neurosporacrassa in *Pichiapastoris* with high purity and its application in the hydrolysis of soybean isoflavone glycosides, *Protein Expr. Purif.* **119:** 75–84. https://doi.org/10.1016/j.pep.2015.11.010.

[5] Sørensen A, Lübeck M, Lübeck PS, Ahring BK (2013) Fungal Beta-glucosidases: a bottleneck in industrial use of lignocellulosic materials, *Biomolecules* **3(3):** 612–631. https://doi.org/10.3390/biom3030612.

[6] Ahmed A, Nasim FU, Batool K, Bibi A (2017) Microbial β-Glucosidase: Sources, Production and Applications, *Appl. Environ. Microbiol* **5(1):** 31-46.

[7] Andrades D, Graebin NG, Kadowaki MK, Ayub M, Fernandez-Lafuente R, Rodrigues RC (2019) Immobilization and stabilization of different β-glucosidases using the glutaraldehyde chemistry: Optimal protocol depends on the enzyme, *Int J Biol Macromol* **129:** 672–678.

[8] Aggarwal S, Chakravarty A, Ikram S (2021) A comprehensive review on incredible renewable carriers as promising platforms for enzyme immobilization & there of strategies, *Int J Biol Macromol* **167:** 962-986.

[9] Naseer S, Ouyang J, Chen X, Pu S, Guo Y, Zhang X, Li D, Yang C (2020) Immobilization of β-glucosidase by self-catalysis and compared to crosslinking with glutaraldehyde, *Int J Biol Macromol* **154:** 1490–1495. https://doi.org/10.1016/j.ijbiomac.2019.11.030.

[10] Esawy MA, Gamal AA, Kamel Z, Ismail AMS, Abdel-Fattah AF (2013) Evaluation of free and immobilized *Aspergillus niger* NRC1ami pectinase applicable in industrial processes, *Carbohydr Polym* **92(2):** 1463–1469.

[11] Hashem AM, Gamal AA, Hassan ME, Hassanein NM, Esawy MA (2016) Covalent immobilization of *Enterococcus faecalis* Esawy dextransucrase and dextran synthesis. *Int J Biol Macromol* **82:** 905-12. doi: 10.1016/j.ijbiomac.2015.09.076.

[12] Das A, Paul T, Ghosh P, Halder SK, Das Mohapatra PK, Pati BR, Mondal KC (2015) Kinetic Study of a Glucose Tolerant β-Glucosidase 3 from *Aspergillus fumigatus* ABK9 Entrapped into Alginate Beads, *Waste Biomass Valori* **6:** 53–61.

[13] Hashem AM, Gamal AA, Mansour NM, Salama BM, Hassanein NM, Awad GEA, Esawy MA (2018) Optimization of *Enterococcus faecalis* Esawy KR758759 dextransucrase and evaluation of some dextran bioactivities, *Biocatal. Agric. Biotechnol* **15:** 348-358.

[14] Sawsan SD, Mohamed EH, Harby EA, Mustafa AE, Mohamed BE (2021) Evaluation of Effectiveness of Covalently Immobilized α Amylase and Lipase in Cleaning of Historical Textiles, *Biointerface Res. Appl. Chem.* **11(3):** 9952 – 9962. https://doi.org/10.33263/BRIAC113.99529962

[15] Shen Q, Yang R, Hua X, Ye F, Zhang W, Zhao W (2011) Gelatin-templated biomimetic calcification for b-galactosidase immobilization, *Process Biochem* **46(8):** 1565–1571.

[16] Ali KA, Hassan ME, Elnashar MM (2017) Development of functionalized carrageenan, chitosan and alginate as polymeric chelating ligands for water softening, *Int. J. Environ. Sci. Technol.* **14:** 2009–2014. https://doi.org/10.1007/s13762-017-1298-y.

