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# Optimization of *Panax notoginseng* root extract hydrolysis by *Cordyceps militaris* derived glycosidase and bioactivities of hydrolysis products

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ABSTRACT

Ginsenosides are saponins found in plants of panax genus. Despite having a wide range of health benefits, they cannot optimally reach their target tissues and organs due to their low intestinal absorption. However, reduction of their sugar moieties can produce more absorbable and bioactive compounds. Even though they are known as a good source of glycosidase, edible and medicinal mushrooms are less investigated for their capability to hydrolyse ginsenosides. An enzyme from *Cordyceps militaris* was used to convert ginsenosides from *Panax notoginseng* roots. Response surface methodology was used to study the effect of temperature (30 – 60 °C), pH (6 – 7), time (30–80 h) and enzyme concentration (0.5 – 1%), on minor ginsenoside production and optimum conditions for maximum ginsenoside hydrolysis. Regression analysis was used to develop a second-degree polynomial model for the production of minor ginsenosides. Statistical significance of the model was evidenced by coefficient of determination ($R^2$ = 95.15%) and (P value = 0.000). Minor ginsenosides production was affected by enzyme concentration, temperature and time respectively in order of magnitude. The effect of pH was found insignificant. Maximal minor ginsenosides were found at 0.86% enzyme concentration, 42.88 °C, 62.63 h and pH 6.62. A significant difference between hydrolysates from different treatments was observed for DPPH scavenging activity and antimicrobial activity. Hydrolysates have shown a strong cytotoxic activity against (SK-LU-1) and (MCF-7) cell lines. The model developed for *Panax notoginseng* hydrolysis by *C. militaris* derived glycosidase can potentially be used for pro-

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ducing minor ginsenosides with improved bioactivities for use in the production of food and medicines.

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## Introduction

Ginsenosides are saponins from ginseng, a collective name given to *Panax* genus plants. They are the most studied of all ginseng compounds and are believed to confer most of the benefits [1]. A structure of ginsenosides has two or three hydroxyl groups at carbon 3, 6 and 20 to which monomeric, dimeric or trimeric sugars can bind [2]. Low intestinal absorption of ginsenosides hinders their full potential which is reportedly due to their higher molecular weights, high total number of hydrogen bonds, topological polar surface area (TPSA) and flexibility [3]. Therefore, transformation; a process of reducing the number of sugar moieties in ginsenosides was adopted to boost their absorption [4].

Ginsenoside transformation is achieved through physical, chemical and biological means. Biological methods involve the use of enzymes and microorganisms to accomplish chemical hydrolysis [5] and are generally preferred for use in food and medicine related purposes over chemical methods which are believed to have undesirable side reactions. The use of microorganisms and many types of fungi has however been associated with the production of toxic by-products such as mycotoxins which cause physiological defects [6]. In attempt to get this solved, several studies have been conducted on the use of food grade microorganisms, such as bacteria from fermented food [7], gut bacteria [8] and different types of fungi [9–11]. Although there is a considerable amount of work done, there is still little knowledge about the use of glycosidases from edible and medicinal mushrooms. It is in this respect that this research sought to find out one which can produce a glycosidase with ability to efficiently convert ginsenosides to minor ginsenosides. Furthermore, this study also reports bioactivities of hydrolysis products along with optimization of hydrolysis conditions which could be utilized for scaled up transformation of *Panax notoginseng* ginsenosides.

## Material and methods

### *Enzyme screening*

A glycosidase with higher activity was assessed from seven mushrooms; *Cordyceps militaris, Coprinellus* sp., *Phellinus* sp., *Ganoderma lucidum, Agrocybe cylindracea, Pleurotus* sp., and *Xerula* sp., through a two-step screening. After 7 days of mycelial growth in YPD and DP liquid media at 220 rmp and 25 °c; PDA containing 1% CMC was used to test glycosidase activity of each of the mushrooms. Lugol detection solution was used to detect a clear halo zone surrounding the sample seats which was considered as the area of enzymatic degradation. Mushrooms showing enzymatic activity were regrown, the liquid culture collected and considered a crude enzyme, the activity of which was tested by DNS method. A reaction mixture (1.0 mL) containing 0.5 mL of salicin (1%) in sodium acetate buffer (pH 4.8) and 0.5 mL of enzyme solution was incubated for 10 min at 37 °C. The reaction was terminated by boiling for 10 min. The reducing sugar was measured immediately using a UV–visible spectrophotometer (UV-1601; Shimadzu, Tokyo, Japan) at 500 nm. The amount of glucose released was quantified using a concentration plot of glucose standard. The enzyme which showed higher activity was filtrated using a 2 $\mu$m pore size Whatman filter paper and condensed by ultrafilter 10 kDa amicon and saturated to 80% with ammonium sulfate. The saturated filtrate was centrifuged and the precipitate diluted with distilled water. This solution was then loaded into a dialysis bag and left overnight in stirring water which was being replaced every four hours to maximize the diffusion of salt ions. After dialysis the dialysate was centrifuged in a 10 kDa amicon ultra-15 centrifugal 126 filter unit. All experiments were done at 4 °C.

### *Ginsenoside extraction*

Thirty grams of *P. notoginseng* powder were extracted three times by ethanol in an ultrasound equipped water bath at 45 °C. The extract was then concentrated to less than 8% moisture content at 45 °C before being fractionated through a cepharose gel at a flow rate of 1 mL/min. The recovered fractions were centrifuged in a 10 kDa amicon ultra-15 centrifugal filter unit. The upper phases were run on (TLC) plates. Fractions with similar TLC results were mixed into three fractions which were then evaporated to dryness in vacuo. The fraction with higher number of saponin content was considered for fermentation experiments. The total extraction yield was calculated as:

$$\text{Extraction yield} = \left(\frac{\text{mt}}{\text{m0}}\right) \times 100\% \quad (1)$$

Where $m_0$ is the mass of material before extraction (mg), mt is the mass of dry extract (mg).

