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Enzymatic hydrolysis of anthocyanin glycosides by *Bifidobacterium infantis* cell-free enzymes: Stability assessment and bioavailability implications

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ABSTRACT: The bioavailability of anthocyanins is crucial for conveying their health benefits, but they are poorly absorbed in the gastrointestinal tract (GIT). The deglycosylation of anthocyanins is the key step in improving their bioavailability and bioactivity. This study investigated the potential of β-glucosidase and β-galactosidase cell-free enzymes from Bifidobacterium infantis, on glycolytic hydrolysis of essential anthocyanins, including Cyanidin-3-O-β-D-glucoside (C3-Glu), Malvidin-3- O-β-D-glucoside (M3-Glu), Cyanidin-3-O-β-D-galactoside (C3-Gal) and Delphinidin-3-O-β-D-glucoside (D3-Glu). According to our previous work, *Bifidobacterium infantis* (*B. infantis*) was chosen for this study due to its high β- glucosidase and β-galactosidase activity. The anthocyanin glycosides' stability was also evaluated to distinguish chemical instability from enzymatic degradation. *B. infantis* β-glucosidase exerted the highest activity toward C3-Glu, forming high levels of protocatechuic acid as an active compound, with lower hydrolytic rates observed for M3-Glu. Additionally, *B. infantis*'s β-galactosidase activity was efficient against C3-Gal. According to this study, *B. infantis* prefers cyanidin glucoside and cyanidin galactoside as substrates. Under experimental circumstances, delphinidin exhibited lower chemical stability, but malvidin and cyanidin glycosides exhibited similar stability. In conclusion, chemical instability is the cause of delphinidin's rate of degradation. According to these results, *B. infantis* may be utilized as a probiotic supplement to improve the health advantages and bioavailability of meals high in anthocyanins, supporting the development of functional foods and medicinal formulations.

KEYWORDS: Anthocyanins; Bioavailability; Deglycosilation; *Bifidobacterium infantis*; β-Glucosidase; β-Galactosidase; Enzymatic hydrolysis, Cell-free Enzyme Stability

1. INTRODUCTION

Anthocyanins are water-soluble natural pigments of fruits, vegetables, and flowers that show all the red, blue, and purple gradations. Being flavonoid pigments, they have been the subject of several studies on their health-friendly effects, generally as antioxidants and anti-inflammatory agents. Research provides a range of evidence that anthocyanins have benefits in preventing and managing certain chronic diseases: cardiovascular diseases, neurodegenerative disorders, and cancers [1–5]. In addition to their protective properties, anthocyanins also possess chemoprotective properties, further justifying their therapeutic potency [6,7]. The anthocyanins are derivatives of the 2-phenylbenzopyrylium ring with hydroxyl and methoxyl substituents on the aromatic ring in different substitutions. They mostly occur in higher plants as glycoside by linking aglycone or anthocyanidin with glucose, arabinose, xylose, and galactose at C3 via β-linkages.

While the sugar moieties are important for the stability and solubility of anthocyanins in plant tissues, they also affect the bioavailability of the pigment in humans. The bioavailability of anthocyanins is generally considered low, which restricts the extent to which these compounds exert their beneficial effects in the body following ingestion [8–10].

Metabolism within the gastrointestinal tract by gut microbiota breaks down anthocyanins into other structures through mechanisms including deglycosylation, ring fission, and hydroxylation. Such microbial

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transformations could enhance or hamper the bioactivity of anthocyanins; deglycosylation has been an important step in increasing their bioavailability. Upon deglycosylation, this increased absorption of the aglycone form exerts several biological activities: antioxidant, anti-inflammatory, and anticancer. The role of probiotics like Bifidobacterium in modifying anthocyanin glycosides is little known [4,5,11,12].

Although extensive literature on the bioconversion of other plant glycosides, such as isoflavones, by probiotics [13–16], the bioconversion of anthocyanin glycosides, especially by single probiotic strains, has been poorly explored. Previous studies have reported the degradation of delphinidin and malvidin glucosides by *Bifidobacterium bifidum*, with differential stability observed between these two anthocyanins [14,17]. On the other hand, hardly any studies have discussed the deglycosylation of cyanidin glycosides, which is common in various fruits and vegetables. This aspect of Bifidobacterium involvement in the deglycosylation process of cyanidin glycosides and its effect on bioavailability has not been studied.

The aglycone fragment of the anthocyanin is accountable for the biological activity of anthocyanins [18,19]. An increase in the biological activity of the aglycones after the removal of glycoside may be explained by two factors: this aglycone structure formed after the removal of glycoside in a better way facilitates hydrogen donation and electron transfer, thus neutralizing the free radicals for flavonols. Besides, the aglycones can chelate metal ions, stabilizing the free radicals and improving the antioxidant activity [14,20].

The awareness of cyanidin is amplified due to the protective action in carcinogenesis of the active metabolite, protocatechuic acid [21,22]. Anthocyanins are not intact by the time they reach the colon. Instead, the colonic microbiota breaks down the unabsorbed anthocyanins into simpler metabolites, which have been demonstrated to support the growth of beneficial bacteria, including *Bifidobacterium* [21,23–26]. The development of probiotic-anthocyanin formulations for enhancing the bioavailability and bioactivity of anthocyanin is of distinct importance for patients who lack β-glucosidase activity due to diet, illnesses, and age [27,28] and for patients who are lactose intolerant because of the deficiency of β-galactosidase [18,29].

