

Article

Production of β -Glucosidase via Fermentation of *Aspergillus kawachii* for the Sustainable Obtaining of the Blue Chromophore from Geniposide in a Simplified Process

Carlos N. Cano-González ^{1,*} , Alexa M. Campos-Jiménez ¹, Ena Deyla Bolaina-Lorenzo ¹ ,
Ana Mayela Ramos-de-la-Peña ²  and Juan Carlos Contreras-Esquivel ^{1,*}

¹ Food Research Department, School of Chemistry, Universidad Autonoma de Coahuila, Saltillo 25250, CO, Mexico; alexa_campos@uadec.edu.mx (A.M.C.-J.); enabolaina@gmail.com (E.D.B.-L.)

² School of Engineering and Science, Tecnológico de Monterrey, Av. Eugenio Garza Sada 2501 Sur, Monterrey 64849, NL, Mexico; ramos.amay@tec.mx

* Correspondence: ccanogonzalez@uadec.edu.mx (C.N.C.-G.); carlos.contreras@uadec.edu.mx (J.C.C.-E.)

Abstract

A. kawachii is of considerable importance in the food industry due to its enzyme production. The objective was to evaluate the feasibility of coupling β -glucosidase production by *A. kawachii*, to partially purify the enzymatic extract by gel filtration chromatography, and to preliminarily apply it to the bioconversion of geniposide into genipin to form a blue chromophore in a simplified process. β -glucosidase production was carried out via liquid fermentation in a glucose-tryptone medium over 54 h, and fermentation process parameters were monitored. Subsequently, the enzyme extract was fractionated by gel filtration chromatography (Hi-Trap G-25 column). The simplified process for the formation of the blue chromophore was carried out by mixing the fraction with β -glucosidase activity, geniposide (10 g/L), and glycine at different concentrations (1.0, 2.5, 5.0, 7.5, and 10.0 g/L), then incubating it at 40 °C for 48 h. The fermentation time for maximum β -glucosidase production (0.059 U/mL) was 48 h. Residual fermentation compounds were removed by chromatography, thereby partially purifying the enzyme (purification fold = 3.88) to prevent it from reacting with genipin. At 7.5 g/L glycine, a yield of 0.96 g/L of the blue chromophore was obtained. In conclusion, the use of enzymes enables a sustainable process for producing a blue chromophore. This chromophore can be considered a potential functional ingredient for the development of nutraceuticals and cosmeceuticals.

Keywords: enzyme; fermentation; functional ingredient; genipin; pigment



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1. Introduction

Genipin is an iridoid monoterpenoid obtained primarily from the fruits of *Genipa americana* and *Gardenia jasminoides*. Its importance lies in its biological activities, which are crucial for the development of nutraceuticals, functional foods, cosmeceuticals, and pharmaceuticals [1,2]. Genipin is biocompatible and non-cytotoxic, and it is attractive mainly for its ability to react with primary amines to produce blue pigments, offering an alternative to synthetic dyes [1,3]. Geniposide is an iridoid glucoside obtained from various plants and has therapeutic effects; upon hydrolysis, Genipin is produced [4]. While various hydrolysis methods exist, enzymatic hydrolysis using β -glucosidase offers several advantages. This approach contributes to the advancement of biotechnology and sustainable solutions in various industries [2].

β -Glucosidase (β -D-glucoside glucohydrolase, EC 3.2.1.21) is a hydrolytic enzyme that cleaves β (1–4) glycosidic bonds in saccharides, yielding glucose monomers as the final product [5]. It participates in the hydrolysis of glycosylated compounds and is therefore used in the production of fruit juice, wine, and derivative industries to enhance organoleptic properties [5,6]. Another application is the production of ethanol from agricultural waste. The wide range of uses for this enzyme underscores its potential to reduce pollution [7,8]. Furthermore, this production method aligns with several Sustainable Development Goals (SDGs), including SDG 7 on affordable and clean energy and SDG 12 on responsible consumption and production. By establishing bioprocesses for product development, environmental sustainability is supported, and a circular economy is fostered, thereby reducing environmental impact and promoting a more sustainable future [9,10]. These enzymes are produced by various microorganisms, including bacteria, yeasts, and fungi, through fermentation.