**Table 1**
Central composite design of ginsenoside hydrolysis.

| Run. | Temperature (oC). | pH. | Time (h). | E/S (%). | Minor ginsenosides (mg/L). |
|---|---|---|---|---|---|
| 1. | 30. | 6. | 30. | 0.5. | 59.27. |
| 2. | 60. | 6. | 30. | 0.5. | 31.89. |
| 3. | 30. | 8. | 30. | 0.5. | 49.29. |
| 4. | 60. | 8. | 30. | 0.5. | 34.25. |
| 5. | 30. | 6. | 80. | 0.5. | 88.38. |
| 6. | 60. | 6. | 80. | 0.5. | 71.69. |
| 7. | 30. | 8. | 80. | 0.5. | 75.66. |
| 8. | 60. | 8. | 80. | 0.5. | 67.3. |
| 9. | 30. | 6. | 30. | 1. | 93.33. |
| 10. | 60. | 6. | 30. | 1. | 60.3. |
| 11. | 30. | 8. | 30. | 1. | 94.27. |
| 12. | 60. | 8. | 30. | 1. | 64.47. |
| 13. | 30. | 6. | 80. | 1. | 87.72. |
| 14. | 60. | 6. | 80. | 1. | 94.43. |
| 15. | 30. | 8. | 80. | 1. | 87.49. |
| 16. | 60. | 8. | 80. | 1. | 84.3. |
| 17. | 15. | 7. | 55. | 0.75. | 49.69. |
| 18. | 75. | 7. | 55. | 0.75. | 25.21. |
| 19. | 45. | 5. | 55. | 0.75. | 93.5. |
| 20. | 45. | 9. | 55. | 0.75. | 87.5. |
| 21. | 45. | 7. | 5. | 0.75. | 30.5. |
| 22. | 45. | 7. | 105. | 0.75. | 93.5. |
| 23. | 45. | 7. | 55. | 0.25. | 29.5. |
| 24. | 45. | 7. | 55. | 1.25. | 87.5. |
| 25. | 45. | 7. | 55. | 0.75. | 103.333. |
| 25. | 45. | 7. | 55. | 0.75. | 109. |
| 27. | 45. | 7. | 55. | 0.75. | 105. |
| 28. | 45. | 7. | 55. | 0.75. | 112.555. |
| 29. | 45. | 7. | 55. | 0.75. | 106.939. |
| 30. | 45. | 7. | 55. | 0.75. | 101.434. |
| 31. | 45. | 7. | 55. | 0.75. | 117.667. |

*Total saponin content determination*

Total saponin of *P. notoginseng* extract was determined employing the vanillin –sulphuric acid method developed by Hiai et al. [11] and improved by Le et al. [12] with minor modifications to fit our working conditions. Briefly, vanillin anhydrous ethanol (8% w/v), sulphuric acid-water (72%, v/v) solutions and different concentrations of aescin in ethanol (100, 80, 60, 40, 20, 10 mg/L) were prepared. The reaction mixture was made of 25 µL *Panax notoginseng root* extract, 25 µL vanillin solution and 50 µL sulphuric acid solution in a 96 well microplate. For vanillin standard curve construction, the reaction mixtures were made of 25 µL of prepared concentrations of aescin, 25 µL vanillin solution and 50 µL sulphuric acid solution. A blank control was prepared by using 25 µL distilled water, 25 µL vanillin solution and 50 µL sulphuric acid solution. The plate was then incubated at 60 °C for 15 min and cooled at room temperature for 5 min before measuring the absorbance at 560 nm using Benchmark plus 96 well plate microplate reader spectrophotometer.

*TLC analysis*

Silica gel 60 $F_{254}$ plate was used in TLC analysis with mobile phase consisting of CHCl3/CH3OH/H2O (65:35:10, v/v/v) and the TLC spots were detected by spraying 10% (v/v) $H_2SO_4$ in ethanol and drying with an ordinary hair saloon hair dryer for several min. The samples were loaded on TLC plates in 1 µL volumes and dried prior to placing them in a TLC tank.

*HPLC analysis*

HPLC samples were prepared by diluting both standards and extracts (1:2 w/v ratio) in aqueous methanol 80% (v/v), vortexed and centrifuged for 5 min and kept at – 20 °C before use. Injection volume was 20 µL. Chromatographic conditions were such that sample flow rate set at 1.2 mL/min, UV detection wave length of 196 nm and column temperature of 40 °C. The mobile phase was made of solution A; Deionized water-acetic acid (100, 0.01 v/v) and solution B; Acetonitrile-water (100, 0.01 v/v). Gradient elution: 20% B at 20 min, 42%B at 60 min, 85% B at 65 min, 85% B at 80 min, 100% B at 81 min, 100% B at 90 min, 20% B at 91 min, 20% B at 95 min. The system was equilibrated for 10 min between experimental runs. All reagents were of analytical grade.

*Ginsenoside fermentation*

Fermentation experiments were designed using Minitab 2019 statistical software with four factors, 2 levels each. Samples were then prepared and subjected to their respective conditions (Table 1). 0.2 M sodium hydroxide and hydrogen chloride was used to set reaction mixtures at desired pH values. After fermentation the total saponin content was measured again.