This study aimed to determine the ability of *Bifidobacterium infantis* on glycolytic hydrolysis of Cyanidin-3-O-β-D-glucoside and Cyanidin-3-O-β-D-galactoside (as the main glycosylated form of anthocyanins, existing in plants) along and to compare with the other previously studied anthocyanins Malvidin-3-O-β-D-glucoside, and Delphinidin-3-O-β-D-glucoside, to upsurge their bioavailability and bioactivity. In addition, the stability of Cyanidin-3-O-β-D- glucoside and -galactoside, Malvidin-3-O-β-D-glucoside, and Delphinidin-3-O-β-D-glucoside has been considered to determine whether the degradation is caused by the presence of probiotic enzymes or due to the chemical instability of anthocyanin under experimental conditions.

2. RESULTS and DISCUSSION

2.1. β-glucosidase and β-galactosidase activity of B.infantis

The β-glucosidase and β-galactosidase activity were determined from the cell-free enzymes extracted from B. infantis used in the present study and were expressed as U/mg protein. One Unit defined the amount of p-nitrophenol or o-nitrophenol produced from the enzyme activity assay reaction per ml min under the experimental conditions. The β-glucosidase activity was shown to be 17.39±1.57 U/mg protein, while the β- galactosidase activity was 15.35±1.11U/mg protein [30]. The differences in enzyme activity observed in the present study related to other data presented earlier can be attributed to the different strains of probiotics [15, 30]. Furthermore, the activity of different probiotic enzymes was influenced by the type and concentration of carbon source on the probiotic growth medium. The differences in enzyme activity reported from different studies can also be attributed to glucose- inhibiting enzyme activity through carbon catabolite repression (CCR) [17].

2.2. β-glucosidase and β-galactosidase activity of B.infantis

In this study, the impact of temperature on the activity of *B. infantis* β-glucosidase and β-galactosidase was investigated at temperatures from 30-80 °C. This study found that the optimal temperature of β-glucosidase and β-galactosidase is 40°C. (Figure 1; A and B). At higher temperatures, β-galactosidase showed higher activity than β-glucosidase activity, with non-significant differences at 50 °C, 96.45%, and 86.64%, of the remaining enzyme activity ($p>0.05$). Remaining activity of β-galactosidase was significantly higher compared to β-glucosidase ($p<0.001$) when the enzymatic assay was done at high temperatures, that is, 65.7% and 43.94% (at 60 °C); 28.38% and 10.93% (at 70 °C); 12.37% and 1.57% respectively (at 80 °C).

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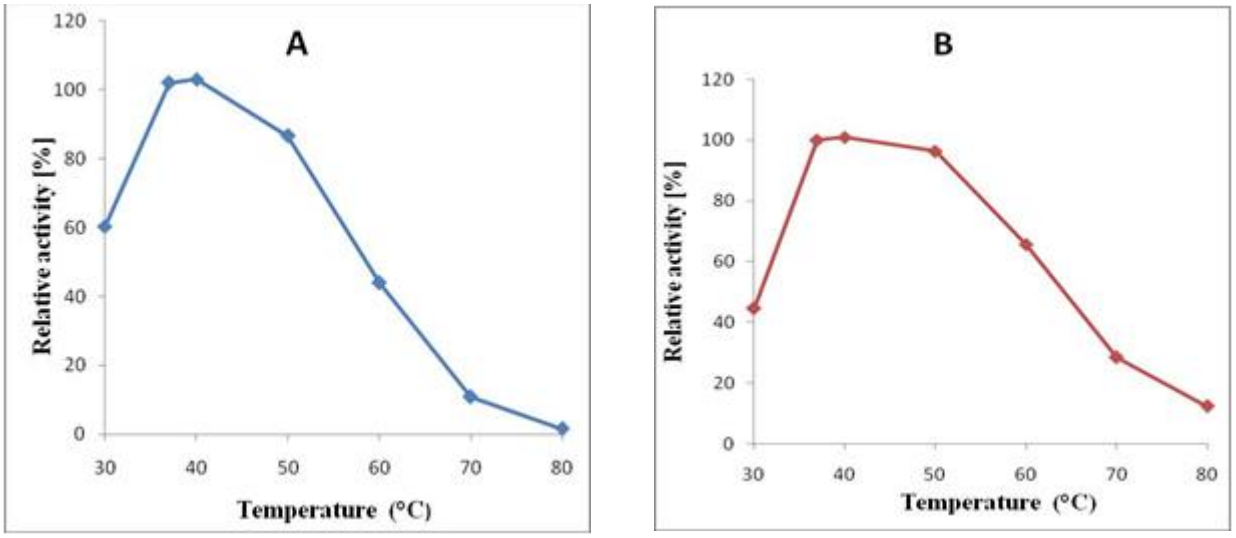
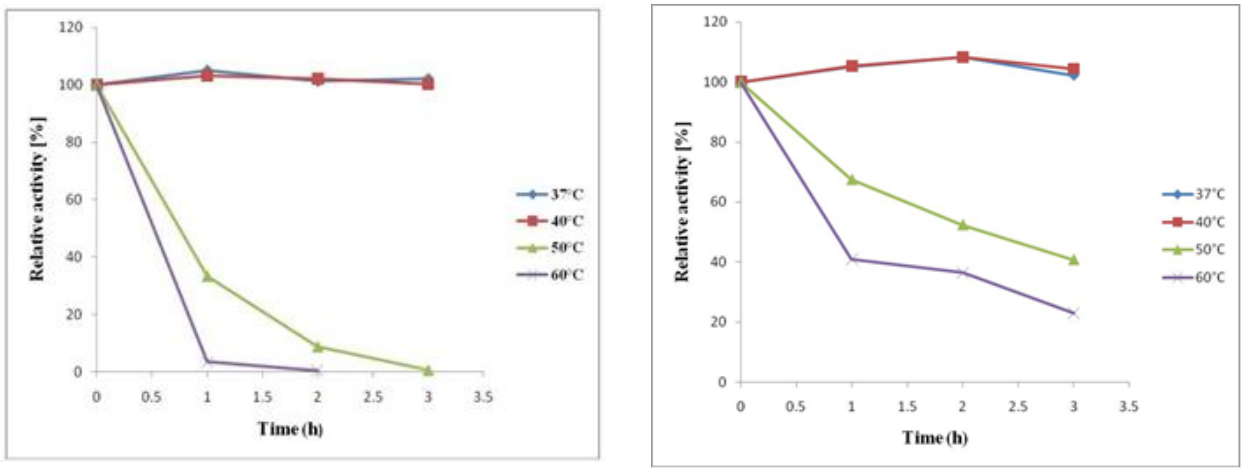