The genus *Aspergillus* is among the most studied groups of filamentous fungi, primarily because of its importance in food biotechnology, medicine, and agriculture [11]. At the molecular level, high divergence has been observed in genes encoding enzymes (as listed in the CAZy database) with the potential to biotransform various glycosylated molecules [12]. *A. kawachii* is a species of great economic importance due to its excellent capacity to produce enzymes such as α -amylase, protease, inulinase, and β -glucosidase. These enzymes play a significant role in food production [13,14]. The genome of *A. kawachii* encodes 247 glycoside hydrolases. This species shares genomic and physiological similarities with *A. niger*, particularly in the industrial production of enzymes and organic acids. *A. kawachii* has long been regarded as a safe industrial microorganism. Previous genomic studies of strain IFO 4308 reported the absence of ochratoxin A production and identified a partial loss of the genomic region associated with OTA biosynthesis, supporting its suitability for food-related applications [15,16].

Therefore, the objective of this study was to evaluate the feasibility of coupling β -glucosidase production by *A. kawachii*, to partially purify the enzymatic extract by gel filtration chromatography, and to preliminarily apply it to the bioconversion of geniposide into genipin to form a blue chromophore in a simplified process.

2. Materials and Methods

2.1. Materials and Reagents

The *A. kawachi* IFO 4308 strain was provided by the Food Research Department of the Autonomous University of Coahuila (Saltillo, Coahuila, Mexico). Potato dextrose agar (PDA) was purchased from BD Bioxon (Mexico City, Mexico). 2-Nitrophenyl β -D-glucopyranoside, p-nitrophenyl, was purchased from Gold Biotechnology Co. (Ashby Rd, Olivette, MO, USA). The reagent for the glucose kit (GOD/POD) was purchased from Randox Laboratories Ltd. (Crumlin, Antrim, UK). All other reagents and analytical substrates were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2. Production of β -Glucosidase from *Aspergillus kawachii* by Fermentation

2.2.1. Strain and Fermentation Culture Conditions

A. kawachii was reactivated in PDA medium. A 1 mL sample of the (preserved) strain was added to the medium plus 1 mL of Tween 80 (0.01% *v/v*). The mixture was homogenized and incubated for 7 days at 30 °C. After reactivation of the strain, spores were obtained with Tween 80 (0.01% *v/v*). Spore concentration was determined by direct microscopic counting using a Neubauer hemocytometer, and the suspension was adjusted with sterile Tween 80 solution to obtain a final concentration of 1×10^6 spores/mL, which was used as the fermentation inoculum.

The liquid culture medium was GT, composed of 1.0 g/L K_2HPO_4 , 0.5 g/L KCl, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $FeSO_4 \cdot 7H_2O$, 2.5 g/L tryptone, and 5.0 g/L glucose. The culture medium was prepared and sterilized without pH adjustment. The initial pH of the medium was approximately 7.0. An inoculum of 1×10^6 spores/mL was used, and fermentation was carried out at 150 rpm and 30 °C for 54 h in Orbital Incubator Shaker (New Brunswick TM Innova[®] 44/44R, Eppendorf, Hamburg, Germany). Samples were collected and filtered every 6 h to obtain biomass, which was then dried in an oven at 50 °C for 48 h. Dry weight was determined gravimetrically, and the fermentation extract was stored at −10 °C [17].