The difference between total saponin before and after fermentation was regarded as minor ginsenoside content. Response surface methodology (RSM) was used to investigate the effects of the factors set to influence the products of ginsenoside hydrolysis by mushroom *C. militaris* enzyme. Experiments were designed basing on central composite design of four factors with two levels namely temperature (30, 60 °C) pH (6, 8) time (30, 80 hrs) and enzyme concentration (0.5, 1% (v/v)). Optimal conditions for enzymatic hydrolysis were determined using central composite design and the polynomial equation produced by Minitab 19 software was used to analyze the data and coefficients for the equation. Lack of fit and coefficient of determination ($R^2$) on the hydrolysis products were determined using analysis of variance.

The quadratic regression equation was predicted as

$$y = \beta_0 + \beta_{11} + \beta_{22} + \beta_{33} + \beta^2_{44+}\beta_{111} + \beta^2_{222} + \beta^2_{333} + \beta^2_{444} + \beta_{1212} + \beta_{1313} + \beta_{1414+}\beta_{2323+}\beta_{2424+}\beta_{3434} \quad (2)$$

Where y represents the dependent variable or the response, $x_{1,}$ $x_{2,}$ $x_{3,}$ $x_4$ independent variables or experimental factors, as temperature, pH, time and enzyme concentration respectively. $\beta_0$ is the intercept and $\beta_n$ is the coefficient.

*Bioactivities*

*DPPH scavenging activity*

Antioxidant activity of both hydrolysates and crude extracts was tested on stable free radical $\alpha$, $\alpha$-diphenyl-$\beta$-picrylhydrazyl (DPPH) using a procedure proposed by Manzocco [13] slightly modified to fit our working conditions. DPPH stock solution was prepared in ethanol at 0.1% concentration and stored at 4 °C. Working solution was prepared by diluting again the stock solution with ethanol in (1:19, v/v) to reach the absorbance of 0.9 ± 0.03 and was mixed with 50 µg/mL of ginsenosides at (1:1, v/v) proportion. The reaction mixture was kept for 30 min in the dark at room temperature after which the absorbance was measured at 517 nm by BIORAD Benchmark plus 96 well plate microplate reader spectrophotometer. The reaction mixture volume equivalent of DPPH was used as negative control and 100 µg/mL of ascorbic acid was reacted to DPPH to act as positive control. The results were computed as

$$\%\ activity = \left(\frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}}\right) \times 100 \quad (3)$$

Where Abs control is the absorbance of DPPH without the sample and Abs sample is absorbance of reaction mixture of DPPH and sample.

*Antibacterial activities*

Three pathogenic microorganisms belonging to both Gram negative (*Pseudomonas aeruginosa*, *Escherichia coli)* and Gram positive (*Staphylococcus aureus*) bacterial strains were grown into nutrient liquid medium made of 5% peptone and 3% meat extract for 24 h before being used in the experiment. In the wells of a 96 well microplate, 90 µL of the bacterial culture were diluted with the same volume of distilled water and incubated with 20 µL of 50 mg/L ginseng extract and hydrolysates, or antibiotics for positive control. Gentamicin was used for *P. aeruginosa*, ciprofloxacin for *E. coli* and Penicillin for *S. aureus*. For negative control, 90 µL bacterial strains were added to 120 µL distilled water. Absorbance of the mixtures were measured right away and incubated for 24 h at room temperature after which the absorbance was re-measured.

*Cytotoxicity assay*

Three hydrolysates subjected to different reaction conditions were considered for cytotoxicity assay in comparison with the crude extract. Sample 1 (T 30 °C, pH 6, time 30 h and E.C 0.5%), Sample 2 (T 45 °C, pH 7, time 55 h and E.C 0.75%) and sample 3 (T 30 °C, pH 6, time 80 h and E.C 0.5%)as described by Skehan et al. [14] and Likhiwitayawuid et al. [15]. Briefly, two cancer cell lines SK-LU-1 line (Human lung carcinoma - Lung cancer) and MCF-7 Series (Human Breast Carcinoma - Breast Cancer) grown in Dulbecco's Modified Eagle Medium (DMEM) and Minimum essential medium with Eagle salts (MEME) respectively were used supplemented with L-Glutamine, Sodium pyruvate, $NaHCO_3$, PSF (Penicillin - Streptomycin sulfate - Fungizone); NAA (Non-essential amino acids); 10% BCS (Bovine Calf serum). $10^4$ cells were plated in each well of a 96 -well plate and left for 24 h to attach to the wall of the plate before treatment with *P. notoginseng* hydrolysis products by *C. militaris* derived glycosidase. 50 $\mu$g/mL of the hydrolysates were then added to the cell monolayers in triplicate and incubated for 48 h at 37 °C in a 5% $CO_2$ environment. Cells were then fixed, washed and stained by Sulforhodamine-B. Acetic acid was used to wash the excess stain and the attached stain was recovered by Tris EDTA buffer. The stain color intensity was measured by an ELISA reader machine at 495 – 515 nm. The same procedure was done for Ellipticine, one of the effective anticancer agents for comparison purposes and 10% DMSO used as negative control. Cell survival value (CS%) was then calculated using the following formula.

$$CS\% = \frac{\text{OD (sample)} - \text{OD (on day 0)}}{\text{OD (DMSO)} - \text{OD ( on day 0)}} \times 100 \quad (4)$$

Results were presented as means ± standard deviation of three independent experiments.

