Acta Scientific MICROBIOLOGY (ISSN: 2581-3226)

Volume 5 Issue 12 December 2022

Research Article

Optimization of Physical Parameters for Bioconversion of Glycyrrhizin into 18α-glycyrrhetinic Acid and 18β-glycyrrhetinic Acid Using Response Surface Methodology

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DOI: 10.31080/ASMI.2022.05.1181

Received: November 14, 2022

Published: November 22, 2022

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Abstract

Therapeutic constituents in the medicinal plants have always been copious in nature that yield useful phytomolecules. One such plant is licorice which is scientifically known as *Glycyrrhiza glabra* and belongs to the Fabaceae family. It is interesting to note that *Glycyrrhiza* has been traditionally utilized in a variety of polyherbal formulations in Japan, known as Kampo medicine. Glycyrrhizin is one of the main constituents of *Glycyrrhiza glabra* which can be biotransformed into biologically and pharmacologically active 18α-glycyrrhetinic acid and 18β-glycyrrhetinic acid simultaneously. Different levels of physical parameters (temperature, pH and time) was optimized by central composite design (central rotatory) of response surface methodology for the production of hydrolytic unit. Maximum hydrolytic unit of 15.678 HU/ml was predicted at an optimum value of temperature 35°C, pH 7.8 and time 45 min using response surface plots and point prediction tool of software Design Expert 8.0.1.3 (Stat-Ease Inc., USA).

Keywords: Glycyrrhizin; 18α-glycyrrhetinic Acid; 18β-glycyrrhetinic Acid; Optimization; Central Composite Design; Response Surface Methodology

Introduction

For centuries plants and their phytochemicals is a source of medicine due to diverse biological nature. Medicinal plants are the most abundant sources of drugs for conventional medical systems, modern medications, nutraceuticals, food supplements, folk remedies, pharmaceuticals and intermediary compounds that allowed for synthesized drugs. One such known medicinal plant is *Glycyrrhiza glabra*. The *Glycyrrhiza* genus, which has more than 30 species, is extensively found around the world. *Glycyrrhiza* name was derived from the Grecian words glykys which means sweet, and rhiza, which means root. The glabra species refers to the smooth husks and is derived from the latin word glaber [1]. *Glycyrrhiza glabra* belongs to family Fabaceae and is commonly known as licorice, sweet root, or mulaithi [2]. Widely utilized in the Asian system as a medicine to cure a variety of diseases. Glycyrrhizin (GL), a triterpenoids saponin, is the primary active component of *Glycyrrhiza glabra* roots and stems. On enzymatic hydrolysis, GL is metabolized into 18β-glycyrrhetinic acid

(18β-GA) which is responsible for its biological activity and has been suggested for use in the treatment of chronic hepatitis C, hepatic lipo-toxicity, and inflammation brought on by lipopolysaccharide (LPS) [3-5]. Licorice is one of the most commercially valuable plants globally, having a wide range of uses in tobacco, cosmetics, the food industry, and pharmaceuticals [6].