2.2.2. Determination of Enzyme Activity (β -Glucosidase)

β -glucosidase activity was performed using the pNP (p-nitrophenol) method, with some modifications [18]. The enzymatic activity of β -glucosidase was determined using a mixture of 30 μ L of enzyme extract and 225 μ L of 2 mM p-nitrophenyl- β -D-glucopyranoside prepared in 50 mM sodium acetate buffer (pH 5.0). After 24 h at 40 °C, 50 μ L of the reaction mixture was mixed with 250 μ L of 0.5 M Na_2CO_3 to stop the reaction and develop color. Absorbance was measured at 400 nm, and enzyme activities were calculated using a standard solution of p-nitrophenol (5–50 mg/L). One unit of enzymatic activity was defined as the amount of enzyme required to generate 1 μ mol of the corresponding nitrophenol in 1 min.

2.2.3. Protein Concentration

Protein concentration was determined by the Lowry et al. [19] method, using bovine serum albumin as a standard (50–500 mg/L). The samples were analyzed by spectrophotometry at 660 nm using a UV–Vis spectrophotometer (GENESYSTM 10S, Thermo Scientific, Waltham, MA, USA).

2.2.4. Residual Glucose and pH

The samples (100 μ L) were treated with 100 μ L of glucose kit reagent (GOD/POD). The reaction was then left to stand in the dark for 30 min. Glucose was used as the assay standard (5–200 mg/L). The samples were analyzed by spectrophotometry at 505 nm using a UV–Vis spectrophotometer (GENESYSTM 10S, Thermo Scientific, Waltham, MA, USA). The pH was determined using a pH meter (Orion 420A, Thermo Scientific, Waltham, MA, USA).

2.3. Purification of β -Glucosidase from *Aspergillus kawachii*

Gel Filtration Chromatography of the Enzyme Extract

For size-exclusion separation, the sample was fractionated on a G-25 HiPrep 26/10 Desalting column (10 cm $\times$ 26 mm, Sigma-Aldrich). A volume of 5.0 milliliters of the sample was injected into the column. The column was equilibrated with 120 mL of sample and 60 mL of eluent, using 0.050 M acetate buffer (pH 5.0) with 0.02% NaN_3 . The procedure was carried out at a flow rate of 5 mL/min, a maximum operating pressure of 1.0 MPa, and 25 °C, yielding 13 fractions of 5 mL each. The fractions were analyzed for β -glucosidase activity (Section 2.2.2) and protein concentration (Section 2.2.3).

2.4. Formation of the Blue Chromophore from Geniposide in a Simplified Process

Glycine served as the primary amine donor, enabling the formation of the blue chromophore. The reaction mixture was prepared by combining 100 μ L of glycine at various concentrations (1.0, 2.5, 5.0, 7.5, and 10 g/L), 100 μ L of geniposide (10 g/L), and 50 μ L of an *A. kawachii* enzyme extract with β -glucosidase activity. After addition, the tubes were mixed by inversion. The samples were incubated at 40 °C for 48 h. Chromophore concentration was determined by the genipin method [20], which is based on the reaction of

genipin with primary amino groups. Glycine was used as the reference amino compound for calibration purposes. Samples were analyzed by spectrophotometry at 589 nm.

2.5. Statistical Analysis

Results were expressed as mean ($\pm$ standard deviation, SD). Statistical analysis was performed using analysis of variance (ANOVA) in SAS 7 (SAS Institute Inc., Cary, NC, USA), followed by Tukey's test. A p -value < 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Production of β -Glucosidase from *Aspergillus kawachii* by Fermentation