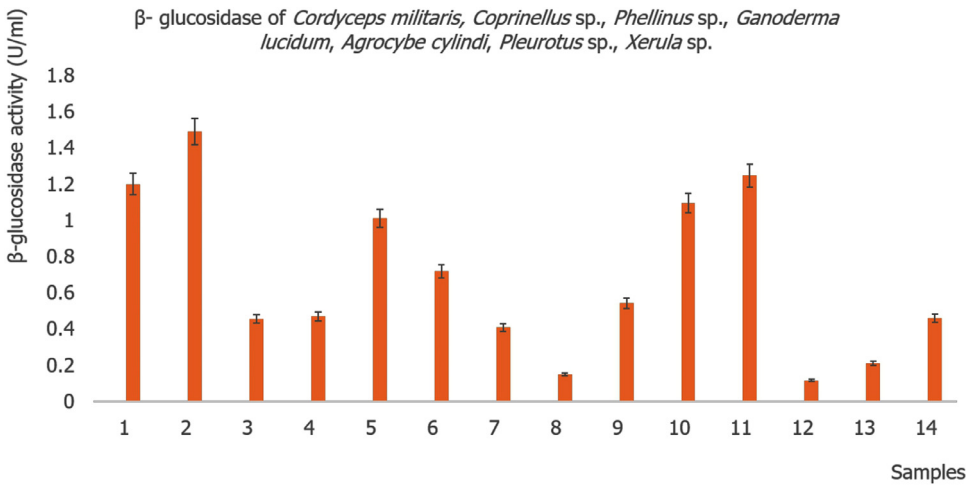


**Fig. 1.** $\beta$-glucosidase activity of mushrooms by DNS method. Mushrooms Cordyceps militaris, Coprinellus sp., Phellinus sp., Ganoderma lucidum, Agrocybe cylindracea, Pleurotus sp., Xerula sp. are in a respective order with odd numbers from YPD medium and even numbers from PD medium.

### *Data analysis*

All data are presented as means ± standard deviation. Experimental design, optimization of parameters and statistical significance was evaluated using Minitab19 software and graph construction by both Minitab 19 and Excel software. Mean differences were assessed considering significance at $P < 0.05$ using ANOVA Tukey's test.

## Results and discussion

### *Mushroom enzyme*

The ability of glycosidase production was evaluated from seven mushrooms grown in two media namely YPD and DP. The results have shown that *C. militaris* was the most $\beta$-glucosidase producer among other mushrooms and was selected for further experiments. Results are presented in (Fig.1).

### *Ginsenoside extraction*

#### *Extraction yield*

Ultrasound assisted extraction was used to extract ginsenosides from the roots of *P. notoginseng*. After drying the extracts to less than 8% moisture, 0.3852 g of weight was found, equivalent to the yield of 1.284%. This yield is comparable to the yield of 0.95% of ginsenosides extracted by Luo et al. [16] from *P. ginseng* using ultrasound assisted extraction in supercritical $CO_2$ reverse micro-emulsion. When the method was used to extract *Canna indica* phytochemicals, the yield also proved to be higher than the conventional extraction [17]. It was also reported by Wu et al. [18] as the most effective method of extraction of ginsenosides from roots of different types of ginseng.

#### *Saponin content of* P. notoginseng *fractions and crude extract*

Saponin content was found using Vanillin-sulphuric acid method which gives a distinctive red- purple color absorbing light at 473–560 nm range. In this study, the experiment was conducted at 560 nm. Results indicated that saponin content in fraction 2 was 129 mg/L, while fraction 1 and 3 contained 92 mg/L and 111.11 mg/L respectively (Supplementary Table 1). Hence, fraction 2 was selected for fermentation experiments.

**Table 2**
ANOVA for the model of ginsenoside hydrolysis.

| Source | DF | Adj SS | Adj MS | F-Value | P-Value |
|---|---|---|---|---|---|
| Model | 14 | 21,221.6 | 1515.83 | 22.44 | 0 |
| Linear | 4 | 8873.9 | 2218.47 | 32.84 | 0 |
| T | 1 | 1286.9 | 1286.86 | 19.05 | 0 |
| pH | 1 | 73.4 | 73.43 | 1.09 | 0.313 |
| Time | 1 | 3648.2 | 3648.2 | 54.01 | 0 |
| E/S | 1 | 3865.4 | 3865.37 | 57.23 | 0 |
| Square | 4 | 11,360.1 | 2840.03 | 42.05 | 0 |
| T*T | 1 | 7384.6 | 7384.64 | 109.33 | 0 |
| pH*pH | 1 | 225.4 | 225.38 | 3.34 | 0.086 |
| Time*Time | 1 | 2821.1 | 2821.06 | 41.77 | 0 |
| E/S*E/S | 1 | 3340 | 3340 | 49.45 | 0 |
| 2-Way Interaction | 6 | 987.6 | 164.6 | 2.44 | 0.072 |
| T*pH | 1 | 12.3 | 12.25 | 0.18 | 0.676 |
| T*Time | 1 | 438.1 | 438.06 | 6.49 | 0.022 |
| T*E/S | 1 | 4.2 | 4.16 | 0.06 | 0.807 |
| pH*Time | 1 | 38.9 | 38.94 | 0.58 | 0.459 |
| pH*E/S | 1 | 23.7 | 23.72 | 0.35 | 0.562 |
| Time*E/S | 1 | 470.5 | 470.46 | 6.97 | 0.018 |
| Error | 16 | 1080.7 | 67.54 | | |
| Lack-of-Fit | 10 | 890.5 | 89.05 | 2.81 | 0.109 |
| Pure Error | 6 | 190.2 | 31.7 | | |
| Total | 30 | 22,302.3 | | | |

*Ginsenoside fermentation*

Ginsenoside hydrolysis experiments were designed using Minitab19 software and optimized by Central composite design of the RSM.The software output equation was as follow:

$$\begin{aligned} Y = &-327 + 4.66\ T + 34.7\ pH + 2.701\ Time + 317.6\ E/S - 0.07142\ T* \\ &T - 2.81\ pH* \\ &pH - 0.01589\ Time* \\ &Time - 172.9\ E/S*E/S + 0.058\ T* \\ &pH + 0.01395\ T* \\ &Time + 0.136\ T* \\ &E/S - 0.0624\ pH* \\ &Time + 4.87\ pH* \\ &E/S - 0.868\ Time*E/S \end{aligned} \tag{5}$$

Where T is Temperature and E/S; enzyme concentration.

