JTE HCMUTE
ISSN: 2615-9740

JOURNAL OF TECHNICAL EDUCATION SCIENCE
Ho Chi Minh City University of Technology and Education
Website: https://jte.edu.vn
Email: jte@hcmute.edu.vn

Enzymatic Conversion of Geniposide to Genipin: A Natural Blue Color Precursor and Biopolymer Film Crosslinker

Thi Nga Vo[1], Dac Loc Ho[1], Hoang Duc Dang[2], Vinh Tien Nguyen[1*]
[1]*Ho Chi Minh City University of Technology and Education, Vietnam*
[2]*Sunrise Global Joint Stock Company, Ho Chi Minh City, Vietnam*

[*]Corresponding author. *Email: tiennv@hcmute.edu.vn*

ARTICLE INFO

Received: 01/05/2024
Revised: 19/05/2024
Accepted: 29/05/2024
Published: 28/12/2024

KEYWORDS

Cellulase;
Genipin;
Chitosan-genipin;
Crosslink;
Blue colorant.

ABSTRACT

The research is motivated by the growing interest in using natural products for biopolymer engineering, particularly in developing bioactive and biocompatible materials. Genipin, a natural blue colorant precursor, has garnered attention due to its unique chemical structure and crosslinking properties with natural polymers. This research focuses on the optimizing conditions for efficient enzymatic conversion of geniposide from *Gardenia jasminoides* into genipin and its subsequent utilization in producing chitosan-genipin films. Geniposide was extracted using 50% ethanol, and its enzymatic conversion to genipin using commercial cellulase was best at pH 4.5, 0.2 g cellulase per gram of geniposide and 6 h of reaction. The synthesized genipin was used to fabricate chitosan-genipin films, which were tested for various properties. The film with 0.01 w/w genipin/chitosan ratio exhibited the highest UV-vis absorbance at 610 nm, indicating significant crosslinking, and demonstrated the greatest mechanical strength at 19.92 N/mm². Additionally, this film showed a moisture content of only 2.01%, significantly lower than that of the control. Increasing the amount of genipin reacting with chitosan significantly reduced the moisture and swelling degree of the chitosan films, indicating their lower hydrophilicity. These results underscore the effectiveness of genipin as a crosslinking agent in biopolymer applications, suggesting its potential to develop sustainable materials with advanced mechanical and moisture-resistant properties.

Doi: https://doi.org/10.54644/jte.2024.1584

1. Introduction

The innovative use of natural products in biopolymer engineering has garnered significant interest in recent years, particularly in the development of bioactive and biocompatible materials [1]. Genipin, a natural blue colorant precursor, has gained significant attention in recent years due to its unique chemical structure and crosslinking properties with natural polymers [2]. It is extracted from various sources, including *Gardenia jasminoides* and *Genipa americana*. Genipin has been recognized for its potential as a bioactive compound, exhibiting antioxidant, antimicrobial, and anticancer properties. Moreover, it is considered a non-cytotoxic crosslinking agent, making it suitable for the production of bio-based materials approved for human contact [3]. The use of genipin in the manufacturing of biopolymers offers opportunities to enhance the physical and mechanical properties of these materials, making them more stable and resistant to degradation.

Several methods have been reported for genipin production, including enzymatic hydrolysis, chemical synthesis, and microbial fermentation. One method of producing genipin involves extracting it from the fruit of Genipa Americana, also known as genipap or caruto. The fruit's core is used to extract genipin-rich genipap oil, which can serve as an inexpensive replacement for commercial genipin powder [4]. This method offers advantages such as high phenolic content, non-hemolytic, antioxidant, and antimicrobial activity. Microbial fermentation utilizes microorganisms to produce genipin from geniposide. This method is environmentally friendly and can be performed under mild conditions.

However, it may have lower yields compared to other methods and requires optimization of fermentation parameters [5].

Enzymatic hydrolysis involves the use of enzymes to break down the precursor geniposide into genipin. This method offers high selectivity and mild reaction conditions, but it can be time-consuming and costly [6]. The primary objective of this research is to optimize the conditions for the conversion of geniposide to genipin using cheap commercial cellulase and to investigate the properties of chitosan-genipin films under various genipin concentrations. The study aims to establish a scalable and efficient methodology for producing genipin and to explore its application in fabricating chitosan-based films with improved physical properties.

2. Materials and Methods

2.1. Extraction and processing of geniposide and genipin

Geniposide extraction: Geniposide was extracted from 10 g of *G. jasminoides* seed powder using 100 mL of 50% ethanol (EtOH). The mixture was covered to prevent evaporation and stirred at 50°C for 1 hour. The mixture was then filtered and the filtrate was stored. This extraction process was repeated three times, pooling the extracts together.

Enzymatic Conversion of geniposide to genipin: The pooled geniposide extract was evaporated to concentrate the geniposide, which was then treated with cellulase in a citric acid-NaOH buffer across a pH range of 4.0 to 7.0. Cellulase (0.1 to 0.5 g per 1 g of geniposide) was added to 100 mL of the buffer containing 0.267 g of geniposide. The reaction was stirred at 50°C for 2 to 10 hours to enzymatically convert geniposide to genipin.

Genipin Extraction: Genipin was then extracted three times from the hydrolysate using 3×50 mL of ethyl acetate. The extracts were combined and evaporated to obtain purified genipin.