Fermentation kinetics were conducted for up to 54 h to determine the time to maximum β -glucosidase production in *A. kawachii*. Figure 1 shows that the highest enzyme activity occurred at 48 and 54 h, with no significant difference between these two times ($p > 0.05$). β -glucosidase activity at 48 h of fermentation was 0.059 U/mL, a significant increase from 6 h, when enzymatic activity was undetectable. The production of β -glucosidase during fermentation reflects the metabolic capacity of *A. kawachii* to synthesize hydrolytic enzymes involved in carbohydrate utilization. These enzymes contribute to nutrient acquisition and support fungal growth and metabolite production under the culture conditions evaluated. Furthermore, the increase in enzyme activity observed between 24 and 48 h suggests an active physiological response associated with the fungus's exponential growth phase. *A. kawachii* can produce a wide variety of hydrolases. A significant advantage of *A. kawachii* is its long history of use in food fermentations, and previous reports, supported by genomic analyses, describe the absence of ochratoxin A production in the IFO 4308 strain. Therefore, this fungus is considered a safe microorganism for the production of industrially important metabolites [15,16]. Established fermentation conditions, such as agitation (150 rpm) and temperature (30 °C), have been reported to be the most suitable for β -glucosidase production [21]. Studies such as that by [22], using *A. niger* CDBB-H-175, demonstrate that β -glucosidase activity increased after 30 h in the presence of glucose and other carbon sources. The increase in β -glucosidase activity after 30 h likely reflects the transition from the adaptation phase to active fungal metabolism, when carbon acquisition becomes essential for sustained growth. As glucose concentrations decline, fungi may activate carbohydrate-hydrolysis systems to maximize nutrient utilization. This metabolic response explains the progressive increase in β -glucosidase activity observed between 24 and 48 h and underscores the enzyme's role in fungal adaptation to carbon-limited environments. Microbial enzymes offer advantages for large-scale bioprocessing, exhibiting high yields and substrate specificity [23]. *Aspergillus* species are of great interest due to their potential applications in biotechnology [24].

The fermentation biomass graph, used to determine fungal growth, is shown in Figure 1. During the first 24 h of fermentation, no significant change in biomass was observed, suggesting the fungus was in an adaptation phase. Between 24 and 36 h of fermentation, the exponential growth phase was observed, and after 42 h, the growth rate decreased. Between 48 and 54 h of fermentation, the stationary phase was observed, with a biomass concentration of 2.64 g/L, and no significant difference was detected ($p > 0.05$). In studies using *A. niger* CDBB-H-175, the highest biomass concentration was observed at 48 h, with values exceeding 4.0 g/L [22]. The secretion of soluble proteins during fermentation reflects metabolic conditions favorable to fungal growth and metabolite production. In the first few hours of fermentation (6–12 h), no protein production is detected, indicating that the concentration of soluble proteins is negligible or very low. However, after 24 h, a significant increase is observed, reaching a maximum of 0.802 g/L at 54 h (Figure 1). This overview of soluble protein production illustrates the metabolic phases the fungus

undergoes to enter the exponential phase and increase protein production. The significant increase in soluble protein indicates the secretion of important proteins, such as biocatalytic, structural, and hormonal proteins, which are necessary for nutrient provision for microbial growth [25,26]. The simultaneous increase in biomass, soluble protein concentration, and β -glucosidase activity suggests that enzyme production is associated with growth. This behavior indicates that extracellular protein secretion is closely linked to the metabolic demand for nutrients during the exponential growth phase. Therefore, biomass production alone is insufficient to explain enzyme synthesis, as extracellular protein secretion appears to be a more direct indicator of biocatalyst production.

