http://dx.doi.org/10.22037/afb.v4i2.15713

Research Article

APPLIED FOOD BIOTECHNOLOGY, 2017, 4 (2):113-121
Journal homepage: www.journals.sbmu.ac.ir/afb

pISSN: 2345-5357
eISSN: 2423-4214

Enzymatic Synthesis of Theanine in the Presence of L-glutaminase Produced by *Trichoderma koningii*

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Abstract

Background and Objective: Since ancient times, it has been said that drinking green tea brings relaxation. The substance that is responsible for a sense of relaxation is theanine. Theanine (γ-glutamylethylamide) is a unique non-protein amino acid. It gives an umami taste and a unique flavor to the tea, and has many physiological and pharmaceutical effects such as anti-tumor, anti-cancer, neuro-protective, anti-hypertensive and anti-obesity effects; it may further help in relaxation and increase focus. So this compound is essential for human body; however, it is not synthesized in the body and should be administrated orally. In the present study, the enzymatic biosynthesis of theanine was examined in the presence of Ethylamine and L-glutamine, and for the first time the enzyme was produced by the fungal strain *Trichoderma koningii*.

Material and Methods: At first**,** solid state fermentation was carried out for the production of L-glutaminase by the fungal strain *Trichoderma koningii* using sesamum oil cake as the solid substrate. Then the biosynthesis of theanine was performed in the presence of extracted enzyme solution, and ethylamine and L-glutamine as substrates. The concentration of effective parameters, namely L-glutamine and ethylamine, and the volume of enzyme solution on theanine production were evaluated based on the response surface methodology coupled with central composite design. 16 experiments were designed by the design expert software and carried out to examine the changes of theanine concentration with changes in the concentration of ethylamine and L-glutamine and the volume of enzyme solution.

Results and Conclusion: This investigation indicated simultaneous synthesis of theanine as well as hydrolysis of L-glutamine and L-glutamic acid. Selected independent variables (including ethylamine concentration, L-glutamine concentration and enzyme solution volume) were effective on theanine concentration. Increase of enzyme solution volume had a significant effect on theanine concentration. The highest theanine concentration (43 mM) was obtained at the ethylamine concentration of 0.9 M, L-glutamine concentration of 0.3 M and enzyme solution of 3 ml.

Conflict of interest: The authors declare no conflict of interest.

Article Information

Article history:
Received 10 Jan 2017
Revised 25 Jan 2017
Accepted 07 Mar 2017

Keywords:
- Enzymatic biosynthesis
- L-glutaminase
- L-Theanine
- Response surface methodology
- *Trichoderma koningii*

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1. Introduction

Since ancient times, it has been said that drinking green tea brings relaxation. Tea is the second most consumed beverage in the world, and contains a number of useful constituents including polyphenols, proteins, amino acids, organic acids, vitamins, minerals and pigments [1], but theanine is responsible for its unique pleasant taste as well as its known relaxation effect. In 1949, this compound was first identified in tea leaves [2], and in 1969, Food and Drug Administration (FDA) confirmed L-theanine as Generally Recognized as Safe (GRAS), and the Japanese Ministry of Health and Welfare approved the use of L-theanine [3] for universal consumption. It comprises 1-2% of the dry weight of tea leaves, makes up approximately 50% ww^{-1} of the amino acids in tea, and is present as the free amino acid only – it does not occur in proteins [4].

In recent years, different studies have been done on theanine to prove its many beneficial physiological and pharmaceutical effects including relaxation [5], increasing concentration and learning ability [6], anti-tumor [7], anti-hypertensive [8], anti-obesity effects [9], and neuro-protection [10]. However, in these studies, the beneficial effects were observed with daily consumption of pure L-theanine of at least 50 mg, an amount equivalent to a minimum of three cups of tea. Besides, many of these studies were done using around 150-250 mg of L-theanine, doses which would not be easily achieved even by the

most willing tea drinkers [11]. Therefore, based on the doses of pure theanine used in the studies showing beneficial effects, there is a perceived demand for L-theanine as a supplement or food ingredient. This has led to exploring efficient and economical extraction techniques for the isolation of natural theanine and investigation into efficient synthetic and biosynthetic means of producing this amino acid. In addition to different natural isolation and chemical methods to produce theanine, recently, biological production of L-theanine using enzymes has attracted much attention [12].