The significance and fitness of the model were evaluated by F and P values as presented in the ANOVA results (Table 2).

From the results, it was realized that the modal is good and fit with the used experimental conditions. The obtained value of $R^2$, 95.15% indicates that 95.15% of the variations in the responses can successfully be explained by the model. The efficacy of the model is further explained by its own F and P values which are 22.44 and (0.000) respectively. Furthermore, the respective values of coefficient of determination ($R^2$) and P value for the test of lack of fit which are 95.15% and 0.716 indicate that the second order model developed by the software in this study (eq. (5)) can be used to predict observations in the future experiments in the range of the design reported in this study. Compared with pure error, the values of lack of fit and (F value 2.81 and P value 0.109) indicate that there was no significant difference between the values and the pure error, making the model a better fit for response estimation as confirmed by the $R^2$ which showed that the variability in the response can be predicted at 95.15% by the model. Interactive influence between factors and response was found to be highly associated satisfactorily as indicated by adjusted determination coefficient ($R^2$·adj. = 90.91%), $R^2$ (95.15) and predicted $R^2$ (75.84) which implies that experimental and predicted response values are highly correlated. F (22.44) and P ($\leq$ 0.0001) (Table 2) values as determined by ANOVA, have indicated that all factors except pH have significantly contributed to variability observed in the responses. The contributions of individual factors as shown by the regression equation and individual P values are such that the enzyme concentration expressed dominancy by exerting major effect on the response. The optimization results are presented as a contour plot produced by Minitab 19 software. (Fig. 2).

Apart from pH, all other factors studied to influence ginsenoside hydrolysis were found to exert both significant linear and quadratic influence with P values $\leq$ 0.0001. (Table 2). In two ways interaction, temperature*time, has had P value of 0.022 and time*enzyme concentration has had P value of 0.018. Comparison of regression coefficients in regression equation, has revealed that enzyme concentration exhibited a high value which reflects its major influence over other factors. When

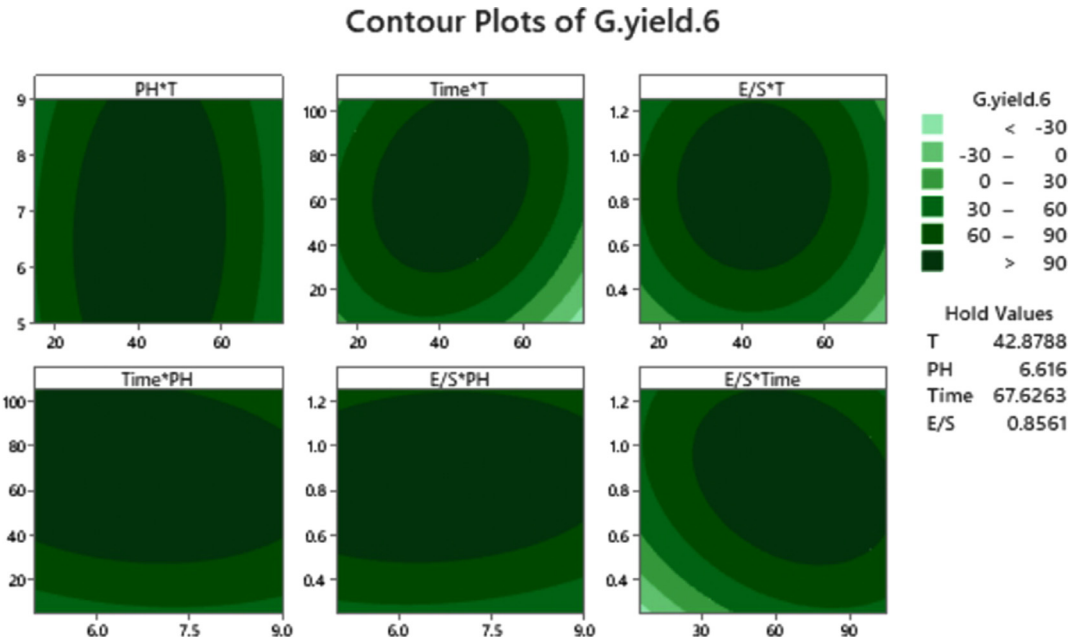


**Fig. 2.** Contour plot showing the effect of interacting factors on hydrolysis of major ginsenosides from P. notoginseng extract by C. militaris mushroom $\beta$-glucosidase with non-interacting factors hold at optimal values.

non-significant factors were removed the regression model became

$$Y = -327 + 4.66\,T + 2.701\,Time + 317.6\,E/S - 0.07142\,T* \\ T - 0.01589\,Time*Time - 172.9\,E/S*E/S + 0.01395\,T*Time - 0.868\,Time*E/S. \quad (6)$$

This equation shows that all the significant factors exerted a positive linear influence whereas their quadratic influence was negative and temperature time interaction had positive effect whereas Time, enzyme concentration interaction had negative effect.