Due to its purported anti-inflammatory, anti-ulcer, anti-allergic, antioxidant, anti-tumor, anti-diabetic, and hepatoprotective properties, it has been utilized in a wide range of polyherbal formulations [7,8]. It is worth mentioning that *Glycyrrhiza* has traditionally been employed in several polyherbal formulations. Yokukansan is a formulation used in Japan, which is traditional Japanese Kampo medicine, consisted of seven different plant species [9]. Glycyrrhetinic acid GA can exist in both trans and cis isomeric forms, the trans form is 18α-glycyrrhetinic acid and the cis form is 18β-glycyrrhetinic acid [10-12]. Although both isomers show biological activity, 18β-glycyrrhetinic acid has received more attention due to its high level in root extract. Hence, 18β-glycyrrhetinic acid was thought to have greater biological activity than 18α-glycyrrhetinic acid [13,14]. Numerous studies demonstrated that microbial transformation is a versatile method for enhancing the structural diversity of plant triterpenoids. Biocatalysis and biotransformation has an upper edge due to their high specificity towards the substrate and requires mild reaction conditions [10]. Therefore finding the right microbe is a major challenge in achieving a desired biotransformation. In light of this, the traditional method of screening, a variety of microbial strains is still the most popular one. A thorough analysis of the microbial metabolism of triterpenoids was previously reported [15]. Phenolphthalein-D-glucuronide (PPG) was used to measure the enzyme activity after screening an isolate of the *Aspergillus parasiticus* that produces β-glucuronidase [16]. A commercial preparation from *Aspergillus niger* was used to enzymatically convert GL into 18β-GA from *G. glabra* [17]. Due to the structural similarities between GL and 18β-GA, they exhibit effects comparable to mineralocorticoids and block the metabolism of adrenocorticosteroids [18]. Biological and physicochemical features are greatly influenced by stereochemistry [19]. In alkaline state, GL exist as β-isomer (18β-GL, 18β-GA) which can be isomerized to their α-isomers (18α-GL, 18α-GA) [20]. Hence screening of different microorganisms is essential to study the bioconversion of GL into 18α-GA and 18β-GA in presence of β-glucuronidase enzyme.

Optimization of process parameters frequently uses response surface methodology (RSM) in combination with central composite design. RSM is a three dimensional experimental design that provides relationship between one or more measured dependent variables and a number of independent factors. The advantages of RSM includes lesser experiment numbers, suitability for multiple factors experiments and ability to search for relativity between the factors to find out the most suitable conditions for the prediction of responses [21-23]. In RSM, contour plots and a model equation fitting the experimental data are constructed using the linear or quadratic effects of experimental variables which enables the optimal value of parameters under investigation to be determined and the prediction of response under optimized conditions [24,25].

This research study focused on the optimization of different physical parameters for simultaneous bioconversion of GL into 18α-GA and 18β-GA in fermented *G. glabra* root extract by enzyme β-glucuronidase. The primary physical parameters that were screened by central composite design (CCD) were optimized using the response surface methodology (RSM). All the bio-transformed molecules were quantitavely analyzed by high-performance liquid chromatography (HPLC) method.

Materials and Methods

Microorganism

The bacterial strain *Escherichia coli* (*E. coli*) MTCC 1652 was obtained from the Institute of Microbial Technology (IMTECH) in Chandigarh, India, and was grown and maintained in Nutrient Agar (NA) and subcultured every 30 days.

Preparation of seed culture

E. coli MTCC 1652 seed cultures were produced by culturing bacterial inoculums in nutrient broth (NB) for 48 hours at 37 ˚C and 150 rpm in an orbital rotary shaker.

Preparation of *Glycyrrhiza glabra* root extract

Clean *Glycyrrhiza glabra* roots were dried and milled in order to prepare the coarse powder. Coarse powder (25 grams) were extracted in 100 % water for 72 hr with soxhlet apparatus. The extract was concentrated at 40 ˚C in a rotating evaporator under reduced pressure.

Production of β-glucuronidase enzyme by submerged fermentation

Submerged fermentation was carried out in 250 ml Erlenmeyer conical flasks. Synthetic media consisting of 0.05 % NH_4Cl, 0.005 % of $(NH_4)_2SO_4$, 0.4 % dextrose, 0.01 % NaCl, 0.01 % $MgCl_2 \cdot 6H_2O$, 0.6 % Na_2HPO_4 and 0.3 % KH_2PO_4, pH was adjusted to 7.2 was used for production of the enzyme from *E. coli* MTCC 1652 [20]. The medium was sterilized at 121 °C and 1.06 kg cm^{-2} for 20 min. 5 ml of bacterial seed culture was inoculated to 50 ml of synthetic media aseptically and fermentation was carried out at 37 °C, 150 RPM for 24 hr for *E. coli.*