The natural sources of the enzymes include plants, animals and microorganisms. In contrast to the plant and animal sources, microbial sources are preferred due to economic as well as other technical benefits such as higher yields obtained within the shortest fermentation time [13]. For industrially biological production of L-theanine, some kinds of bacterial enzymes display potential as ideal biocatalysts. In 2005, Yamamoto et al. produced theanine by couple fermentation with energy transfer employing *Pseudomonas (P.) taetrolens* Y-30 L-glutamine synthetase [14]. In 2002, Suzuki et al. used *Escherichia coli* γ-glutamyl transepeptidase to produce theanine from ethylamine and L-glutamine [15]. In 2012, Wang et al. [16] reported a new method of theanine synthesis using this enzyme, and replaced L-glutamine with L-glutamine-Zn (II) complex as glutamyl donor, which successfully reduced the side autotranspeptidation reaction and led to higher yield of theanine. L-glutaminase is the other biocatalyst that catalyzes the hydrolysis of γ-amido bond of L-glutamine to L-glutamic acid and free ammonia. Additionally, a few L-glutaminases can also catalyze the transfer reaction of γ-glutamyl moiety of L-glutamine to a hydroxylamine as an acceptor molecule. So far, only *P. nitroreducens* L-glutaminase has been reported as L-theanine producing L-glutaminase [17,18]. In these reports, in which L-glutamine (as γ-glutamyl donor) and ethylamine (as γ-glutamyl acceptor) have been used to produce L-theanine by free *P. nitroreducens* L-glutaminase, the γ-glutamyl transfer reaction (Fig. 1) and the hydrolysis of L-glutamine (Fig.2) have happened simultaneously. No L-glutaminase with other microbial sources has been used to produce theanine.

In this paper, for the first time, synthesis of theanine was examined in the presence of L-glutaminase enzyme produced by *Trichoderma (T.) koningii* fungus. At first, production of L-glutaminase enzyme by *T. koningii* using sesamum oil cake under solid state fermentation was evaluated. Then L-theanine production by L-glutamine and ethylamine as substrates and extracted enzyme solution was reported. The effective parameters, namely L-glutamine and ethylamine, and the concentration and volume of enzyme solution on theanine production were evaluated based on the response surface methodology coupled with central composite design.

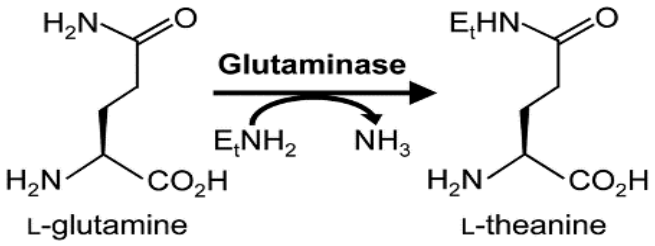

Fig. 1. Theanine synthesis reaction catalyzed by L-glutaminase [19].

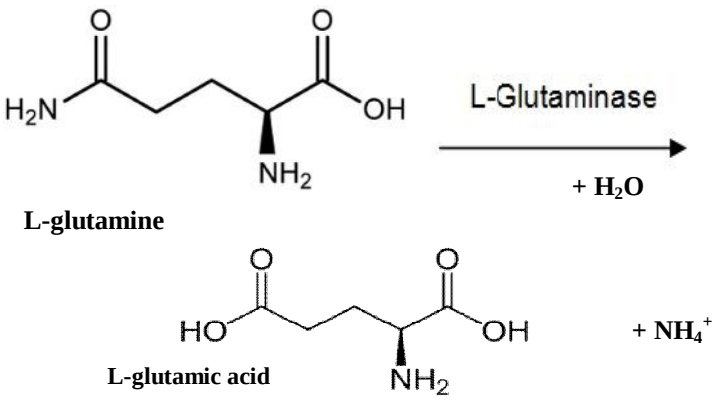

Fig. 2. L-glutamine hydrolysis catalyzed by L-glutaminase [20].

The fungal strain *T. koningii* PTCC 5139 used in this study was supplied from the Biotechnology and Environmental Research Institute of Sharif University of Technology, Tehran, Iran. The main culture was maintained on potato dextrose agar (PDA) medium purchased from Merck Company. Other chemicals also were purchased from Merck Company and used as received without any purification. Inoculated slants were grown in an incubator at 30℃ for 6 days. Then the slants were stored at 4℃ and sub-cultured once every 4 weeks.

2.2. Enzyme Production and Extraction

Sesamum oil cake purchased from local market of Isfahan, Iran was used as solid substrate for the production of L-glutaminase by *T. koningii*. 5 grams of dried crushed sesamum oil cake was taken in a 250 ml Erlenmeyer flask and moistened with 10 ml of salt solution containing glucose (0.6% w v^{-1}), KH_2PO_4 (0.1% w v^{-1}), $MgSO_4.7H_2O$ 0.05% w v^{-1}) and KCl (0.05% w v^{-1}). The completely mixed flask was autoclaved at 121℃ for 20 min and then cooled down to room temperature [21]. Conidial suspension was prepared from a 6 day-old culture of *T. koningii* on PDA slant by suspending in 2 ml of 0.85% w v^{-1} sterile saline solution. Autoclaved flask was inoculated with 2 ml of the prepared fungal conidial suspension. The contents were mixed thoroughly, and the flask was placed in an incubator at 30°C for 5 days.