Figure 1. Optimum temperature of (A) β-glucosidase (A) and (B) β-galactosidase extracted from *Bifidobacterium* infantis cells. Enzymatic activity was determined by incubation for 15 min at different temperatures, pH 6.5, using p-NPGlu and o-NPGal as substrates.

The thermostability (degree of deactivation) of β-glucosidase and β-galactosidase was assessed at 30-60 °C. Figures 2 A and 2 B show that both enzymes have high stability at 37°C and 40°C temperatures. β-galactosidase activity was more stable during incubation for 1h at 50°C, with 67.5% remaining activity, while β-glucosidase showed 33.29% of initial activity. At 60°C, β-glucosidase lost most of its initial activity (3.3% activity remaining), while β-galactosidase activity was at 40.94% of its initial activity.



A) B)

Figure 2. Thermostability of β-glucosidase (A) and β-galactosidase (B) extracted from *Bifidobacterium infantis* cells, incubated at different temperatures at different times, followed by enzyme activity analysis at 37°C pH 6.5, incubation 15 min.

2.3. The impact of pH on the β-glucosidase and β-galactosidase activity and stability. The optimal pH and pH stability

In this study, the impact of different pH values on the properties of β-glucosidase and β-galactosidase were evaluated. The optimal pH for both enzymes was pH 6 (pH 6.5) (Figure 3).

The results show that both enzymes had maximum activity at pH 6. Both enzymes had high activity in the pH values 5-7 range. At pH 5, the retention of β-galactosidase activity was significantly lower than β-glucosidase, meaning 75.12% and 91.38%, respectively ($p<0.001$).

To assess the pH stability, β-glucosidase and β-galactosidase released from the cells were incubated at 37°C for 2 hours across a range of pH values, and the retention of activity was determined by the standard

enzymatic activity assay reaction using p-NPGlu and o-NPGal. Both enzymes had the highest stability at pH 6 (and 6.5) and were quite stable in the pH range from 5-7 (more than 90%). At pH 4, retention of β-galactosidase activity was lower than that of β-glucosidase, meaning 56.2% and 67.5%, respectively ($p<0.05$).

The determination of the optimal temperature is important for determining the analysis and processing conditions in the future, while the thermostability results are important for the future applications of different thermal drying technologies (spray drying and fluidized bed drying). The results obtained from optimal pH and pH stability are beneficial for using these enzymes in various food applications, including fruit juices with low pH.