2.2. Reaction of genipin with amine-containing compounds

The extracted genipin (8 mg) was reacted with different amine-containing compounds (ethanolamine, glycine, urea, diethanolamine, n-pentylamine, monosodium glutamate - MSG) in a solution of 50% EtOH at 75°C for 2 to 12 hours. The amine-to-genipin molar ratio ranged from 1 to 3. Post-reaction, the mixture was dried in a vacuum dryer to yield the pigment powder.

2.3. Chitosan-genipin film formation

A 2% w/v chitosan solution was prepared in a 1% w/v acetic acid containing 0.6% w/v glycerol. An aqueous solution of genipin was added and the mixture was thoroughly stirred before being poured in Petri dishes (90 mm diameter) and dried at 40°C for 72 hours to form chitosan-genipin films. These films were manually peeled and conditioned in a closed chamber with 75% RH at room temperature at least 2 days before further characterization.

2.4. Colorimetric measurements

The absorption spectra of solutions were recorded using an UH5300 spectrophotometrer (Hitachi, Japan).

To quantify the color of genipin-based colorants, their aqueous solutions were absorbed on a white filter paper and the CIE Lab* color space of the colored paper was measured using a Minolta colorimeter (Konica, Japan). The surface color of chitosan-genipin films was also measured using the same instrument. Within this color space, the L*-value represents the lightness or luminance of a color, ranging from 0 (black) to 100 (white). The a*-value indicates the position of the color along the red-green axis, with positive values representing red and negative values representing green. The b*-value, on the other hand, represents the position of the color along the yellow-blue axis, with positive values indicating yellow and negative values indicating blue.

The colorimetric measurements were conducted in triplicate. Statistical analysis was performed using IBM SPSS Statistics version 20.0. Analysis of variance (ANOVA) with Duncan's multiple range test at $p < 0.05$ was carried out to compare the mean values.

3. Results and Discussion

3.1. Factors influencing the hydrolysis of geniposide

3.1.1. pH

Cellulase activity, crucial for geniposide hydrolysis, shows strong pH dependence [7]. While the enzyme (sourced from *Trichoderma reesei*) operates between pH 4.5 and 7.5, optimal conditions for genipin yield were investigated due to side reactions at specific pH levels causing blue product formation and potential genipin degradation in acidic conditions. Because genipin strongly absorbs 325 nm light, we used absorbance at this wavelength as the indicator of genipin production [8]. Figure 1 illustrates variations in blue pigment intensity across a pH range of 4 to 7, with the darkest blue at pH 4.5, suggesting optimal enzymatic activity and minimal side reactions at this pH.

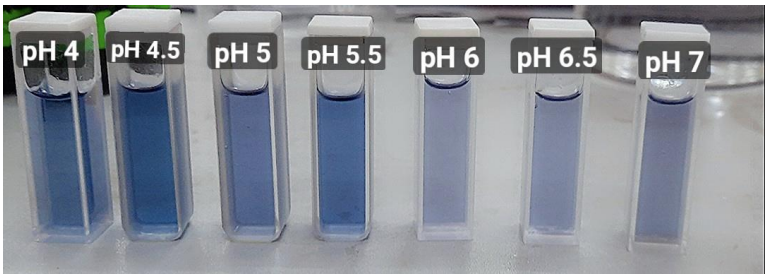


Figure 1. *Gardenia blue solution in different pH of enzymatic reaction.*

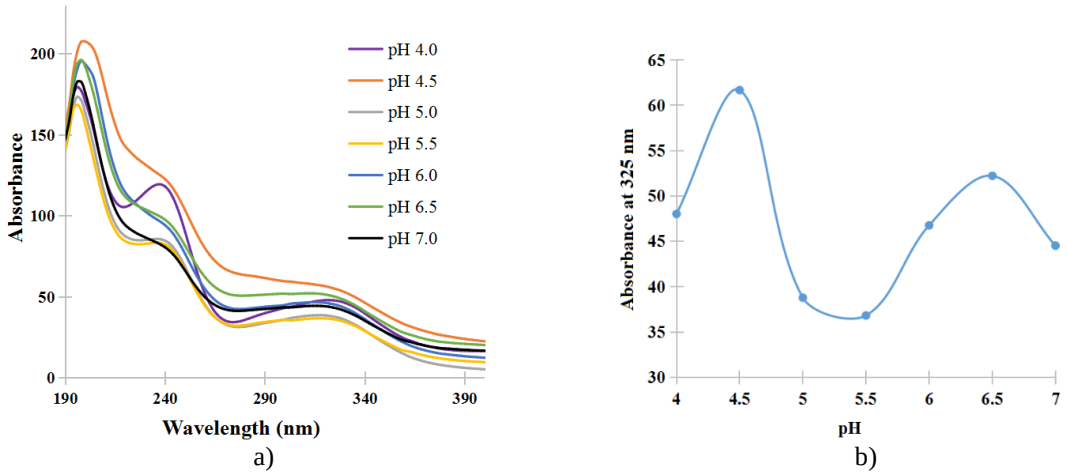


Figure 2. *(a) UV spectra and (b) absorbance at 325 nm of genipin solutions after enzymatic reaction at different pH conditions.*