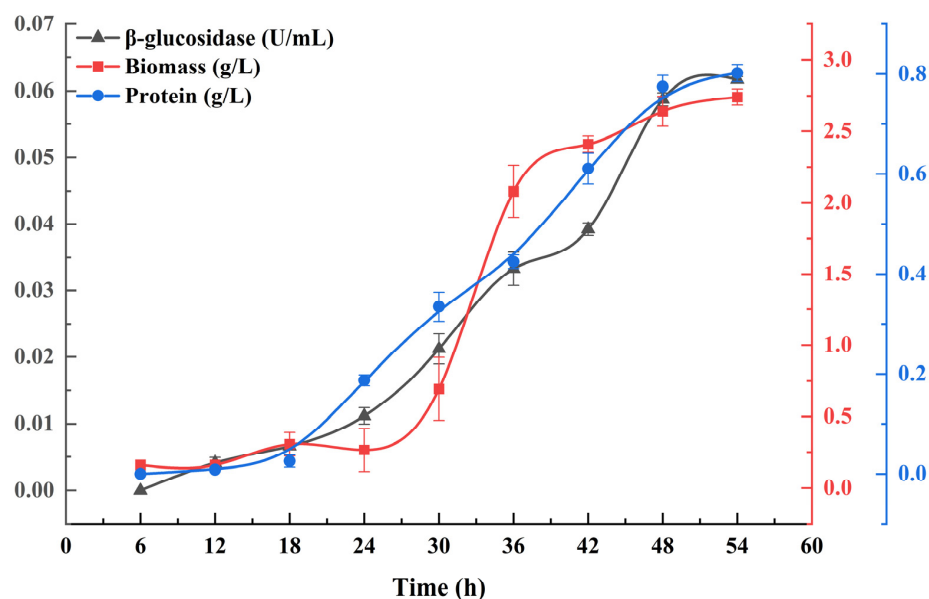


Figure 1. Kinetics of β -glucosidase (black line), protein (blue line), and biomass (red line) production from the fermentation of *A. kawachii*. Values are expressed as the mean $\pm$ standard error ($n = 3$). Significant differences are indicated at $p < 0.05$.

The pH was monitored throughout the 54 h fermentation to assess its relationship to β -glucosidase production (Figure 2). The pH remained near 7.0 but decreased between 30 and 42 h, with a pronounced drop at 36 h, when it reached an acidic pH of 4.47. This behavior is associated with the fungus's exponential growth phase and results from the synthesis and secretion of organic acids and metabolites linked to cell growth. After this period, the pH gradually increased toward neutrality. While cell lysis may have contributed to this behavior, the relatively constant biomass concentration suggests that additional mechanisms may have been involved. The pH recovery could also be associated with the consumption of previously accumulated organic acids and the metabolism of nitrogenous compounds present in the culture medium. In particular, the utilization of tryptone and other nitrogen sources can generate alkaline metabolites, such as ammonia, which can contribute to an increase in pH during the later stages of fermentation. Notably, these pH fluctuations reflect the metabolic behavior of *Aspergillus* spp. [22].

Residual glucose in the fermentation extracts was quantified using an enzymatic method. Figure 2 shows that glucose levels in the medium decreased over time. Glucose served as a carbon source for the microorganism, and as fermentation progressed, it was depleted from the medium because it was the primary energy source for growth. After 12 h of fermentation, the glucose concentration decreased significantly, reaching 0.039 g/L at 48 h and 0.026 g/L at 54 h; no significant difference was observed ($p > 0.05$). The gradual decrease in glucose in the fermentation medium is due to factors such as optimal pH and temperature, which favor fungal metabolic activity. An environment with favorable

conditions that efficiently utilizes glucose not only promotes microbial growth but also enhances enzyme production [27,28].

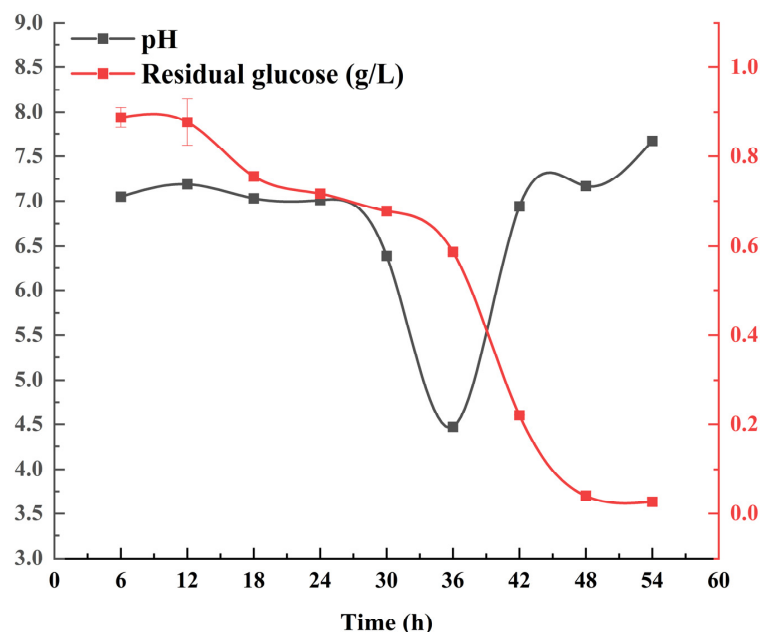


Figure 2. *A. kawachii* fermentation effects on residual glucose concentration (red line) and pH (black line) of growth media. Values are expressed as the mean $\pm$ standard error ($n = 3$). Significant differences are indicated at $p < 0.05$.