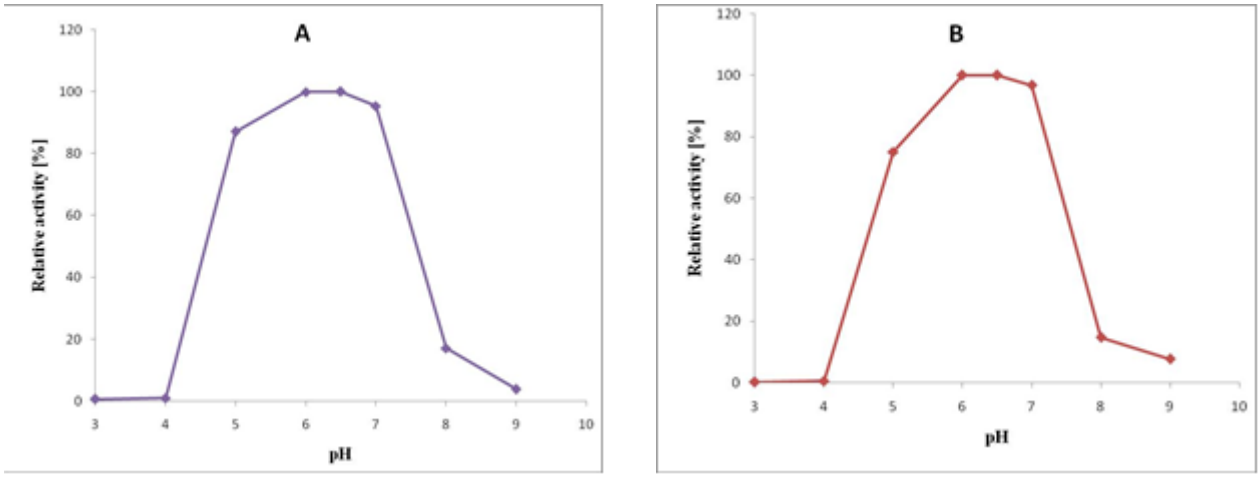


Figure 3. Optimal pH of cell-free β-glucosidase (A) and β-galactosidase (B) from Bifidobacterium infantis cells. The enzyme activity was determined at different pH, with 15 min incubation at 37°C, using p-NPGlu and o-NPGal as substrates.

2.4. Biotransformation of anthocyanin glucosides and galactosides

In the current research, the activity of cell-free extract of B. infantis containing β-glucosidase and β-galactosidase on deglycosylation rate of C3-Glu, C3-Gal, M3-Glu, and D3-Glu was determined.

Analysis of control samples, at baseline (time 0 h), showed that anthocyanin glucosides and galactosides were not recovered completely. The recovery was 84% for cyanidin glucosides (83.8 µM ± 3.9 µM) and 82% (82.5 µM ± 5.6 µM). This can be attributed to the absorption of anthocyanins on the denaturated proteins caused during cell disruption [12].

The elevated intracellular β-glucosidase activity in *B. infantis* did not correspond to the rate of glycolytic hydrolysis of different anthocyanin glycosides used in this study. These results suggest that the activity of enzymes, determined toward general substrates such as p-NPG and o-NPG, cannot predict the activity toward different groups of plant polyphenols nor different structure molecules of the same phenolic group.

Figure 4 presents the deglycosylation of cyanidin glucoside in the presence of the cell-free enzyme of *B. infantis* throughout the first hour of incubation, a higher rate of hydrolysis was observed for C3-Glu, followed by C3-Gal 18.1%, M3-Glu (17.89%), and D3-Glu (10%).

The main metabolites observed during the first hour of incubation were protocatechuic acid for C3-Glu and C3-Gal, syringic acid for M3-Glu, and gallic acid for D3-Glu. It has been demonstrated that microbiology degradation of anthocyanin glycosides leads to the formation of phenolic acids [31,32]. Phenolic acids were identified by comparing their UV spectra and elution characteristics against standards.

This study suggests that the preferred substrates for *B. infantis* are C3-Glu and C3-Gal. After 2h of incubation, the highest deglycosylation rate was observed for cyanidin glucoside (64.9%). During the 1h of incubation, no significant differences in the deglycosylation rate were observed for M3-Glu and C3-Gal ($p>0.05$). These differences became evident during the 2nd hour of incubation with a higher rate of hydrolysis for C3-Gal (39.3%) compared to M3-Glu (26.1%). No significant differences in the degradation rate between M3- and D3-Glu were observed after 2 hours of incubation, namely 26.1% and 24.9%, respectively ($p>0.05$).

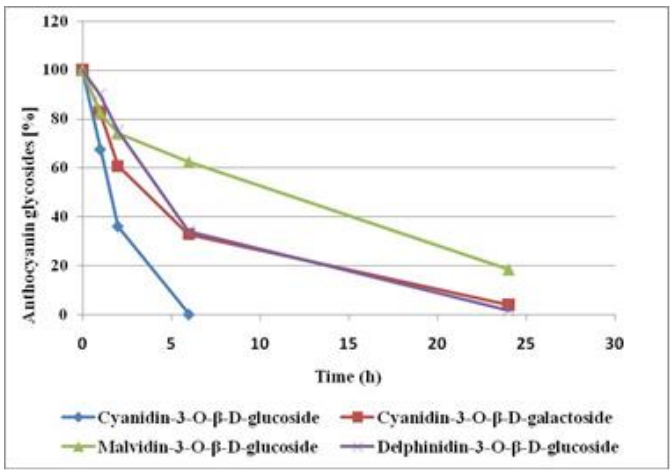


Figure 4. Comparison of deglycosylation rate of Cyanidin-3-O-β-D-glucoside, Cyanidin-3-O-β-D- galactoside, Malvidin-3-O-β-D-glucoside, and Delphinidin-3-O-β-D-glucoside in the presence of *Bifidobacterium infantis* cell-free enzymes.