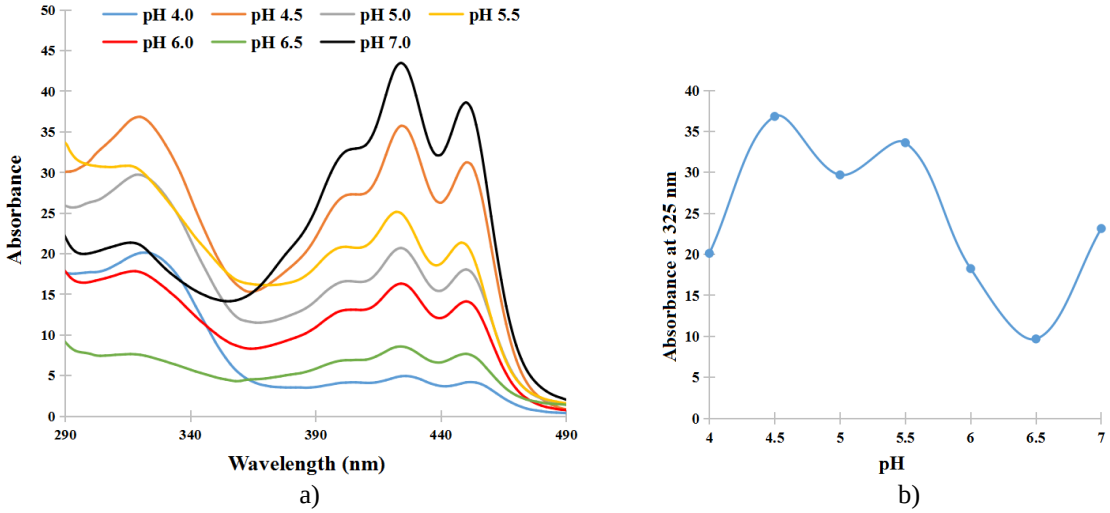


Figure 3. *(a) UV-vis spectra and (b) absorbance at 325 nm of genipin solutions after ethyl acetate extraction and enzymatic reaction in different pH conditions.*

This finding was supported by UV-VIS spectra and color measurements indicating the highest genipin concentration at pH 4.5 (Figure 2 and Figure 3), corroborated further by color metrics indicating significant differences in blueness, with pH 4.5 showing the deepest blue tone (Table 1).

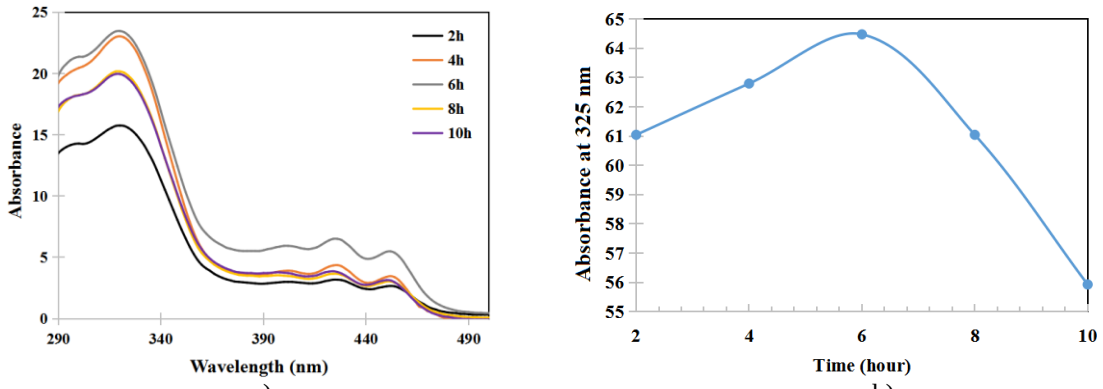
Table 1. *Color measurement of gardenia blue solutions in different pH of enzymatic reaction.*

pH	L value	a value	b value
4.0	61.99 ± 4.28^{b}	-1.03 ± 0.66^{bc}	-2.95 ± 1.79^{b}
4.5	50.14 ± 2.75^{a}	-2.06 ± 0.76^{abc}	-6.12 ± 0.36^{a}
5.0	58.28 ± 3.79^{b}	-0.76 ± 1.63^{c}	-2.75 ± 0.87^{b}
5.5	57.39 ± 3.24^{b}	-3.26 ± 1.47^{a}	-4.03 ± 1.35^{b}
6.0	60.13 ± 6.02^{b}	-2.55 ± 0.44^{ab}	-1.97 ± 1.55^{b}
6.5	61.23 ± 3.03^{b}	-0.56 ± 0.44^{c}	1.2 ± 0.58^{c}
7.0	63.60 ± 3.12^{b}	-0.25 ± 0.26^{c}	1.23 ± 0.33^{c}

Note: different superscript letters in a column illustrate significant differences ($p < 0.05$).