[17] Abeer AA, Faten AM, Mohamed EH, Eman RH, Mona AE (2017) Covalent immobilization of *Alternaria tenuissima* KM651985 laccase and some applied aspects, *Biocatal. Agric. Biotechnol* **9:** 74–81.

[18] Baranauskaite J, Ockun MA, Uner B, Tas C, Ivanauskas L (2021) Effect of the Amount of Polysorbate 80 and Oregano Essential Oil on the Emulsion Stability and Characterization Properties of Sodium Alginate Microcapsules, *Molecules* **26:** 6304. https://doi.org/10.3390/molecules26206304.

[19] Handa C, Couto U, Vicensoti A, Georgetti S, Ida E (2014) Optimisation of soy flour fermentation parameters to produce β-glucosidase for bioconversion into aglycones, *Food Chem* **152:** 56- 65.

[20] El-Shazly A, Noor El-Deen A, Ibrahim N, Abdelwahed N, El-Beih A, Shetaia Y, Farid M (2017) Assessment of genistein and daidzein production by some local fungal and bacterial isolates. Egypt. *J. Microbiol.* **52(1):** 49-61.

[21] Stanbury PF, Whitaker A, Hall SJ (1999) The Isolation, Preservation and Improvement of Industrially Important Microorganisms. In: Stanbury, P. F., Whitaker, A., & Hall, S. J. (Ed.), Principles of Fermentation Technology

(Second Edition), Amsterdam: Pergamon, 35-91.

[22] Jensen V (1968) The plate count method, In: Gray TRG and Parkinson D (Eds), The ecology of soil bacteria, An international symposium, Liverpool university press, Liverpool, 158-170.

[23] Gargas A & Taylor JW (1992) Polymerase Chain Reaction (PCR) Primers for Amplifying and Sequencing Nuclear 18s rDNA from *Lichenized Fungi*, *Mycologia **84*(4):** 589–592. https://doi.org/10.2307/3760327

[24] 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. *J Appl Microbiol* **103(4):** 910-917.

[25] Yadav S, Pandey AK, Dubey SK (2021) Molecular modeling, docking and simulation dynamics of β-glucosidase reveals high-efficiency, thermo-stable, glucose tolerant enzyme in *Paenibacilluslautus* BHU3 strain, *Int J Biol Macromol* **168:** 371–382. https://doi.org/10.1016/j.ijbiomac.2020.12.059

[26] Karam EA, Hassan ME, Moharam ME, Kansoh AL (2018) Immobilization of Inulinase Produced by *Rhizopus oligosporus* NRRL 2549 for continuous fructose production, *J. Mater. Environ. Sci.* **9:** 2315–2321.

[27] Mostafa FA, El Aty AAA, Hassan ME, Awad GEA (2019) Immobilization of xylanase on modified grafted alginate polyethyleneimine bead based on impact of sodium cation effect, *Int J Biol Macromol* **140:** 1284-1295.

[28] Elnashar MM (2011) Carriers for Immobilization and Reusability of Enzymes. *US Patent*, application No. 12 / 866, 748. http://www.wipo.int/pctdb/ wo/2009/155 937, Publication No. US 2011/0076737 A1.

[29] Wahba MI & Hassan ME (2017) Agar-carrageenan hydrogel blend as a carrier for the covalent immobilization of β-D-galactosidase, *Macromol. Res.* **25:** 913.

[30] Yoav S, Stern J, Salama-Alber O, Frolow F, Anbar M, Karpol A, Hadar Y, Morag E, Bayer EA (2019) Directed Evolution of *Clostridium thermocellum* β-Glucosidase A Towards Enhanced Thermostability, *Int. J. Mol. Sci* **20(19):** 4701. https://doi.org/10.3390/ijms20194701.

[31] Hu S, Wang D, Hong J (2018) A Simple Method for Beta-glucosidase Immobilization and Its Application in Soybean Isoflavone Glycosides Hydrolysis. *Biotechnol Bioproc* **E 23:** 39–48. https://doi.org/10.1007/s12257-017-0434-3.