In order to extract L-glutaminase enzyme from the fermented solid substrate, it was mixed with 41 ml of 0.1M sodium phosphate buffer (pH= 8). The flask was then kept on a rotary shaker at 150 rpm for 30 min. The slurry was centrifuged at 11963 ×g for 15 min at 4°C in a cooling centrifuge. The supernatant was first filtered by 0.4 micron filter and then by 0.22 micron syringe filter. The clear filtrate was centrifuged again at 11963 ×g for 5 min at 4°C to obtain a completely transparent solution.

2.3. Enzyme Assay

L-glutaminase was assayed according to Imada et al. [22]. The enzymatic reaction mixture containing 0.5 ml of L-glutamine (0.04 M), 0.5 ml of 0.1M Tris-HCl buffer (pH 8.0), 0.5 ml of distilled water and 0.5 ml of enzyme solution was incubated at 37°C for 30 min. The enzymatic activity was stopped by the addition of 0.5 ml of 1.5 M Trichloroacetic acid. Then to 3.7 ml of distilled water, 0.1 ml of the above mixture and 0.2 ml of Nessler's reagent were added, and the developed color was read after 10-15 min at 480 nm in a spectrophotometer. One unit (U) of L-glutaminase was defined as the amount of enzyme that liberates 1 µmol of ammonia under optimal assay conditions.

2.4. Theanine Synthesis

Theanine production was done in the presence of L-glutamine and ethylamine as reaction substrates and extracted enzyme solution.

2.5. Theanine Analysis

The concentration of produced theanine in the presence of ethylamine, L-glutamine and L-glutaminase enzyme solution was determined by HPLC (Sykam Chromatography, Germany, ODS C18 (4.6 × 250 mm, 5 µm)) having 2 discrete pumps and a UV detector at 220 nm. Water was used as a mobile phase with a flow rate of 1.0 ml min^{-1}.

2.6. Experimental Design and Mathematical Model

The design of experiments and statistical analysis of the obtained data were carried out by Design Expert Software (ver. 7.0). The selection of independent variables of major effects on the system was done according to the study objectives. Ethylamine and L-glutamine concentrations (A and B, respectively) and the volume of enzyme solution (C) were independent variables selected in the experiments design. The levels of variables were selected as different values of a variable at which the experiments must be carried out. The range of variables was selected considering the previous studies. Central composite design (CCD) as one of the methods under response surface methodology (RSM) was employed to explore the relationship between process response and variables (A, B and C) in terms of actual units and experimental data obtained for the response. This design consists of the following parts: (1) a full factorial or fractional factorial design; (2) an additional design, often a star design in which experimental points are at a distance from its center; and (3) a central point. A total of 16 experiments were designed, and the amount of theanine concentration was calculated as response (Table 1). The response (theanine concentration) is a function of the levels of Ethylamine and L-glutamine concentrations and the volume of enzyme solution (Eq. 1):

$$Y = f(A, B, C) + \varepsilon \quad \text{Eq. 1}$$

Where, A, B and C are independent variables (Ethylamine and L-glutamine concentrations and enzyme solution volume, respectively), and ε represents the noise or error observed in response y [23].

Table 1. Experimental conditions and results for theanine synthesis

Run	thesis			
	Factor 1 A: Ethylamine concentration (mol l^{-1})	Factor 2 B: Glutamine concentration (mol l^{-1})	Factor 3 C: Enzyme solution volume (ml)	Response: Theanine concentration (mmol l^{-1})
1	0.6	0.3	1	11
2	0.9	0.4	1	10
3	0.9	0.2	1	8
4	0.3	0.4	5	27
5	0.6	0.3	3	33
6	0.9	0.4	5	33
7	0.3	0.3	3	30
8	0.9	0.2	5	36
9	0.3	0.2	5	17
10	0.9	0.3	3	43
11	0.6	0.2	3	14
12	0.6	0.4	3	20
13	0.3	0.4	1	8
14	0.3	0.2	1	4
15	0.6	0.3	3	32
16	0.6	0.3	5	35

3. Results and Discussion

In the presence of L-glutaminase enzyme solution, and ethylamine and L-glutamine as substrates, two reactions happened: theanine synthesis (as shown in Fig. 1) and hydrolysis of L-glutamine, and production of glutamic acid (as shown in Fig. 2). According to the standard curve of theanine, one representative HPLC Chromatogram for one of the 16 samples is shown in Fig. 3. Peak 1 shows the theanine exit time from the column, concentration of which is determined from the standard curve of peak area ratios for increasing the concentration of ammonium sulfate.