After 6h of incubation, C3-Glu completely disappeared, leading to a high concentration of protocatechuic acid, with health-promoting properties. This is the first study to show the degradation of cyanidin glucoside by a single probiotic. Furthermore, no studies have been reported on probiotics bioconversion of anthocyanin galactosides. Aura et al. [12] stated the deglycosylation of cyanidin-rutinoside and cyanidin glucoside by gut microflora, giving the protocatechuic acid as the main metabolite.

C3-Gal was degraded faster in comparison than M3-Glu during 6h of incubation in the presence of the cell-free enzyme. The high degradation rate of D3-Glu after 6h of incubation, comparable to C3-Glu and higher compared to M3-Glu, can be attributed more to the chemical instability of D3-Glu under pH 6.5 and incubation temperature (Figure 5).

Similar results were observed in previously reported studies, which showed that M3-Glu is more stable in control samples than D3-Glu. These differences in the stability of degradation can be justified by the methoxyl groups located in the ring B of malvidin [32]. It is evident that among the studied anthocyanin glycosides, C3-Glu and C3-Gal were shown to be the preferred substrate for *Bifidobacterium infantis*, leading to a high concentration of protocatechuic acid, as a bioactive metabolite, which has been reported to exert antioxidant activity, anti-inflammatory and protective effect on colon cancer (Figures 6-8). These results suggest that the activity of enzymes, determined toward general substrates such as p-NPG and o-NPG, cannot predict the activity toward different groups of plant polyphenols nor different structure molecules of the same phenolic group.

The findings demonstrated that aglycone forms of anthocyanins quickly underwent further degradation. So, the bioavailability in the colon may be very small because they are quickly degraded to further metabolic compounds. The further degraded compounds of anthocyanin are phenolic compounds, which are known to exhibit antioxidant activity, anti-inflammatory effect, and chemoprotective effect. Thus, interest in identifying metabolites of anthocyanin probiotic degradation has increased [33].

A diet containing anthocyanins in fruits and vegetables significantly impacts gut microbiota composition. However, anthocyanins exhibit limited bioavailability due to their partial absorption in the small intestine, with a percentage of total polyphenol ranging 5-10% intake being absorbed. Notably, most dietary anthocyanins reach the colon in the unchanged form. In the colon, these anthocyanins encounter the microbiota and go through a biotransformation process before being taken up by the

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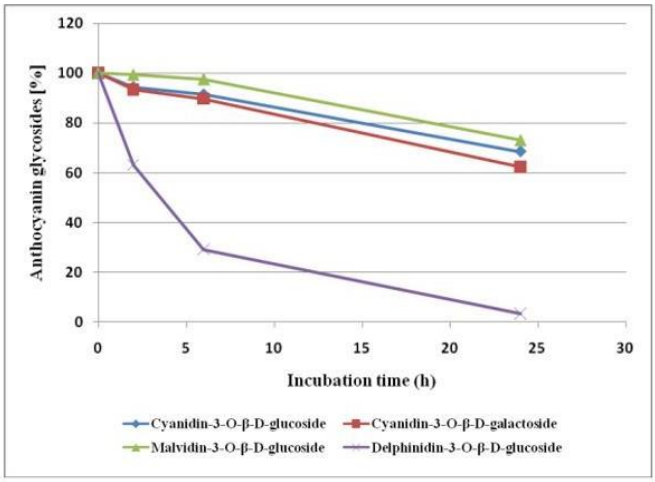

Figure 5. Stability of Cyanidin-3-O-β-D-glucoside, Cyanidin-3-O-β-D-galactoside, Malvidin-3-O-β-D- glucoside, and Delphinidin-3-O-β-D-glucoside in control samples, during incubation at 37°C for 24h.

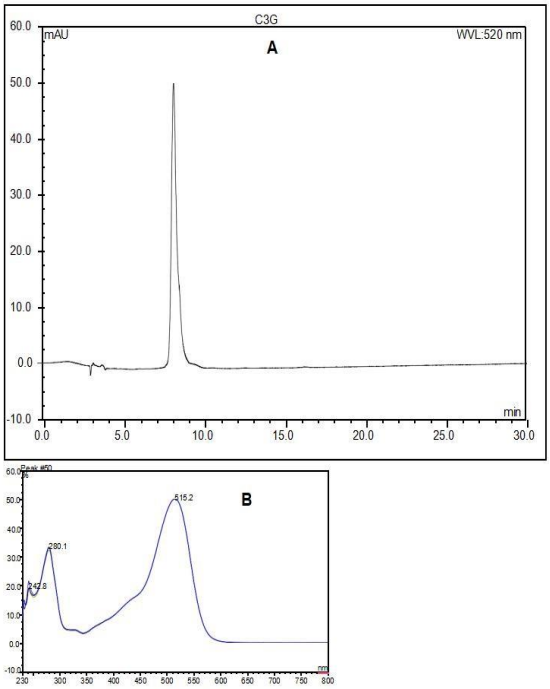

Figure 6. (A) Chromatogram recorded with RP-HPLC at 520 nm; the sample prepared from *Bifidobacterium infantis* cell-free enzyme and cyanidinglucoside before incubation (time 0h). The Peak represents cyanidin glucoside. (B) Cyanidinβ-glucoside UV spectrum.