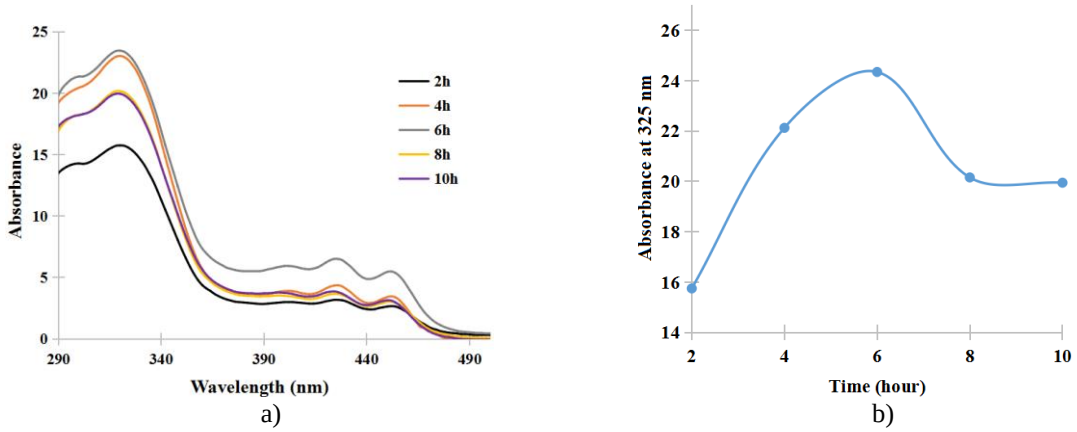
Therefore, we chose pH 4.5 as the most favorable for genipin production using cellulase [10].

3.1.2. Time



a) b)

Figure 4. *(a) UV-vis spectra and (b) absorbance at 325 mn of genipin solutions after enzymatic reaction at different times of enzymatic reaction.*



a) b)

Figure 5. *(a) UV-vis spectra and (b) absorbance at 325 nm of genipin solutions after ethyl acetate extraction in different times of enzymatic reaction.*

The reaction time profoundly impacts genipin yield, with optimal conditions needing balancing to enhance efficiency and minimize ineffective durations. Analysis of blue pigment intensity across varying times (2 to 10 hours) identified 6 hours as potentially optimal, supported by UV-VIS spectra (Figures 4 and 5). Extended times showed diminishing returns in genipin content, suggesting enzymatic activity peaks and declines due to substrate depletion [11]. This duration effectively maximizes genipin yield while minimizing potential losses from prolonged exposure to enzymatic processes and side reactions.

3.1.3. Enzyme concentration

Enzyme concentration significantly influences the efficiency of the enzymatic hydrolysis of geniposide. Optimal enzyme usage is crucial for maximizing yield while minimizing waste. Figure 3.10 illustrates variations in blue pigment intensity across different enzyme concentrations ranging from 0.1 to 0.5 g per 1 g of geniposide. The darkest blue observed at 0.1 g suggests this concentration might be optimal. However, further experiments, including UV-VIS spectra and color measurements, were conducted for verification.

Figures 6 and 7 display the effects of enzyme concentration on genipin yield. An increase in genipin was noted from 0.1 to 0.2 g of enzyme per gram of geniposide, followed by a decrease from 0.2 to 0.5 g. The optimal genipin content was observed at 0.2 g, as evidenced by the UV-VIS spectra at 325 nm and the corresponding absorbance measurements.

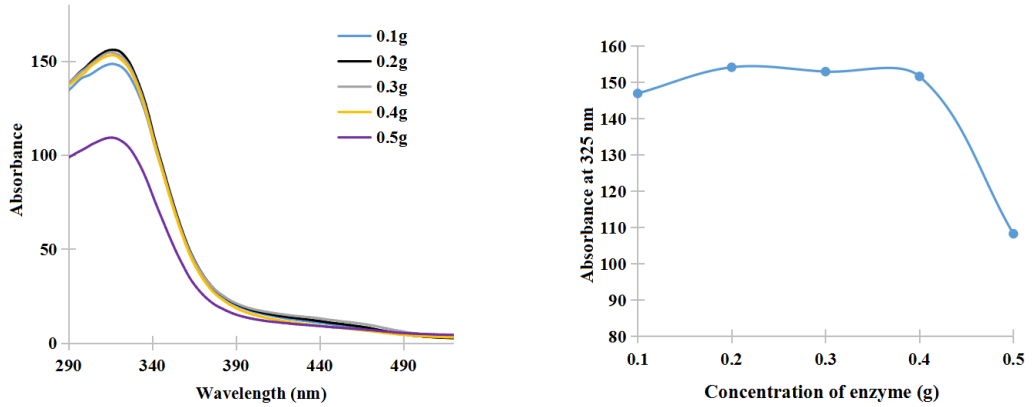


Figure 6. *(a) UV-vis spectra and (b) absorbance at 325 nm of genipin solutions after enzymatic reaction in different enzyme concentration.*