[32] Maitan-Alfenas GP, De A Lage LG, De Almeida MN, Visser EM, De Rezende ST, Guimarães VM (2014) Hydrolysis of soybean isoflavones by *Debaryomyceshansenii* UFV-1 immobilised cells and free β-glucosidase, *Food Chem* **146:** 429–436. https://doi.org/10.1016/j.foodchem.2013.09.099

[33] Silva LH, Celeghini RMS, Chang YK (2011) Effect of the fermentation of whole soybean flour on the conversion of isoflavones from glycosides to aglycones, *Food Chem* **128(3):** 640-644.

[34] Sun N, Liu X, Wang X, Shi H, Zhang H, Li L, Na W, Guan Q (2021) Optimization of fermentation conditions for the production of acidophilic β-glucosidase by *Trichoderma reesei* S12 from mangrove soil, *Biotechnology & Biotechnological Equipment* **35(1):** 1838-1849.

[35] Kim EY, Kwon CW, Chang PS (2021) Purification and characterization of a novel acid-tolerant and heterodimeric β-glucosidase from pumpkin (Cucurbita moschata) seed, *J. Biosci.Bioeng.* **132(2)**:125-131.

[36] Asati V & Sharma PK (2019) Purification and characterization of an isoflavones conjugate hydrolyzing β-glucosidase (ICHG) from Cyamopsis tetragonoloba (guar), *Biochem. Biophys. Rep.* **20:** 100669,

[37] Mahapatra S & Manian R (2022) Enhancement, production, and immobilization of beta-glucosidase from *Zobellelladenitrificans* VIT SB117 and its utilization in bioethanol production from lignocellulosic feedstock, Biomass Convers. *Bioref.* **12:** 447–458.

[38] Neculai A, Ivanov D, Bernards MA (2018) Partial purification and characterization of three ginsenoside-metabolizing β-glucosidases from *Pythium irregular. J. Ind. Microbiol. Biotech* 45: 369–378.

[39] Nishida VS, De Oliveir RF, Brugnari T, Correa RCG, Peralta RA, Castoldi R, De Souza CGM, Adelar BRM (2018) Immobilization of *Aspergillus awamori* β-glucosidase on commercial gelatin: An inexpensive and efficient process. *Int J Biol Macromol* **111:** 1206-1213.

[40] Figueira JA, Fernanda FG, H´elia HS, Pedro F (2011) Screening of Supports for the Immobilization of β- Glucosidase, *Phytochemistry* **70:** 1948-1957.

[41] Boudabbous M, Ben Ameur R, Belhaj I, Cheffi M, Belghith H, Allouche N, Chamkha M (2020) A novel sec-butyl-β-D-cellobioside and sec-butyl-β-D-glucoside biosynthesized by immobilized β-glucosidase of *Fusarium solani* with surfactant properties, *Appl. Catal. A: Gen.* 602.

[42] Esawy MA, Gamal AA, Helal MM, Hassan ME, Hassanein NM. Hashem AM (2016) Enzymatic synthesis using immobilized *Enterococcus faecalis* Esawy dextransucrase and some applied studies. *Int J Biol Macromol*, **92:** 56-62. doi: 10.1016/j.ijbiomac.2016.06.041.

[43] Elnashar MM, Yassin MA, Kahil T (2008) Novel thermally and mechanically stable hydrogel for enzyme immobilization of penicillin G acylase via covalent technique, *Journal of Applied Polymer Science* **109(6):** 4105-4111.

[44] Cao H, Zhang Y, Shi P, Ma R, Yang H, Xia W, Cui Y, Luo H, Bai Y, Yao B (2018) A highly glucose-tolerant GH1 β-glucosidase with greater conversion rate of soybean isoflavones in monogastric animals. *J. Biosci. Bioeng. J BIOS* **125:** 185-191.