A total of 16 experiments with different combinations of ethylamine concentration (A), L-glutamine concentration (B) and enzyme solution volume (C) were designed by the software and conducted to obtain response in accordance with Table 1. The experimental data were then fitted to the following second order polynomial equation:

$$Y = a_0 + a_1A + a_2B + a_3C + a_{11}A^2 + a_{22}B^2 + a_{33}C^2 + a_{12}AB + a_{13}AC + a_{23}BC + \varepsilon \quad \text{Eq.2}$$

Where, Y is the predicted response, ε is the calculated error, a_0 is the value of the fitted response at the center point of the design, and a_i ,a_{ii} and a_{ij} are the linear, quadratic and cross point coefficients, respectively. These coefficients can be used to characterize the relation between the variables (i.e. concentration of ethylamine and L-glutamine and volume of enzyme solution) and the response (theanine concentration).

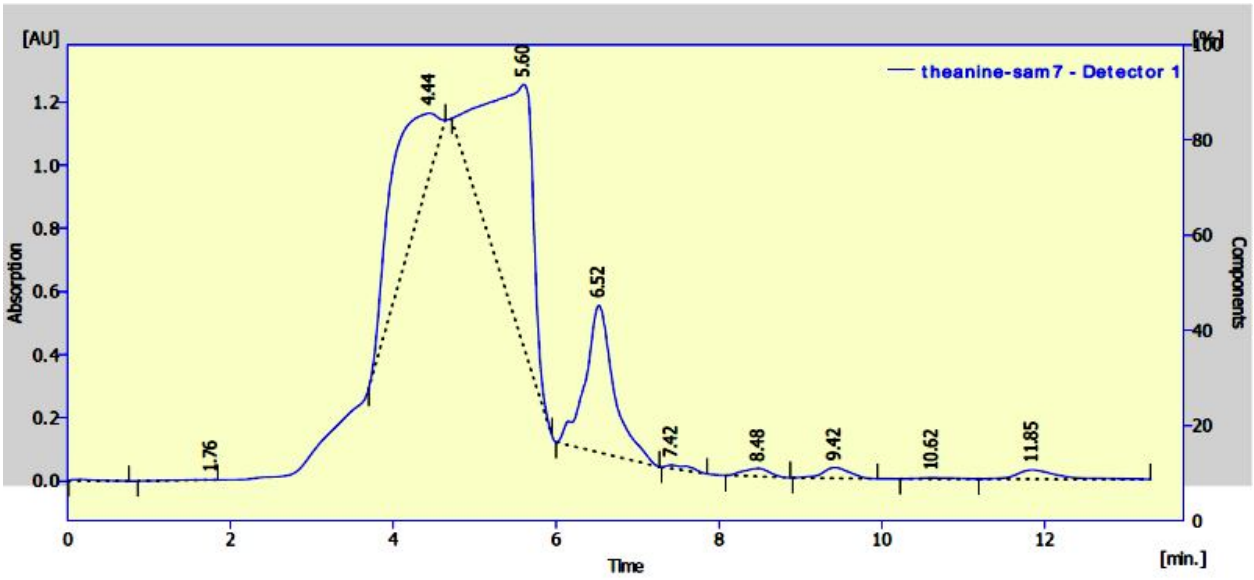

Fig. 3. Representative HPLC chromatogram for one of the 16 samples according to the experiment design.

Table 2 illustrates the reduced models on terms of coded factors with significant model terms and analysis of variance (ANOVA) results for the response. The actual and predicted plot for the response (Fig. 4a) and the normal plot of residuals (Fig. 4b) are shown in Fig. 4. These figures show good agreement between the actual and modeled data.

Values obtained from the ANOVA analysis determine the rank of significance degree. P-value was computed to determine the significance of the model terms. In Table 2, the model was developed with the very low probability value ($p \leq 0.0002$). This indicates that the model terms were significant. The lack of fit value was also insignificant ($p \geq 0.05$) for the studied response. Coefficient of determination (R^2) and adjusted R^2 were 0.98 and 0.95, respectively. Furthermore, adequate precision measures the signal to noise ratio, and a ratio greater than 4 is desirable. In this case, the value of adequate precision was 18.683. Therefore, the proposed model could be adequately used to describe the response under a wide range of operating conditions.