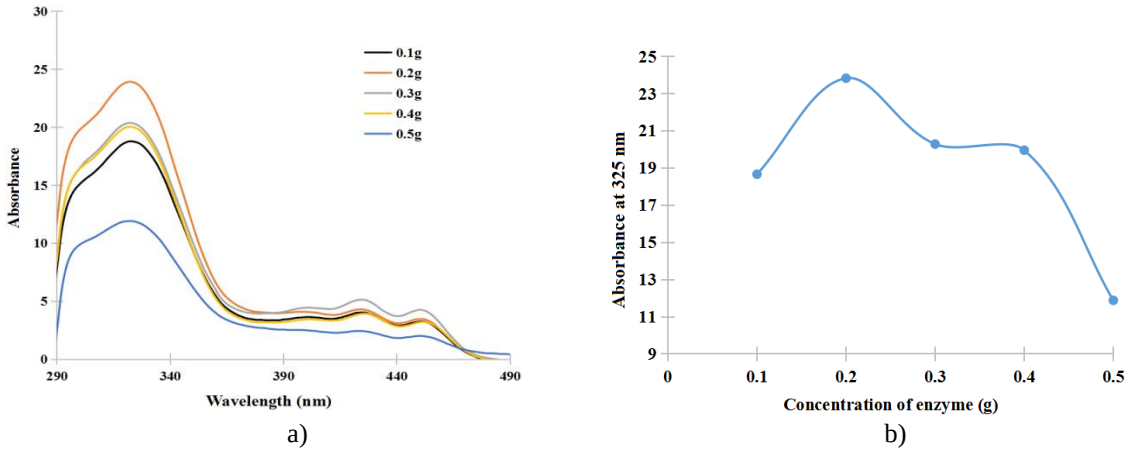
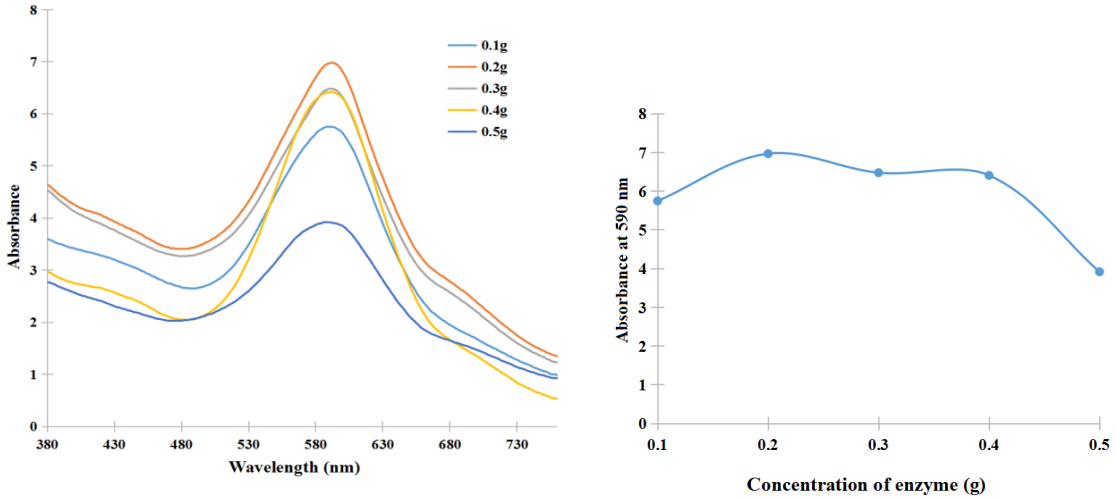


Figure 7. *(a) UV-Vis spectra and (b) absorbance at 325 nm of genipin solution after ethyl acetate extracted in different enzyme concentration.*

Figure 8 further supports these findings, showing a peak in the production of gardenia blue pigment at an enzyme concentration of 0.2 g per gram of geniposide, particularly noted at 590 nm, the UV λ max for the blue pigment of gardenia blue.



a) b)

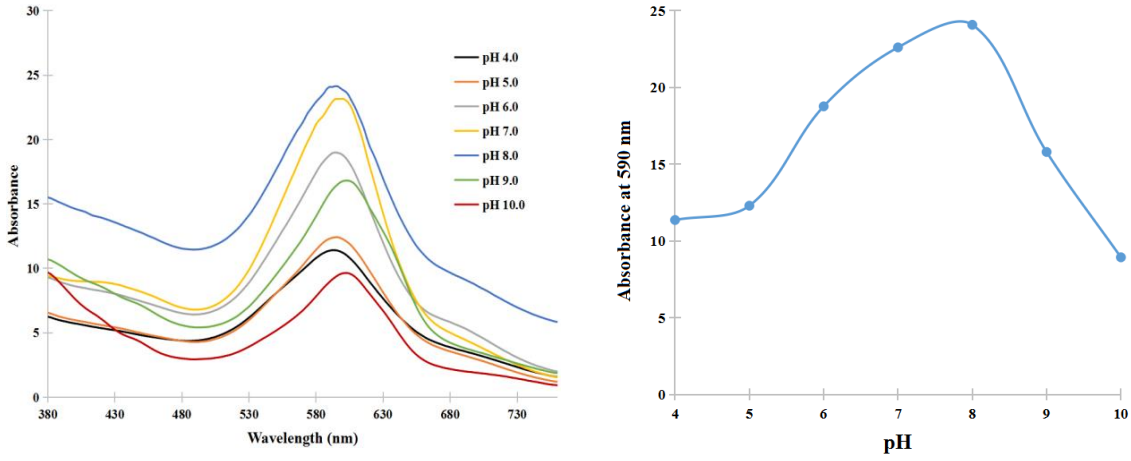
Figure 8. *(a) UV-vis spectra and (b) absorbance of 325 nm of gardenia blue solution in different enzyme concentrations.*

These results suggest that lower enzyme concentrations prevent excessive enzyme-substrate interactions that might lead to undesirable side reactions, thus optimizing genipin yield. Conversely, higher concentrations may induce rapid interactions between genipin and the amino groups in cellulase, leading to reduced pigment production.