Purification of *E. coli* β-glucuronidase enzyme

Fermented broth of *E. coli* MTCC 1652 was centrifuged at 4000 RPM for 10 min. The supernatant obtained was discarded and pellets were retained. The cell pellet (18 g) were suspended in buffer A (1:1 w/v) (50 mM sodium phosphate buffer, pH 3.5,10 mM β-mercaptoethanol and 0.1% triton X-100) and disrupted by sonication (Sonics vibra cell disruptor, W-185, with power setting at 50 W for 5 min with 1 min interval on crushed ice). The cell homogenate was centrifuged at 4000 RPM for 10 min. The fragmented cell pellet was discarded and only supernatant (25 ml) was taken. The original pH of supernatant was 5.98 and adjusted to 3.5 by using 50 mM sodium phosphate buffer, pH 3.5 to make the enzyme positively charged. To the 25 ml of this supernatant, 7.5 g of CELITE 545 was added and incubated for 2 hr at 22 °C. Then the resulting mixture was subjected to centrifuge at 3000 RPM for 5 min. The supernatant (S_1) was collected for analysis and the pellets (pH 4.8) were treated with 50 mM sodium phosphate buffer, pH 6.5 to make the enzyme negatively charged. The pH treated pellets were kept under shaking condition at 22 °C for 2 hr and then centrifuged at 3000 RPM for 5 min [26]. The supernatant (S_2) was collected and analyzed by HPLC.

Assay for β-glucuronidase enzyme

Hydrolytic Unit (HU) was used for enzyme activity by incubating the enzyme with 3 mM GL solution for 10 min at 35°C. The enzymatic reaction was stopped by adding glycine buffer solution (200 mM) of pH 10.4. The amount of 18α-GA and 18β-GA formed was analyzed by high performance liquid chromatography (HPLC). One HU is defined as microgram (µg) of 18α-GA and 18β-GA produced per 10 min from 1 µg/µl of pure GL solution.

Optimization of physical parameters for enzymatic bioconversion

The physical parameters (incubation temperature, pH and time of experiment) were chosen for the study. The various levels of physical parameters have been summarized in table 1. Experimental design for optimization of physical parameters was planned as per the central composite design (central rotatory) tool of response surface methodology (RSM) using software Design Expert 8.0.1.3 (Stat-Ease Inc., USA). An experimental design of 20 runs containing 6 central points (Table 2) was made according to the central composite rotatable design (CCRD) of response surface methodology for selected three physical parameters using software Design Expert 8.0.1.3 (Stat-Ease Inc., USA). An optimum value of the factors for ideal value of bioconversion of GL into 18α-GA and 18β-GA was determined by point prediction tool of software Design Expert 8.0.1.3.

Physical parameters	Levels				
	-2	-1	0	+1	+2
Temperature °C	33	35	37	39	41
pH	4.8	5.8	6.8	7.8	8.8
Time (min)	30	45	60	75	90

Table 1: Levels of physical parameters used in experiment.

Run	Temperature (°C)	pH	Time (min)
1	35	5.8	45
2	39	5.8	45
3	35	7.8	45
4	39	7.8	45
5	35	5.8	75
6	39	5.8	75
7	35	7.8	75
8	39	7.8	75
9	33	6.8	60
10	41	6.8	60
11	37	4.8	60
12	37	8.8	60
13	37	6.8	30
14	37	6.8	90
15	37	6.8	60

16	37	6.8	60
17	37	6.8	60
18	37	6.8	60
19	37	6.8	60
20	37	6.8	60

Table 2: Central composite response surface design for three physical parameters.

Bioconversion of glycyrrhizin to 18α-glycyrrhetinic acid and 18β-glycyrrhetinic acid

Fermented broth of *E. coli* MTCC 1652 was taken and added to GL extract in presence of phosphate buffer at various pH (5.5, 6.5, and 7.5) and incubated at 35 °C. The samples were withdrawn at different time intervals and analyzed by HPLC.