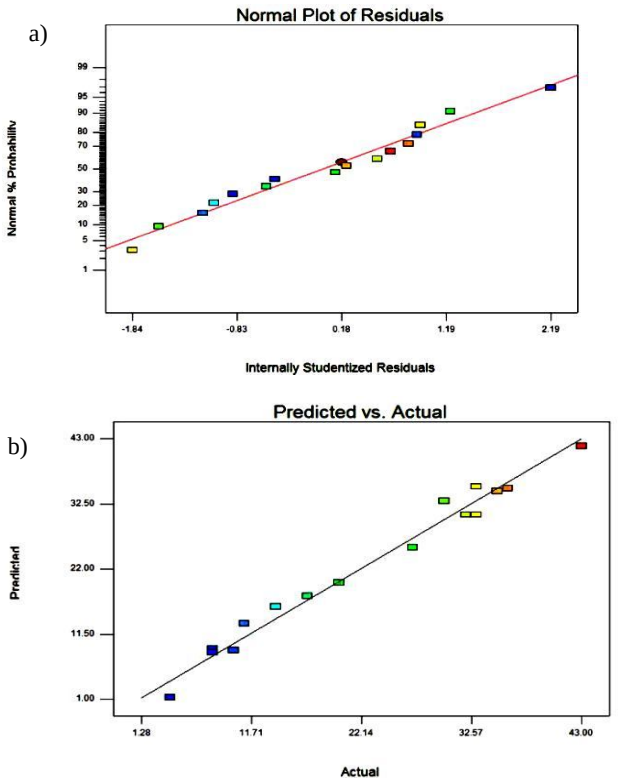

Fig. 4. (a) Predicted vs. actual values for theanine concentration, and (b) normal plot of residuals.

Table 2. Anova results for the model of design expert 7.0.0 for studied response

Response	Theanine concentration (mmol l^{-1})
Modified equation with significant terms (in terms of coded factors)	$30.74+4.40A+1.9B+10.70C-1.88AB+2.38A\,C+0.13BC+6.64A^2+12.86B^2-6.86C^2$
Model p-value	0.0002
Lack of fit	0.1777
R^2	0.9804
Adj.R^2	0.9511
Adeq. precision	18.683
S.d.*	2.74
Press	557.84
C.V**	12.15

*Standard Deviation

**Coefficient of Variation

Table 3. Comparision of maximum theanine concentration in different researches

Authors	Enzyme and source	Substrates for theanine synthesis	Year	concentration of theanine (mmol l^{-1}) *	Reference
Sakhaei, Alemzadeh (Present work)	L-glutaminase from Trichoderma koningii	Ethylamine, L-glutamine	2016	43	
Takashi Tachiki et al.	Glutaminase from Pseudomonas nitroreducens IFO12694	Ethylamine, L-glutamine	1998	270	[18]
Hideyuki Suzuki et al.	γ-glutamyltranspeptidase from Escherichia coli K-12 strain SH642	Ethylamine, L-glutamine	2002	120	[15]
Sachiko Yamamoto et al.	Glutamine synthetase from Pseudomonas. taetrolens Y-30	Ethylamine, Sodium Glutamate	2005	170	[14]
Hao-Qi Wang et al.	γ-glutamyltranspeptidase from Bacillus subtilis NX-2 strain	Ethylamine, L-glutamine-Zn(II) Complexes	2012	61.3	[16]

* Maximum concentration of theanine (mmol l^{-1}) at optimum conditions

Figure 5 shows the simultaneous effect of ethylamine concentration and L-glutamine concentration on the response (theanine concentration) in the enzyme solution volumes of (a)1 ml, (b) 3 ml and (c) 5 ml. As shown in Fig. 5, by increasing the concentration of ethylamine and L-glutamine (from 0.6 to 0.9 and from 0.2 to 0.3, respectively) the response increases. But in the concentration range of L-glutamine (0.3-0.4), the concen-tration of theanine decreases. Furthuremore, L-glutamine concentration has a significant effect on theanine concentration but the effect of ethylamine concentration on theanine response is mild. By comparing three curves with different volumes of enzyme solution, it can be concluded that by increasing the enzyme solution volume, theanine concentration changes happen in higher range of concen-tration.