3.2. Partial Purification of an Active Fraction of β -Glucosidase from *Aspergillus kawachii*

The fermentation extract was subjected to gel filtration chromatography to obtain a fraction enriched in β -glucosidase activity. The enzyme extract was subjected to size-exclusion chromatography (gel filtration) on a G-25 HiPrep 26/10 Desalting column (Figure 3). β -glucosidase activity was observed in fractions 3–5, with fraction 4 exhibiting the highest enzyme activity, reaching 0.026 U/mL. The protein concentration in fraction 4 was quantified at 0.088 g/L; This fraction showed the highest enzyme activity, suggesting an increase in β -glucosidase activity and a decrease in low-molecular-weight compounds present in the fermentation extract, including residual metabolites, sugars, and other components that could interfere with subsequent bioconversion reactions, thereby reducing potential interference during the reaction between genipin and glycine. Furthermore, a large portion of the residual glucose in the fermentation extract that the fungus did not utilize after 48 h of fermentation could be removed. Size-exclusion chromatography separates molecules based on molecular weight; it is particularly useful for purifying enzymes, as it separates the desired molecules from impurities in the fermentation extract [29,30].

β -glucosidase is widely used in the food industry and requires high purity. Using gel filtration chromatography, the reported total activity was 0.026 U/mL, with an enzyme yield of 44.63% and a purification factor of 3.88. The results of filtration chromatography using a G-25 column are presented in Table 1, along with the purification criteria for β -glucosidase: total activity, total protein concentration, specific activity, yield, and purification factor. The crude extract from the culture medium was partially purified by gel filtration, a widely used method for purifying molecules, including enzymes of various classes [31]. One of its advantages is the removal, by molecular weight, of compounds of no interest present in the fermentation extract, whether they be fungal metabolites or culture medium components [32]. In addition to reducing low-molecular-weight compounds, the chromatographic step played a crucial role in preventing unwanted compounds in the fermentation broth from participating in side reactions with genipin. The removal

of residual sugars, amino acids, and low-molecular-weight metabolites was essential to ensure that pigment formation was driven primarily by the intended reaction between genipin and glycine. Consequently, this purification step improved the process selectivity and increased the reliability of the subsequent chromophore formation assay.