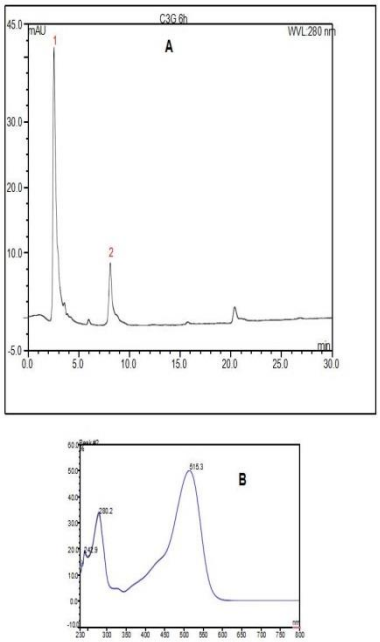

Figure 7. (A) RP-HPLC chromatogram recorded at 280 nm, showcasing the sample prepared from Bifidobacterium infantis cell-free enzyme and Cyanidin-3-O-β-D-glucoside after incubation at 37°C. Peak 1- protocatechuic acid, Peak 2- cyanidin galactoside. (B) UV spectrum of Cyanidin-3-O-β-D-glucoside.

intestinal mucosa [34–37]. Patients with inflammatory bowel disorders (IBD) or similar illnesses have lower amounts of *Bifidobacterium* and *Lactobacillus* in their gut microbiota [27,34,38]. Consequently, there is decreased activity of ß-glucosidase and β-galactosidase in these strains. Probiotics have been shown to alleviate the symptoms of irritable bowel syndrome and IBD [4,18]. This study's results suggest that a mixed formulation of anthocyanin glycosides and probiotics could be beneficial. Combining anthocyanin glycosides with probiotics before consumption could initiate hydrolysis of the anthocyanin glycosides, yielding a more bioactive product. This conversion could continue within the human gut. Beyond their therapeutic potential, anthocyanins have been found to support gut microbiota balance, highlighting their

potential as a prebiotic source [4,18]. Thus, these probiotic bacteria and anthocyanins colonizing the gut may present a chance for continuous biotransformation in the human gut.

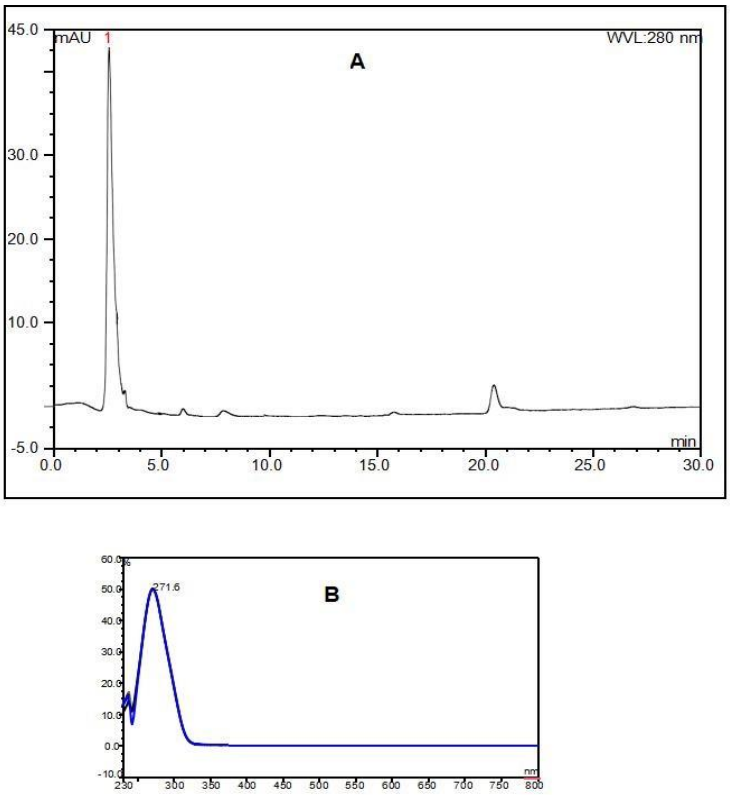

Figure 8. (A) RP-HPLC chromatogram recorded at 280 nm, showcasing the sample prepared from *Bifidobacterium infantis* cell-free enzyme and Cyanidin-3-O-β-D-glucoside after 6 h incubation at 37°C. Peak 1- protocatechuic acid, Peak 2- Cyanidin-3-O-β-D-glucoside. (B) UV spectrum of Protocatechuic acid.

3. CONCLUSION

To the best of our knowledge, this study is the first to investigate the role of *Bifidobacterium infa*ntis β-glucosidase and β-galactosidase in the glycolytic hydrolysis of Cyanidin β-glucoside and Cyanidin β-galactoside, and to distinguish between degradation caused by probiotic enzymes and chemical instability of anthocyanins under experimental conditions.