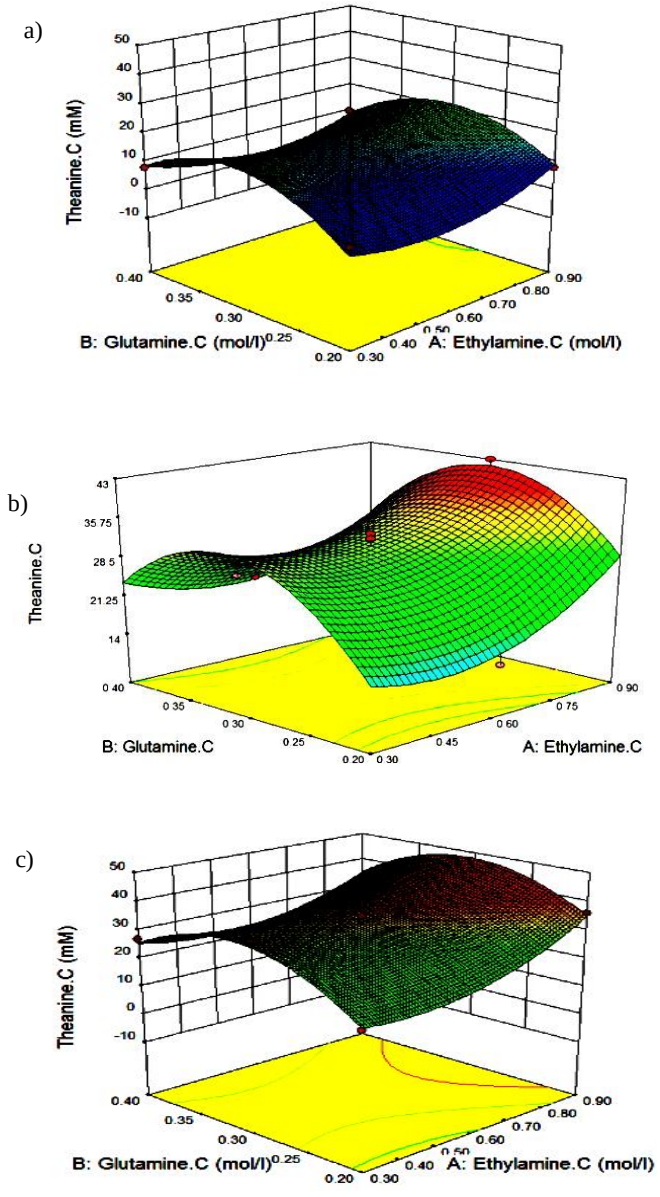

Fig. 5. Three-dimensional plots for theanine concentration vs. ethylamine and L-glutamine concentration with the enzyme solution volume of (a) 1 ml; (b) 3 ml and (c) 5 ml.

Figure 6 shows the simultaneous effect of the variables of ethylamine concentration and enzyme solution volume on the response (theanine concentration) in the L-glutamine concentrations of (a) 0.2 mol l^{-1}, (b) 0.3 mol l^{-1} and (c) 0.4 mol l^{-1}. Fig. 6 indicates that increase of both ethylamine concentration and enzyme solution volume causes the increase of theanine; however, the effect of enzyme solution volume is more significant. As can be seen in Table 2, increasing effect of factor C (enzyme solution) on the response is shown with positive coefficient, 10.70, indicating more dependency of the response on C than on A (ethylamine concentration) and B (L-glutamine con-cenntration) with the coefficients of 4.40 and 1.9, respectively. Figures 7a, 7b and 7c illustrate the effects of ethylamine concentration, L-glutamine concentration and enzyme solution volume on theanine concentration, respectively.