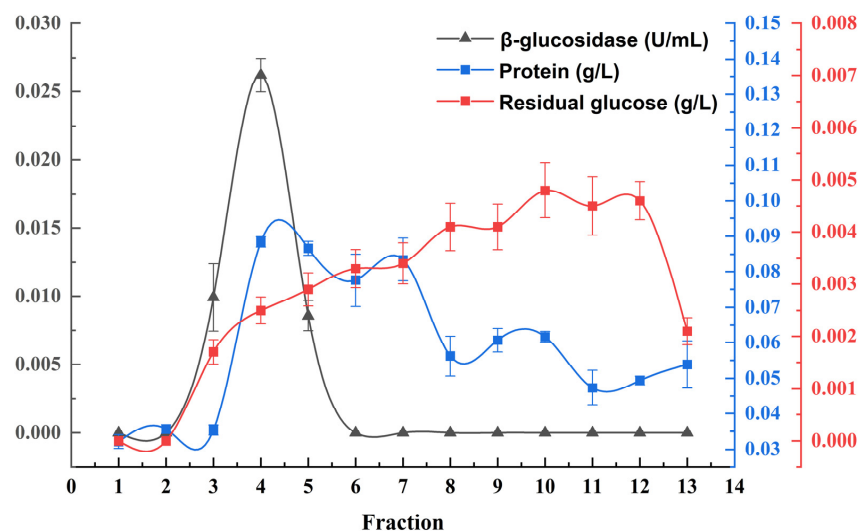


Figure 3. Chromatogram of the partial purification of β -glucosidase from *A. kawachii* by gel filtration chromatography on a G-25 column (column volume: 30 mL). Determination of enzymatic activity (black line), protein concentration (blue line), and residual glucose (red line). Values are expressed as the mean $\pm$ standard error ($n = 3$). Significant differences are indicated at $p < 0.05$.

Table 1. Purification step for β -glucosidase from *A. kawachii*.

Purification	Total Activity	Total Protein	Specific Activity	Yield	Purification
Step	(U)	(mg)	(U/mg)	(%)	(Fold)
Culture filtrate	0.059	768.51	0.000076	100	1
G-25	0.026	88.44	0.000296	44.63	3.88

The β -glucosidase activity obtained in this study (0.059 U/mL) was lower than that reported for other *Aspergillus* species. Singh et al. [24] reported that *Aspergillus japonicus* VIT-SB1 increased β -glucosidase production from 0.40 to 10.13 U/mL through statistical optimization of the fermentation process. Similarly, Karami et al. [33] achieved a β -glucosidase activity of 0.497 U/mL in *Aspergillus niger*, indicating optimal culture conditions. Previously, Pushalkar et al. [34] reported a β -glucosidase production of 2.18 U/mL for *Aspergillus terreus*. These studies focused primarily on maximizing enzyme production through medium optimization, process engineering, or statistical experimental designs. Conversely, the objective of this work was not to maximize β -glucosidase productivity, but rather to demonstrate the feasibility of producing an active enzyme fraction by submerged fermentation, partially purifying it by gel filtration chromatography.

3.3. Formation of the Blue Chromophore from Geniposide in a Simplified Process

A simplified method for the formation of the blue chromophore is established using molecules with a primary amino group, in this case, glycine and geniposide, in the presence of β -glucosidase from *A. kawachii*. Figure 4 shows the chromophore concentration as a function of different glycine concentrations. The glycine concentration showed no significant differences above 7.5 g/L, and the chromophore concentration was 0.96 g/L. β -glucosidase hydrolyzes geniposide to produce genipin and glucose. The released genipin reacts covalently with the primary amino groups of glycine to form the blue chromophore,

which is stable under various conditions [3,35,36]. The released genipin reacts covalently with the primary amino group of glycine, promoting the formation of intermolecular cross-links. These reactions lead to the generation of oligomeric structures responsible for the characteristic blue coloration. The stability of the chromophore has been attributed to the formation of these cross-linked molecular networks under mild reaction conditions. The most important and stable reaction that produces the blue color occurs at carbon 3, where the heterocyclic ring opens, and the primary amine attaches to the carbon, while at the other end, a partial aldehyde is formed; this is where the secondary amine is located. The secondary amine attacks the aldehyde carbon [35], initiating heterocycle reformation, where the amine remains in the ring. The missing double bond is formed by an elimination reaction. This reaction is slow and involves nucleophilic attack of a primary amine on the ester group of genipin, resulting in an amide [1,37,38]. Given the importance of blue in the food industry, the blue hue of the genipin complex could serve as a functional ingredient in various products, such as beverages and foods, providing significant visual appeal to consumers [35,37]. This study demonstrated the feasibility of producing β -glucosidase from *A. kawachii* and its application in the bioconversion of geniposide to genipin for blue pigment formation. Although the results demonstrate the feasibility of using β -glucosidase produced by *A. kawachii* for geniposide bioconversion and pigment formation, additional studies are required to optimize process variables, including pH, temperature, substrate loading, enzyme dosage, and reactor operating conditions. Previous studies have reported the conversion of geniposide to genipin by β -glucosidase and the subsequent formation of blue pigments using different amino acids for food applications. However, these processes have generally been carried out as independent steps [37,38]. As in Hao et al. [39], β -glucosidase effectively catalyzed the hydrolysis of geniposide, thereby enabling the subsequent formation of the blue chromophore. However, the recombinant β -glucosidase was produced in *Escherichia coli*. They do not report pigment concentrations. In contrast, the present study proposes a simplified process in which the β -glucosidase produced by *A. kawachii* is applied directly to the bioconversion of geniposide and the formation of the blue chromophore, demonstrating the feasibility of integrating both stages into a single workflow.