3.2. Factors affecting gardenia blue pigment formation

3.2.1. pH

The formation of gardenia blue pigment is sensitive to the pH of the reaction environment. UV-VIS spectra (Figure 9) and colorimetric data (Table 2) confirm that the reaction between genipin and amino groups is pH-dependent [12]. The interaction is less efficient under acidic conditions, with no significant pigment formation. Under basic conditions, the reaction shifts away from blue pigment production, yielding mauve or dark red colors instead [13]. Meanwhile, pH levels 7 and 8 yield the darkest blue pigment, suggesting these conditions are most favorable [14].



a) b)

Figure 9. *(a) UV-vis spectra and (b) absorbance at 590 nm of gardenia blue solution in different pH of the formation of garnedia blue reaction.*

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Table 2 highlights that the highest amount of gardenia blue is found at pH 8, where the b-value of -9.39 surpasses other pH levels. This peak suggests a critical point where the unprotonated amino groups most effectively react with genipin. As the pH increases beyond 8, saponification of genipin occurs, leading to different color productions as genipinic acid forms and reacts with the amino groups [13].

Table 2. *Color measurement of gardenia blue solution from genipin produced in different pH.*

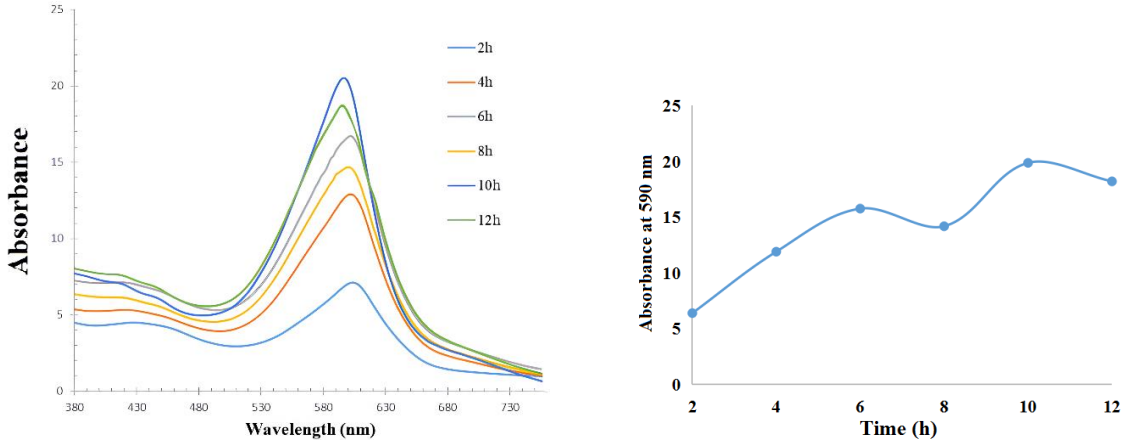
pH	L value	a value	b value
4.0	49.13 ± 0.99^{cd}	-2.58 ± 0.67^{d}	-7.88 ± 0.38^{a}
5.0	49.13 ± 2.30^{cd}	-4.02 ± 0.38^{cd}	-5.88 ± 0.45^{b}
6.0	45.42 ± 0.48^{bc}	-3.84 ± 0.58^{cd}	-7.40 ± 1.42^{a}
7.0	41.96 ± 1.72^{ab}	-4.99 ± 0.68^{bc}	-8.43 ± 0.24^{bc}
8.0	41.40 ± 1.87^{a}	-4.56 ± 0.35^{c}	-9.39 ± 0.51^{cd}
9.0	44.68 ± 0.33^{ab}	-8.56 ± 0.12^{a}	-3.38 ± 0.23^{c}
10.0	51.48 ± 0.29^{d}	-6.66 ± 1.04^{b}	-2.07 ± 0.67^{d}
pH	L values	a values	b values

Note: different superscript letters in a column illustrate significant differences ($p < 0.05$).

In summary, the optimal pH for producing gardenia blue is identified as pH 8, based on the combined results of spectral and colorimetric analysis, aligning with current literature which suggests that neutral to slightly basic conditions favor the production of gardenia blue pigments. This finding will guide further experimental settings and help in scaling up the production process for industrial applications.

3.2.2. Time

Optimal reaction time for producing gardenia blue pigment was evaluated by examining genipin interaction with $-NH_2$ groups over intervals ranging from 2 to 12 hours. UV-vis spectra (Figure 10) indicated that absorbance at 590 nm, the peak characteristic of gardenia blue pigment, varied significantly with time. Notably, the intensity of the blue pigment peaked at 10 hours, suggesting this duration maximizes genipin's effective interaction with $-NH_2$ to produce a vibrant blue color. Colorimetric data (Table 3) supported this finding, with the 10-hour sample showing the deepest blue (lowest b value).



a) b)

Figure 10. *(a) UV-vis spectra and (b) absorbance at 590 nm of gardenia blue solutions in different times of the formation of garnedia blue reaction.*

Table 3. *Color measurements for reaction time variability.*

Time	L value	a value	b value

2h	51.73±3.08[b]	-3.22±0.86[d]	2.64±0.36[b]
4h	47.03±0.92[a]	-5.94±0.63[a]	-5.16±1.09[a]
6h	46.42±1.04[a]	-5.02±0.61[a]	-5.95±0.86[a]
8h	45.27±1.65[a]	-5.00±0.98[ab]	-4.81±0.34[a]
10h	43.32±2.06[a]	-5.00±0.46[ab]	-6.26±0.89[a]
12h	44.50±1.79[a]	-3.85±0.28[cd]	-5.50±2.07[a]

Note: Values in a column with different lowercase letters indicate significant differences ($p < 0.05$).

3.2.3. Amine variability

After optimizing reaction pH and time between genipin and the amino group, we further explored the reaction of genipin with various amine-containing compounds to find potential genipin-based colorants. Figure 3.11 displayed distinct color changes when genipin reacted with different amines, and Figure 12 highlighted the spectral differences of the resultant colors.