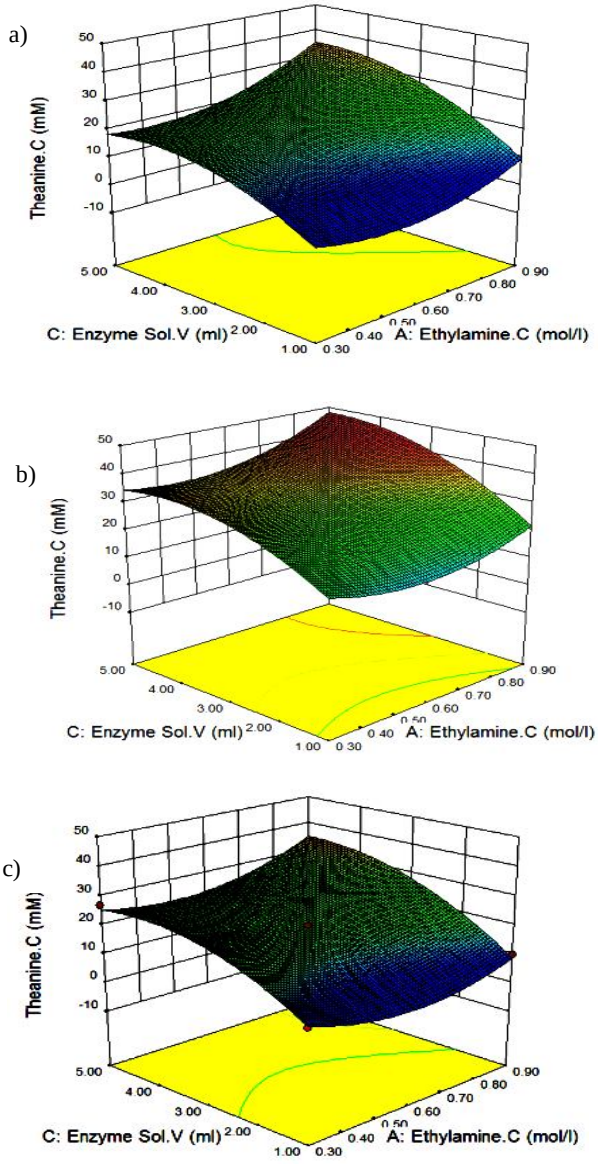

Fig. 6. Three-dimensional plots for theanine concentration vs. ethylamine concentration and enzyme solution volume with the L-glutamine concentration of (a) 0.2 mol l^{-1}; (b) 0.3 mol l^{-1} and (c) 0.4 mol l^{-1}.

As illustrated in Fig. 7a, by increase of ethylamine concentration, the concentration of theanine increases mildly. According to Fig. 7b, by increase of L-glutamine concentration from 0.2 to 0.3 mol^{-1}, theaine concentration increases; however, from L-glutamine concentration of 0.3 to 0.4, theanine concentration reduces. The reson behind this that in higher concentrations of L-glutamine, hydrolysis of L-glutamine and production of glutamic acid increase (Fig. 2). So L-glutamine concentration as a substrate for theanine synthesis reduces, and consequantly, theanine concentration reduces, too. According to the obtained results, the highest obtained theanine concentration was 43 mM at ethylamine concentration of 0.9 M, L-glutamine concentration of 0.3 M and enzyme solution of 3 ml. Table 2 compares the maximum concentrations of theanine under optimum conditions in the present research and those, reported in a number of previous studies in which the biological method has been used to produce theanine.

4. Conclusion

L-theanine is a free amino acid with many health benefits, and recently, potential demand in industry for this amino acid is expected. This study indicates theanine synthesis from L-glutamine and ethylamine through the transpeptidation reaction of L-glutaminase produced by *T. koningii*. At first, production of enzyme by *T. koningii* using sesamum oil cake under solid state fermentation is evaluated. Then L-theanine production by L-glutamine and ethylamine as substrates and extracted enzyme solution is reported. For this purpose, the concentration of effective parameters, namely L-glutamine and ethylamine, and volume of enzyme solution on theanine production were evaluated based on the response surface methodology coupled with central composite design. In addition to theanine production reaction, the hydrolysis reaction of L-glutamine in the presence of L-glutaminase enzyme solution happened as an undesirable one. Among the three independent variables, the enzyme solution volume had the most increasing effect on theanine concentration. The highest theanine concentration was obtained as 43 mM at ethylamine concentration of 0.9 M, L-glutamine concentration of 0.3 M and enzyme solution of 3 ml.

5. Acknowledgements

The authors gratefully acknowledge the experimental support by Biochemical and Bioenvironmental Engineering Research Center (BBRC) at Sharif University of Technology, Tehran, Iran

6. Conflict of Interest

The authors have declared no conflict of interest.