It is clear from the experimental table of factor level and responses that higher temperature is associated with low production when all other factors are on hold (Effect of pH can be neglected). However, the amount of ginsenosides was found to increase gradually with the increase of temperatures from 30 °C to optimal temperature at which the maximum amounts were obtained. (Linear effect). Further increase in temperatures beyond 43 °C has left the records of minor ginsenosides reduced significantly (Quadratic effect) (Fig. 2). This tendency is explained by the physicochemical characteristics of the enzyme as an active protein.

The optimum temperature and pH of Beta glucosidase from mushroom *C. militaris* was found to be 42.88 °C and pH 6.62 respectively. However, there was no significant change in response as a result of pH variations from 5 – 9. At pH 5 the response was 93.8% of the optimal response for the optimal parameters of all factors whereas at pH 9, the response turned 86.8% of the optimal response. This explains the flexibility of the enzyme to hydrogen ions which is an excellent property. The properties of this enzyme are similar to those of some bacterial enzymes such as *Paenibacillus mucilaginous*[19], *Microbacterium esteraromaticum* [20] which also proved to be successful in ginsenoside transformation. The same tendency of effect was also observed for enzyme concentration. Enzyme concentration has had a higher effect than other factors. It exhibited a sound linear effect whose significance is confirmed by (p value $\leq$0.0001) and a standardized regression coefficient of 317.6 making it at the same time an important and significant factor. With enzyme concentration variability, the response increased as the ratio increased from 0.25% to 0.82 – 0.89% (positive linear effect) to which minor ginsenosides were at their optimal production and then declined as the ratio increased beyond 0.89 (negative quadratic effect). The reported positive linear effect and negative quadratic effect of time in ginsenoside hydrolysis experiments only means that reactions ended before the maximum time which is produced by minitab 19 software as increase in minor ginsenosides with time (linear effect) and a fall in their numbers after the optimum time; which is theoretically the time at which the reaction substrate is exhausted.

Ginsenoside Rg3 was the most abundant hydrolysis product of mushroom *C. militaris* derived enzyme to *P. notoginseng* root extract. Rg3 is extensively known for its anti-cancer properties [21]. All the available means of Rg3 production are however less fruitful with respect to obtaining reasonable amounts of standard substance, time and cost effectiveness[22]. TLC analysis in (Fig. 3) shows the appearance of Rg3 after hydrolyzing P. notoginseng root extract with mushroom C. militaris enzyme extract and HPLC chromatograms in (Fig.4) give an idea of the amount of the metabolite produced. *C. militaris* is

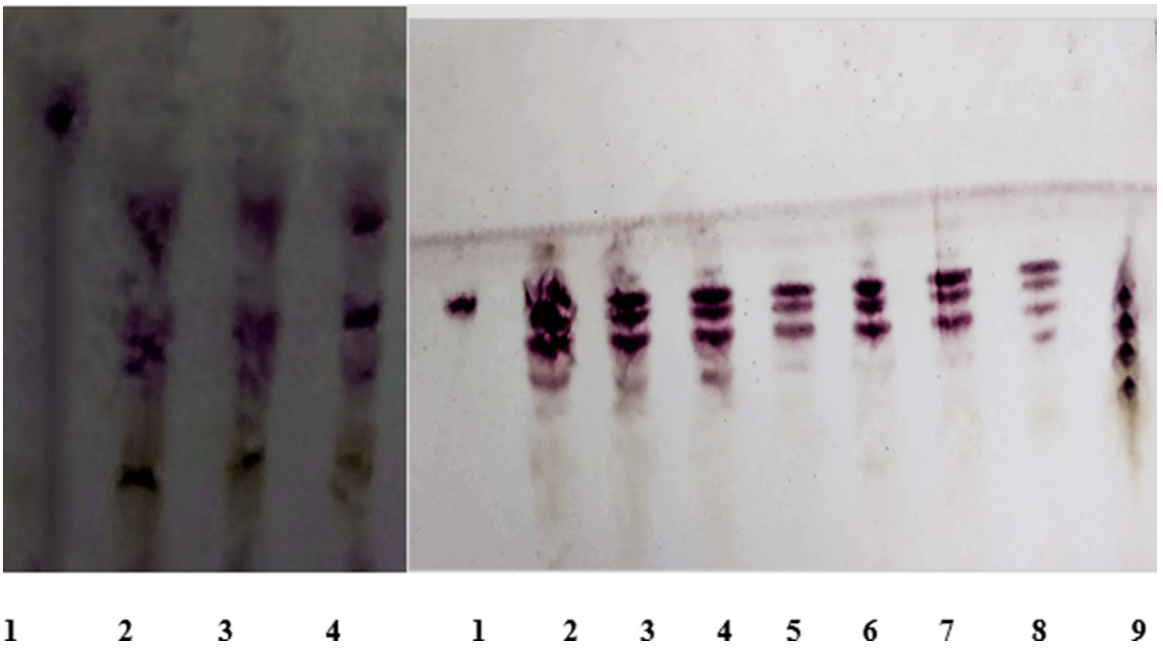


**Fig. 3.** TLC analysis of ginsenosides content in extract (left) and some chosen hydrolysates (right) with minor $\beta$-ginsenoside Rg3 as standard. **Left**: 1 Minor ginsenoside Rg3 standard, 2, 3 and 4 are respectively fraction 1, 2, and 3 of P. notoginseng crude extract. **Right**: 1: Rg3 standard, 2 – 8: hydrolysates. 2: (T30 °C, pH6, t 30 h, E/S 0.5). 3: (T60 °C, pH8, t 30 h, E/S 0.5). 4: (T30 °C, pH8, t 60 h, E/S 1). 5:(T60 °C, pH8, t 80 h, E/S 1). 6:(T15 °C, pH7, t 54 h, E/S 0.75). 7:(T75 °C, pH7, t 45 h, E/S 1.25). 8: (45 °C, pH7, t 40 h, E/S 0.75). 9: Crude extract.