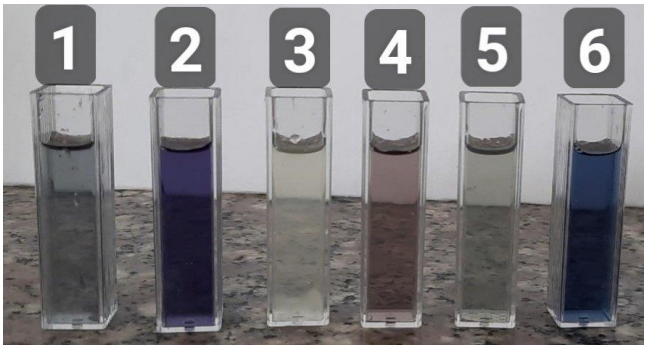


Figure 11. *Color difference of solutions after reacting with different amine-containing compounds:. (1) ethanolamine (2) glycine (3) urea (4) diethanolamine (5) n-pentylamine (6) MSG.*

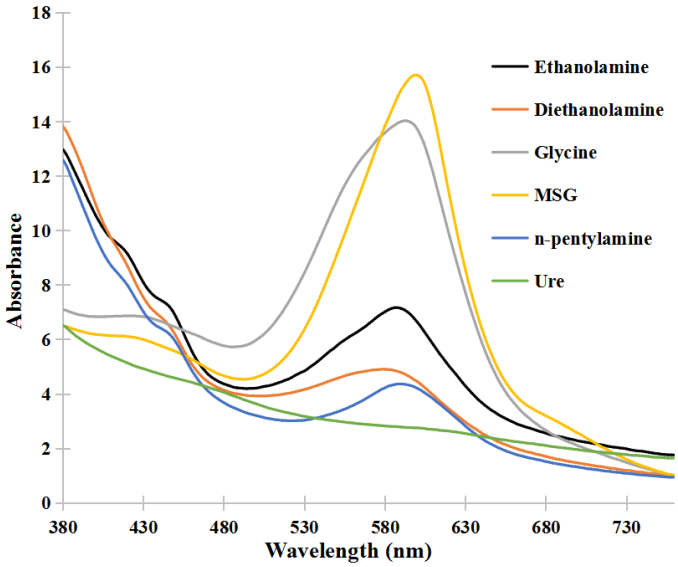


Figure 12. *UV-vis spectra of gardenia blue solution in types of amine-containing compounds.*

The colorimetric results, as summarized in Table 4, showed that MSG and glycine produced the most intense blue colors, indicating optimal interactions at these conditions.

Table 4. *Color measurements across different amines.*

Compound	**L value**	**a value**	**b value**

MSG	44.19±1.92^{b}	-4.46±1.68^{a}	-8.87±0.29^{b}
Ure	62.81±0.42^{d}	0.78±0.68^{b}	2.40±0.10^{c}
Ethanolamine	55.3±1.07^{cd}	-2.87±0.45^{a}	7.26±0.49^{f}
Glycine	41.07±1.31^{a}	-0.16±0.43^{b}	-10.24±0.09^{a}
Diethanolamine	54.18±0.80^{c}	-0.43±0.23^{b}	6.03±0.57^{e}
n-pentylamine	56.54±0.46^{d}	-4.11±1.14^{a}	3.72±0.39^{d}

Note: different superscript letters in a column illustrate significant differences ($p < 0.05$).

3.3. Properties of chitosan-genipin films

After assessing factors affecting blue pigment production from genipin, we investigated the properties of chitosan-genipin films, particularly focusing on optical properties, crosslinking behavior, moisture content, mechanical properties, and swelling characteristics.

3.3.1. Optical properties and crosslinking behavior

UV-vis spectroscopy (Fig. 13) revealed the influence of genipin concentration on the crosslinking of chitosan films. The absorption spectra exhibited significant changes across the genipin concentration range from 0.0025 to 0.01. Films showed intense absorption in the 260-700 nm range, peaking around 600-610 nm, indicative of the radical polymerization of genipin and its crosslinking with chitosan [3]. Notably, higher genipin concentrations correlated with increased absorption at both 280-290 nm and 600-610 nm, suggesting more extensive crosslinking [15].

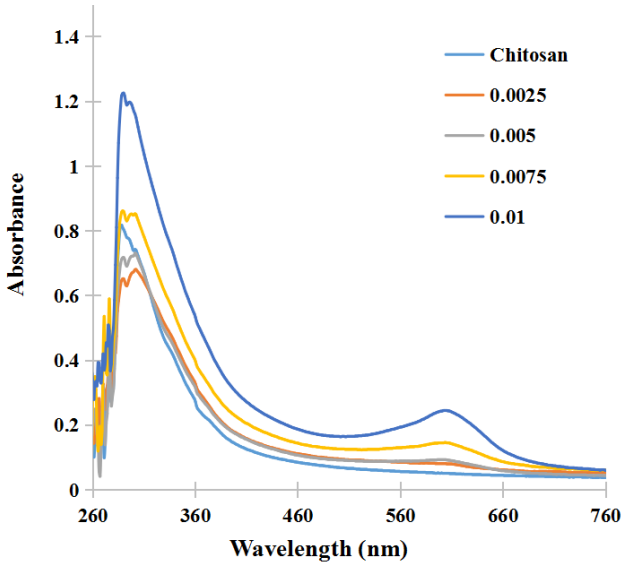


Figure 13. *UV-vis spectra of genipin-chitosan films.*

3.3.2. Moisture content analysis (Table 5) of the films indicated a clear trend: increased genipin concentration led to lower moisture content, suggesting that higher crosslinking density reduced the hydrophilicity of the films.