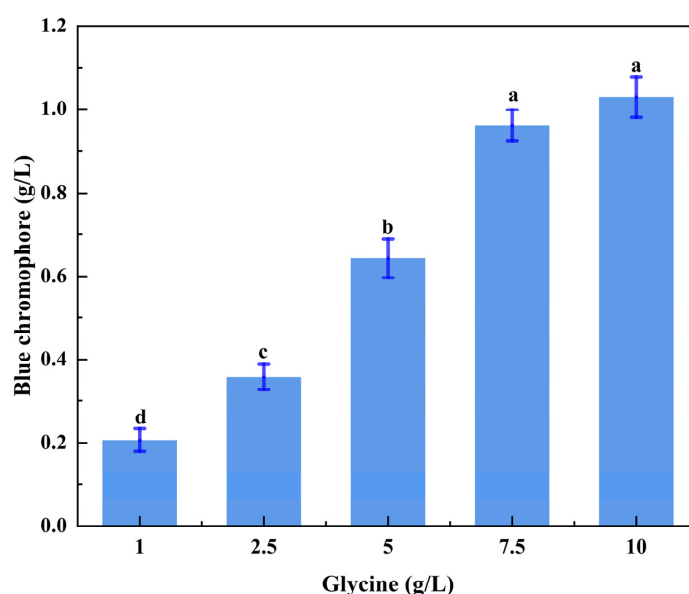


Figure 4. Determination of the formation of the blue chromophore in a single step with glycine at different concentrations (1.0, 2.5, 5.0, 7.5, and 10.0 g/L) using geniposide and β -glucosidase from

the extract of *A. kawachii* (0.026 U/mL). Values are expressed as the mean $\pm$ standard error ($n = 3$). Different letters indicate significant differences according to Tukey's test ($p < 0.05$).

4. Conclusions

A. kawachii is a fungus with a long history of use in food-related fermentations and has been reported as a safe industrial microorganism in previous studies. However, the present work focused on enzyme production and pigment formation, and analyses of mycotoxin production were not performed. This fungus can synthesize the enzyme β -glucosidase through submerged fermentation. β -glucosidase is essential for the hydrolysis of glycosides, enabling the release of value-added compounds across various biotechnological areas, including food, cosmetics, and pharmaceuticals. The β -glucosidase fraction obtained in this work catalyzed the bioconversion of geniposide under the evaluated conditions, thereby enabling the formation of the blue chromophore with glycine through a simplified, efficient process. The resulting blue chromophore has significant potential as a natural food colorant, helping reduce synthetic dye use while meeting increasing consumer demand for safer, cleaner-label ingredients. Beyond improving the visual appeal of food products, this pigment may support the development of functional foods, nutraceuticals, and cosmetics. Moreover, the enzymatic production of genipin-derived pigments aligns with the principles of green chemistry and sustainable biotechnology by minimizing the use of hazardous chemicals and reducing environmental impact. The main contribution of this study lies in the integration of β -glucosidase production, the partial purification of an active enzyme fraction, and its preliminary application to the bioconversion of geniposide, thereby directly forming blue chromophores in a simplified process. These findings demonstrate the feasibility of coupling enzyme production and pigment synthesis within the same biotechnological framework and provide a basis for future studies on process optimization and scale-up.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

SDGs	Sustainable Development Goals
PDA	Potato dextrose agar
GOD/POD	Glucose Oxidase Peroxidase
Hi Prep	High-Performance Preparative
MPa	Megapascal

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