Chromatographic condition and analysis of GL, 18α-GA and 18β-GA

Samples containing GL were analysed by high performance liquid chromatography (Shimadzu, Japan). The chromatography was carried out by RP C18 column (temperature 25°C), the mobile phase consisting of methanol: water (85:15 v/v) at a flow rate of 1 ml/min with run time of 10 min and detection at 254 nm. 18α-GA and 18β-GA was simultaneously analysed by HPLC method with the mobile phase acetonitrile: tetrahydrofuran: water (10: 80: 10 v/v) at flow rate of 1 ml/min with run time of 10 min. The detection of 18α-GA and 18β-GA was carried out by UV detector at 254 nm [27].

Results and Discussion

Bioconversion of glycyrrhizin to 18α-GA and 18β-GA

Enzymatic bioconversion has always advantages over conventional chemical methods as it allows the sugar molecules to be separated from the glycoside under mild reaction conditions and prevents the generation of additional phytomolecules and other microbial by-products. Enzymes originating from animal livers and human intestinal flora have already been used to bioconvert GL into GA [16]. β-glucuronidase enzymes belongs to the glycosidase family, group-II of enzymes that have ability to hydrolyse the glycosidic bond between two or more carbohydrate or between a carbohydrate and non-carbohydrate moiety [28].

In the present research study enzymatic conversion of glycyrrhizinic acid (GL) from root extract of *Glycyrrhiza glabra*, into 18α-GA and 18β-GA, was completed by optimization of three key physical parameters using β-glucuronidase from bacterial strain *E. coli* MTCC 1652. This enzymatic bioconversion was studied in terms of hydrolyzing unit (HU). Simultaneous and quantitative determination of 18α-GA and 18β-GA was performed by using HPLC with reverse phase C-18 column and the detection of 18α-GA and 18β-GA was carried out by UV detector at 254 nm (Figure 1).

Optimization of physical parameters for enzymatic bioconversion

Response surface methodology (RSM) is considered to be an optimization tool for major scientific process that is used to determine the optimum values of a variety of significant factors for the process. It is a compilation of statistical techniques to design the experiments, evaluate the effects of variables and thereby, seeking the optimum conditions. It is widely used in optimization of different types of biotransformation and related bioprocesses. The robustness of RSM is the confined sets of experimental runs that are required to provide sufficient data for statistically acceptable results, in addition, its suitability for multiple factor experiments and examination of common relationship between various factors under experiment towards finding the most suitable production conditions for the biotransformation and forecast the response [29]. Experimental design for enzymatic bioconversion of pure GL to 18α-GA and 18β-GA by crude β-glucuronidase enzyme was designed according to central composite rotary design (CCRD) tool of RSM using Design Expert software (Stat-Ease Inc., USA) for selected three physical parameters (temperature, pH and time). The various levels of physical parameters were summarized in table 3. An experimental design of 20 runs containing 6 central points was

Figure 1: HPLC chromatogram of standard glycyrrhizin (a), 18α-glycyrrhetinic acid (b), 18β-glycyrrhetinic acid (c) and standard mixture of 18α-GA and 18β-GA (d).

made according to central composite design (central rotatory) of response surface methodology for three physical parameters.

The individual and interactive effects of these physical parameters were studied by conducting the experiment run at

Std Run	Temperature (°C)	pH	Time (min)	Hydrolyzing Unit/ ml	
				Actual	Predicted
1	35	5.8	45	0.47	1.16
2	39	5.8	45	00	0.41
3	35	7.8	45	2.66	2.75
4	39	7.8	45	2.27	2.35
5	35	5.8	75	0.88	0.68
6	39	5.8	75	0.26	0.05
7	35	7.8	75	2.08	1.56
8	39	7.8	75	2.08	1.28
9	33	6.8	60	2.25	2.16
10	41	6.8	60	0.94	1.14
11	37	4.8	60	0.31	-0.09
12	37	8.8	60	2.21	2.72
13	37	6.8	30	3.33	2.63
14	37	6.8	90	0.28	1.08
15	37	6.8	60	0.28	0.30
16	37	6.8	60	0.28	0.30
17	37	6.8	60	0.28	0.30
18	37	6.8	60	0.28	0.30
19	37	6.8	60	0.28	0.30
20	37	6.8	60	0.28	0.30

Table 3: CCD design of selected physical parameters with actual and predicted values of hydrolyzing unit.

different levels (Table 1) of all three parameters. The bioconversion response was measured in terms of hydrolyzing unit after the experiment was over. The results of experimental data and simulated values are listed in table 4. Data collected for hydrolyzing unit in each experiment run were analyzed using software Design Expert 8.0.1.3.