Our results show that Cyanidin β-glucoside is hydrolyzed more efficiently by B. infantis cell-free β-glucosidase than other anthocyanin glucosides, such as Malvidin β-D-glucoside and Delphinidin β-D-glucoside. In addition, Cyanidin β-galactoside was also successfully hydrolyzed by β-galactosidase from *B. infantis*. Based on the above results, *B. infantis* could become a functional probiotic that increases the bioavailability and bioactivity of anthocyanins in the gastrointestinal tract and food products. The mixture of *B. infantis* with cyaniding glucosides and galactosides looks promising and can be developed further for various medicinal formulations and functional foods for health benefits.

4. MATERIALS AND METHODS

4.1. Chemicals

Cyanidin-3-O-β-D-glucoside, Cyanidin-3-O-β-D-galactoside, Malvidin-3-O-β-D-glucoside, Cyanidin chloride, and Malvidin chloride were sourced from Extrasynthese (Genay, France). Protocatechuic acid, gallic acid (3,4,5–trihydroxyphenylacetic acid), p-coumaric acid (trans-4-hydroxycinnamic acid), and syringic acid (3,5- dimethoxy-4-hydroxybenzoic acid) were obtained from Sigma Aldrich (Sigma-Aldrich, USA). The HPLC-grade solvents used in the experiments were provided by VWR International GmbH (Austria).

4.2. Bacterial growth conditions

Bifidobacterium infantis was activated and cultured anaerobically in Reinforced Clostridial Medium (RCM, Oxoid Ltd, United Kingdom) in the anaerobic chamber using AnaeroGen kits (Oxoid Ltd, United Kingdom).

4.3. Enzyme extraction

Cells were harvested from early stationary phase cells to obtain cell-free enzymes. The obtained cells were washed two times with a phosphate buffer at a concentration of 50 mM, pH 6.5, following resuspension in the buffer. For mechanical disruption, the cell suspension was mixed with glass beads (200–300 mL), followed by vortexing. in five cycles, with 2-minute intervals on ice. The suspensions were centrifuged at 17,000 g (30 minutes at 4 °C) by a Sorvall RC5C centrifuge (Thermo Scientific, USA). The collected supernatant was filtered through a 0.45 µm microfilter (Merck Millipore, Germany), as previously described.

4.4. Determination of β-glucosidase enzyme activity

The assay of activity of β-glucosidase was assessed according to the methods described [30]. 150 µL of 5mM p-nitrophenyl-β-D-glucopyranoside (p-NPGlu) in phosphate buffer with 50 mM, pH 6.5 was mixed with 600 µL of the intended dilutions of crude enzyme. The incubation was performed at different periods to optimize the conditions. The enzyme reaction was terminated on ice through the addition of 375µL of cold solution of 0.1M NaOH. The release of p-nitrophenol was quantified using a Hitachi U-1100 spectrophotometer (Hitachi, Japan) at an absorbance of 410 nm. Under the given reaction conditions, the enzyme activity was measured in units (U) using the quantity of p-nitrophenol emitted per milliliter per minute. Standard solutions of p-Nitrophenol (Sigma-Aldrich, USA) were used to create a calibration curve, plotting the absorbance of p- nitrophenol (at 410 nm) vs. the p-nitrophenol concentration. A blank solution was prepared by mixing 150 µL 5mM pNPGlu in 50 mM phosphate buffer pH 6.5 with 600 µL of 50 mM PB pH 6.5, incubated at the same conditions with working samples, followed by the addition of 375 µL cold 0.1M NaOH.

4.5. Determination of the activity of β-galactosidase enzyme

The assay of activity of β-galactosidase enzyme was determined according to previously described method [17,30] with some modifications. 150 µL of 15mM o-nitrophenyl-β-D-galactopyranoside (oNPGal) in phosphate buffer, with a concentration 50 mM and pH 6.5, was combined with 600 µL of the intended dilutions of the cell-free enzyme. The reaction was incubated and halted by adding 1 mL of cold 0.1M NaOH on ice. The release of p-nitrophenol was measured at 410 nm using a Hitachi U-1100 spectrophotometer (Hitachi, Japan). Under the given reaction conditions, the enzyme activity was measured in units (U) using the quantity of o- nitrophenol emitted per milliliter per minute. Standard solutions of o-Nitrophenol (Sigma-Aldrich, USA) were used to create a calibration curve by plotting the absorbance of o-nitrophenol at 410 nm against the p- nitrophenol concentrations. A blank solution was prepared by mixing 100 µL of 50 mM phosphate buffer pH 6.5 with 900 µL 1mM pNPGlu in 50 mM phosphate buffer pH 6.5, incubated under the same conditions as the working samples, followed by the addition of 1mL of cold 0.1M NaOH.

4.6. Effect of temperature on cell-free β-glucosidase and β-galactosidase. Optimal temperature and thermal stability

The optimal temperature of cell-free β-glucosidase and β-galactosidase was tested by measuring the activity at different temperatures (30-80 °C) with the standard assay using p-NPGlu and o-NPGal as substrates. Thermal stability was evaluated by pre-incubating the free cell extract at different temperatures (30-60 °C) for 1h, 2h, and 3h, followed by a standard enzymatic assay at 37 °C, 15 min pH 6.5.