then a safe source of enzyme for Rg3 production which had never been investigated before and is hereby reported of being efficient

*Bioactivities*

The free radical scavenging, antibacterial and cytotoxic activities were studied for hydrolysis products of ginsenosides by the mushroom derived enzyme. All runs as in RSM design were used for DPPH scavenging and antibacterial activities. For cytotoxic activity, a crude extract was compared to hydrolysates treated at 1, (T 30 °C, pH 6, time 30 h and E.C 0.5%) 2, (T 60 °C, pH 7, time 80 h and E.C 1%) and 3, (T 30 °C, pH 6, time 30 h and E.C 0.5%). It was found that antioxidant activity of products of ginsenoside hydrolysis were different with (Pvalue = 0.000). The variability observed may be due to different compounds or different proportions of hydrolysis products. The maximum activity recorded in the 31 hydrolysis runs was 33. 9% and the minimum was 13. 8% with average of 25%. These numbers are a little bit more than the activity reported by Chung et al. [23]for white ginseng which was 26.67% and less than 42.06% for red ginseng.

Significant differences were also observed among hydrolysis treatments for antibacterial activities. P-values for the mean differences for activities against three pathogenic bacteria studied are all less than 0.001% within the confidence interval of 0.05. Nevertheless, the difference between activities towards the three species is also remarkable. Maximum values of inhibition were found to be 10.3% in *P. aeruginosa*, 30.3% in *S. aureus* and 31.9% in *E. coli.* Their respective minimal values were 0.2%, 9.07% and 7.9%. These findings show promising activities of ginseng enzymatic products against these pathogens but are in contrast with results reported by Wu et al. [24]who reported that growth of *P. aeruginosa* is unaffected by ginsenosides even if they eradicate its virulence by inhibiting their formation of biofilms. Moreover, treatment with ginseng was found to clear *P. aeruginosa* infection from lungs through the increase of immune response and exerting a negative effect on its quorum sensing network [25]. Inhibitory effect of *C. militaris* derived enzyme hydrolysates of *P. notoginseng* root extract on *S .aureus* was found to be higher than that of ginsenoside crude extract as investigated by Na et al. [25], however, both reports have shown that the effect differs with different ginsenoside treatments. In addition, S. *aureus* was found to be more susceptible to ginsenoside Rg3 which was the final product of heat induced hydrolysis which better explains why all the hydrolysates in this report exhibited more inhibitory effect than the crude extract. There was no significant difference between the effects of ginseng hydrolysis products by *C. militaris* on inhibiting *S. aureus and E. coli* but the susceptibility of these species were almost two times higher than that of *P. aeruginosa.* When Kochan et al. [21] evaluated the inhibitory effect of ginsenosides extracted from cultures of hairy roots of ginseng on *E. coli*, it has shown a remarkable sensitivity to these compounds relatable to the results presented in this report.

Products of *P. notoginseng* hydrolysis by *C. militaris* enzyme have shown a great cytotoxic activity against SK-LU-1 and MCF-7 cells. There is however a remarkable difference in susceptibility of these two types of cancer cell lines on minor gin-