The data fitted into a multiple non-linear regression model

Source	Sum of Squares	DF	Mean Square	p-value Probe> F
Model	17.68	09	1.96	0.0048
Temprature	1.05	1	1.05	0.1026
pH	7.94	1	7.94	0.0006
Time	2.40	1	2.40	0.0218
AB	0.060	1	0.060	0.6761
AC	7.021E-003	1	7.021E-	0.8862
BC	0.26	1	003	0.3939
A2	2.88	1	0.26	0.0140
B2	1.62	1	2.88	0.0501
C2	3.383	1	1.62	0.0065
Residual	3.26	10	3.83	
Lack of Fit	3.26	5	0.33	
Pure Error	0.000	5	0.65	
Corrected Total	20.94	19	0.000	

Table 4: Analysis of variance of the predicted model for bioconversion of glycyrrhizin to 18α-glycyrrhetinic acid and 18β-glycyrrhetinic acid.

proposes following equation (in the coded factor) for hydrolytic unit production.

Hydrolytic Unit/ml = 0.30 – 0.26A + 0.70B - 0.39C + 0.087AB + 0.030AC - 0.18BC + $0.34A^2$ + $0.25B^2$ + $0.39C^2$ where A, B and C represents temperature, pH and time respectively.

The effects of all physical parameters for bioconversion rate was compared with the help of perturbation plots (Figure 2). The lines in the graph represent influences and sensitivity of the respective factors for bioconversion of GL to 18α-GA and 18β-GA.

Figure 2: Perturbation plot showing the effects of all physical parameters on of hydrolytic unit where A, B, and C represents temperature, pH and time respectively.

This model resulted in three response surfaces with contours. The response surface plots of calculated model for hydrolytic unit production are shown in figure 3 (a-c). The analysis of variance of regression for bioconversion of GL to 18α-GA and 18β-GA is summarized in Table 4. All the response surfaces/contour could be analyzed for determining the optimized value of the factors, but it was difficult to analyze all these simultaneously. Hence, point prediction tool of design expert software was used to determine the optimum values of the factors for maximum hydrolytic unit. Finally an optimum value of temperature 35 °C, pH 7.8 and time 45 min resulted in hydrolytic unit production of 15.678 HU/ml. These optimized values were further validated and an average of 14.237 HU/ml was obtained. This shows 90.80% validity of the predicted model.

Figure 3: Response surface showing the relative effects of two physical parameters on bioconversion of GL to 18α-GA and 18β-GA while keeping other parameters constant (a-c).

Conclusion

Biotransformation of natural plant based compounds is a dominant approach to produce several new phytomolecules which may be used in various human disease in the form of herbal formulation. In the present study, glycyrrhizin of *Glycyrrhiza glabra* root extract was biotransformed to 18α-glycyrrhetinic acid and 18β-glycyrrhetinic acid. This biotransformation process was statistically examined by using central composite design of response surface methodology which reduces the number of experiment, suitability for multiple factors experiments and more importantly interaction between the factors to predict the responses. The hydrolytic unit production was finally predicted as 15.678 HU/ml at an optimum value of temperature 35°C, pH 7.8, and time 45 min. When these optimized values of process parameters were validated by further biotransformation study in duplicate, an average of 14.237 HU/ml was obtained with 90.80% validity of the predicted model. This biotransformation study may be used in the biotransformation of other phytomolecules.

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