Table 5. *Moisture, swelling, and mechanical properties of chitosan-genipin films.*

Genipin/ chitosan (g/g)	Moisture (%)	Thickness (mm)	Tensile Strength (N/mm^2)	Elongation at break (%)	Swelling degree (%)
0	9.21±0.03^{d}	0.104±0.013^{a}	21.77±6.56^{a}	227.12±14.06^{b}	165.17±10.27^{c}
0.0025	7.87±0.02^{c}	0.098±0.003^{a}	18.42±0.99^{a}	161.72±79.8^{a}	148.41±21.47^{b}
0.005	5.41±0.01^{b}	0.107±0.010^{a}	16.43±1.19^{a}	163.22±18.77^{a}	129.69±3.70^{a}

0.0075	3.47±0.01[a]	0.106±0.007[a]	23.46±2.75[a]	179.72±0.48[a]	121.31±1.79b[c]
0.01	2.01±0.02[a]	0.099±0.001[a]	19.92±3.50[a]	182.64±5.12[a]	109.62±4.04[d]

Note: different superscript letters in a column illustrate significant differences ($p < 0.05$).

3.3.3. The tensile strength and elongation of the films (Table 5) were measured to assess their mechanical integrity and flexibility. Results showed that films with a genipin/chitosancontent of 0.0075 exhibited superior mechanical properties compared to other concentrations, suggesting an optimal balance of flexibility and crosslinking.

3.3.4. Swelling behavior of the films (Table 5) was inversely proportional to the genipin concentration, indicating that higher crosslinking density effectively reduced the film's ability to absorb and retain water [16].

In conclusion, chitosan-genipin films demonstrated a dependence of optical and mechanical properties on the genipin concentration, with an optimal balance achieved at intermediate concentrations. This balance enhances film stability and decreases hydrophilicity, making these films suitable for various applications where moisture sensitivity is a critical factor.

4. Conclusion

This study successfully demonstrated an optimized method for the extraction of geniposide from Gardenia jasminoides and its enzymatic conversion to genipin, with subsequent application in creating enhanced chitosan-genipin films. The optimal extraction of geniposide was achieved using 100 mL of 50% ethanol for each 10 g of seed powder, with the best conversion occurring at pH 4.5 for reduced side reactions and maximum yield. The resultant genipin was effectively used to produce chitosan-genipin films, where the film containing 0.01 genipin/chitosan mass ratio showed significant properties, including the highest UV-vis absorbance at 610 nm and superior mechanical strength of 19.92 N/mm², with a significantly reduced moisture content of 2.01%. These findings highlight the potential of genipin as a viable crosslinker in biopolymer applications, promising for medical and environmental uses due to its enhanced mechanical properties and moisture resistance.

Acknowledgments

The authors acknowledge Ho Chi Minh City University of Technology and Education for facility support in completing this study.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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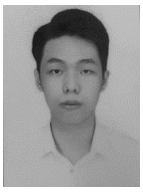
Vo Thi Nga received a PhD degree at Vietnam National University HCMC, VNUHCM- University of Science. Her fields of interest: Investigation of chemical constituents of the herb, including extraction, isolation and elucidation of natural product structures; and in-vitro and in-vivo biological activity assay of natural products. Working for Ho Chi Minh City University of Technology and Education since 2001.Email: ngavt@hcmute.edu.vn. ORCID: https://orcid.org/0000-0003-2369-3260

Ho Dac Loc graduated from the Ho Chi Minh City University of Technology and Education (HCMUTE) in 2023 with a Bachelor's degree in Food Technology. Loc currently works as a Food Industry Engineer. Loc's research interests include food technology, food engineering, food testing, and food quality management. Email: 18116022@student.hcmute.edu.vn. ORCID: https://orcid.org/0009-0009-3978-5574



Dang Hoang Duc holds a Bachelor's degree in Food Technology Engineering, which he obtained from the Ho Chi Minh City University of Technology and Education (HCMUTE) in 2023. Currently, Duc is employed as a Food Industry Engineer at Sunrise Global Joint Stock Company, Ho Chi Minh City, Vietnam. His research interests span across various aspects of the food industry, including food technology, food engineering, food testing, and food quality management. Email: ducdang29122000@gmail.com. ORCID: https://orcid.org/0009-0009-9378-0758

Nguyen Vinh Tien received the specialist degree in chemistry from Tula State University, Tula, Russia in 2009 and the Ph.D degree in chemistry also from Tula State University in 2014.
From 12/2013 to 12/2014, he was a probationary lecturer at the department of Chemical Technology, faculty of Chemical and Food Technology, Ho Chi Minh City University of Technology and Education, Ho Chi Minh City, Vietnam. From 12/2014 till now, he worked as a full-time lecturer in the same faculty. His research interests include nanomaterials and polymeric materials applied in food technology, synthesis of curcumin derivatives and analogues.
Email: tiennv@hcmute.edu.vn. ORCID: https://orcid.org/0000-0002-1863-4138