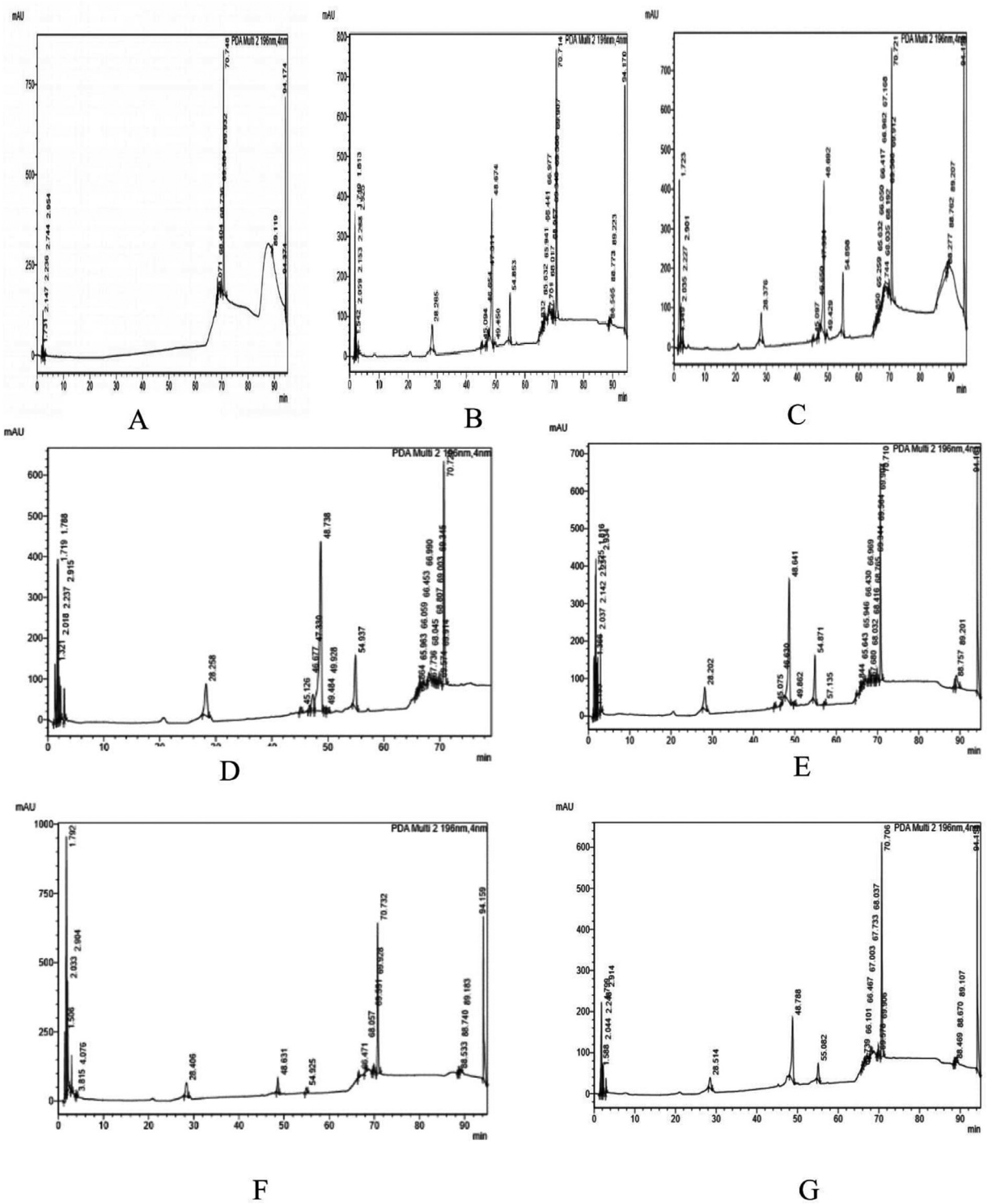


**Fig. 4.** HPLC chromatograms of P. notoginseng root extract hydrolysis products by C. militaris. Where: A is a standard $\beta$-ginsenoside Rg3, B-G; hydrolysis products with factors and levels as indicated in blackets, B (T30 °C, pH6, t 30 h, E/S 0.5), C (T60 °C, pH8, t 30 h, E/S 0.5), D (T30 °C, pH8, t 60 h, E/S 1), E (T60 °C, pH8, t 80 h, E/S 1), F (T15 °C, pH7, t 54 h, E/S 0.75), G (45 °C, pH7, t 40 h, E/S 0.75).

senosides. Maximal cell survival values were observed for a hydrolysate treated at (T 30 °C, pH 6, time 30 h and E.C 0.5%) and were 44.63% ±3.04 for MCF-7 and 26.31% ±2.19 for SK-LU-1 whereas minimal cell survival values were found with a hydrolysate treated at (T 60 °C, pH 7, time 80 h and E.C 1%) and were 23.18%±2.25 for MCF-7 and 3.35±0.27 for SK-LU-1. The difference between these values can be mainly attributed to hydrolysis products which are dependent on the hydrolysis factors. High numbers recorded are due to suboptimal parameters which may have resulted into products with less cytotoxicity towards tested cell lines. The hydrolysate which gave maximum cancer cell cytotoxicity was close to optimal enzyme concentration and temperature, the major determinants in *P. notoginseng* root extract hydrolysis by *C. militaris* as revealed by the model regression equation, (eq.6) with reasonable amount of time. Hydrolysis of *P. notoginseng* ginsenosides is at the base of their cytotoxic activity as confirmed by the negative effect of crude extract. Cytotoxicity-structure relationship was revealed by Lee et al. [26]and the difference is explained by differences in cell uptake. Their results are in accordance with the results obtained in this investigation. PPD ginsenoside group was reported to exhibit ability of suppressing proliferation of MCF-7 than their PPT counterparts. The major hydrolysis product of *P. notoginseng* as revealed by HPLC studies is ginsenoside Rg3. This explains the general role of ginsenosides hydrolysis for the cytotoxic effects. Studies of potential inhibition of breast cancer resistance protein have however excluded ginsenoside Rg3 from compounds with that ability, but exert apoptotic effects by activating caspase-3 pathway. Several studies have shown that compounds with less polar structures have higher cancer cell cytotoxicity explaining that ginsenoside Rh2; hydrolysis product of Rg3, one glucose molecule removed at C3 is reported more pharmacologically active than its parent Rg3 [26]. Steaming notoginseng has improved the content of Rh2, Rk1, Rk3, and Rg3 which significantly increased its anti-proliferative effects against liver cancer cell lines such as SNU449, SNU182, and HepG2 [27].

## Conclusion

The model developed in this study is highly reliable for predicting the responses. Predicted and experimental values have not shown significant difference, which proves that the model can be used to transform ginsenosides from *P. notoginseng* root extract using glycosidase enzyme extracted from *C. militaris* as described in this report. Obtained results confirm the high bioactivity potential of the products. These findings suggest the possible use of the developed model for minor ginsenoside production and exploitation of their bioactivities for use in food and medicines.

## Authors' contribution

LE Thi Hoang Yen and NGUYEN Duc Doan planned and designed the study, Jean Baptiste Simurabiye performed laboratory experiments and wrote the manuscript. TRINH Dac Hoanh, VU Duy Nhan, TRAN Huyen Thanh DONG Thi Hoang Anh assisted in laboratory works, Jean Bosco Nshimiyimana helped in editing and revising the manuscript.

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## Declaration of Competing Interest

The authors declare no conflict of interest.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sciaf.2021.e01082.

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