4.7. Effect of pH on cell-free β-glucosidase and β-galactosidase. Optimal pH and pH-stability

The optimum pH for cell-free enzymes was determined using a range of pH buffers (pH 3 to 9), specifically employing acetate and phosphate buffers. The assay was done by mixing 25 µL of cell-free enzyme with 25 µL of 20 mM pNPGlu (or oNPGal) in phosphate buffer with 50 mM and pH 6.5, and 950 µL of buffers with different pH (pH values from 3-9), at 37°C, incubation time 15 min. The samples were put on ice to end the reaction, and 1 mL of cold 0.1 M NaOH was added. The concentration of p-nitrophenol (o-nitrophenol) released from the reaction was measured in a spectrophotometer at 410 nm. P-nitrophenol and o-nitrophenol were used to prepare the standard curves. For pH-stability evaluation, enzymes released

from cells were preincubated in different buffers (pH range 3-8) for 2h, followed by a standard assay. The remaining activity is expressed as a percentage of the initial concentration.

4.8. Enzymatic hydrolysis of Cyanidin-3-O-β-D-glucoside, Cyanidin-3-O-β-D-galactoside, Malvidin-3-O-β-D- glucoside and Delphinidin-3-O-β-D-glucoside

Glycolitic hydrolysis of four anthocyanin glycosides in the presence of *Bifidobacterium infantis* cell-free extract containing β-glucosidase and β-galactosidase was measured, with the optimized method [39].

Cell-free enzymes were filtered through a microfilter (Merck, Austria) and used for enzymatic reactions. Cyanidin glucoside, malvidin glucoside, and cyanidin galactoside were prepared by dissolving them in DMSO at a concentration of 10 mM. Glycolitic hydrolysis of anthocyanins was undertaken by mixing 10 µL of the individual anthocyanin stock solution with 990 µL of the appropriate diluted cell-free enzyme. Control samples were prepared by mixing 10 µL of each anthocyanin stock solution (10 mM) and 990 µL of phosphate buffer with a concentration of 50 mM and pH 6.5. In control samples, the stability of anthocyanin glycosides during incubation in PB pH 6.5 was determined. The samples and control were incubated at 37°C for 1, 2, 6 and 24h. In all assays, the initial concentration of anthocyanin glycosides was 100 µM. 300 µL of aliquots were taken at different incubation times, and the reaction was terminated by adding 300 µL of 0.1% HCl in methanol (methanol containing 0.1% HCl). The samples were used for HPLC analysis.

4.9. Chemical stability of Cyanidin-3-O-β-D-glucoside, Cyanidin-3-O-β-D-galactoside, Malvidin-3-O-β-D- glucoside and Delphinidin-3-O-β-D-glucoside

The chemical stability of C3-Glu, C3-Gal, M3-Glu, and D3-Glu was determined to determine whether anthocyanin glycoside degradation is carried out chemically or by the presence of probiotic enzymes. This determination was carried out by mixing 10 µL of individual anthocyanin stock solution (10 mM) and 990 µL of phosphate buffer, 50 mM and pH 6.5, and then incubated at 37°C for 1, 2, 6, and 24h. After the incubation, 300 µL of aliquots were mixed with 0.1% HCl in methanol to stop the reaction. The chemical stability of anthocyanins was analyzed using the HPLC method.

4.10. HPLC analysis

The deglycosylation of anthocyanin glycosides and their metabolites in the reaction mixture was analyzed using the Dionex ICS-3000 HPLC system. The system was equipped with a quaternary pump, an autosampler, and a PDA-100 diode array detector. Samples were analyzed on LiChrospher® RP-18 reverse-phase column (250 mm x 4.6 m; particle size, 5 µm) as the stationary phase, with protection from a pre-column of the same material. The HPLC method was optimized according to Ávila et al. 2009 [16].

The mobile phases used for the analysis consisted of 4.5% (v/v) formic acid in water (solvent A) and acetonitrile of HPLC grade (solvent B) with a flow rate set at 0.5 mL/min. The gradient program was 0-min-10% solvent B, 0.5-20 min-20% solvent B, 20-30 min, and 25% B.

The mobile phases were prepared from HPLC-grade solvents provided by Sigma Aldrich. Standard solutions of cyanidin-glucoside, cyanidin-galactoside, and malvidin glucoside were prepared by diluting the stock solution in methanol, which were then used to establish the standard curve. Data acquisition was carried out using the Dionex Chromeleon ICS-3000 software. A diode array UV-visible detector was used, set at 280, 320, and 520 nm detection wavelengths.

The areas of the identified peaks were compared to a calibration curve generated from standard stock solutions. These calibration curves were constructed by plotting the concentration of standards (µM/L) against their respective peak areas. The curves demonstrated linearity, with the regression coefficients R2 of 0.999.

4.11. Statistical analysis

Every experiment was carried out three times, and the results were reported as a mean value ± SD (n = 3). GraphPad Prism 3.0 was used for statistical analyses, especially one-way ANOVA and Tukey cross-comparison tests for every research group. Significant values were defined as $p < 0.05$.

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