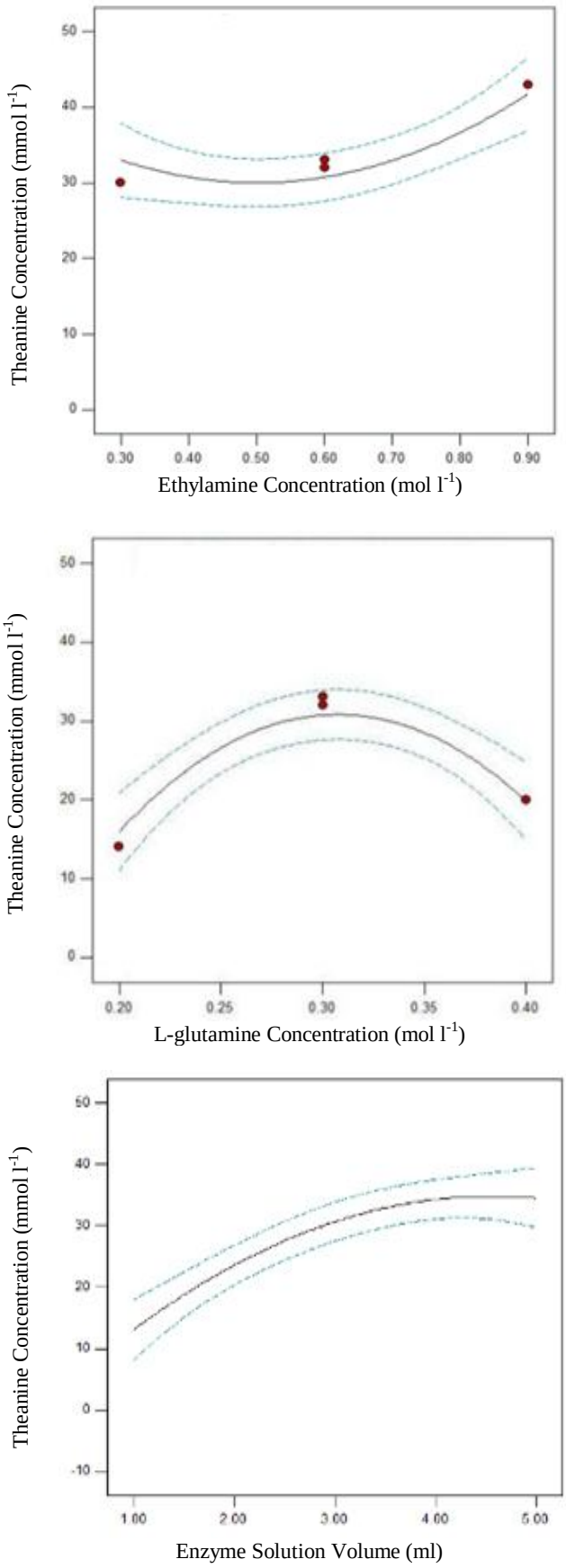

Fig. 7. Theanine concentration vs. (a) Ethylamine concentration; (b) L-glutamine concentration and (c) enzyme solution volume.

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http://dx.doi.org/10.22037/afb.v4i2.15713

Research Article

APPLIED FOOD BIOTECHNOLOGY, 2017, 4 (2):113-121
Journal homepage: www.journals.sbmu.ac.ir/afb

pISSN: 2345-5357
eISSN: 2423-4214

سنتز آنزیمی تئانین در حضور ال-گلوتامیناز تولید شده توسط *تریکودرما کونینجی*

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چکیده

سابقه و هدف: از زمان‌های قدیم گفته شده است که نوشیدن چای سبز، آرامش به ارمغان می‌آورد. ترکیبی که این حس آرامش را ایجاد می‌کند، تیانین نام دارد. تیانین(گاما-گلوتامیل اتیل آمید) یک آمینواسید غیرپروتئینی منحصر به‌فرد است. این ترکیب به چای طعم اومامی و عطر و طعم منحصر به فرد می‌دهد و اثرات دارویی و فیزیولوژیکی بسیاری مانند اثرات ضد تومور، ضد سرطان، محافظت نورونی، ضد پر فشاری خون و ضد چاقی دارد و موجب ایجاد حس آرامش و افزایش تمرکز می‌شود. بنابراین، این ترکیب برای بدن انسان لازم است؛ اما در بدن تولید نمی‌شود و باید به صورت خوراکی مصرف شود. در مطالعه حاضر، بیوسنتز آنزیمی تیانین در حضور اتیل آمین و ال-گلوتامین و توسط آنزیم ال-گلوتامیناز تولید شده توسط گونه قارچی *تریکودرما کنینجی*، برای اولین بار، مورد بررسی قرار گرفت.

مواد و روش‌ها: در ابتدا، تخمیر حالت جامد برای تولید ال-گلوتامیناز توسط گونه قارچی *تریکودرما کنینجی* با استفاده از کیک روغن کنجد به عنوان سوبسترای جامد، انجام شد. سپس، بیوسنتز تیانین در حضور محلول آنزیم استخراجی و اتیل آمین و ال-گلوتامین، به عنوان سوبسترا، صورت گرفت. غلظت پارامترهای مؤثر نظیر اتیل آمین و ال-گلوتامین و حجم محلول آنزیمی بر تولید تیانین، بر اساس روش سطح پاسخ و طرح مرکب مرکزی، ارزیابی شد. 16 آزمون با استفاده از نرم افزار دیزاین اکسپرت طراحی و به منظور بررسی تغییرات غلظت تیانین، درصورت تغییر غلظت اتیل آمین و ال-گلوتامین و حجم محلول آنزیمی اجرا شد.

یافته ها و نتیجه گیری: نتایج نشان می‌دهد که سنتز تیانین و هیدرولیز ال-گلوتامین و تبدیل آن به ال-گلوتامیک اسید همزمان اتفاق افتاد. متغیرهای مستقل انتخاب شده (شامل غلظت اتیل آمین، غلظت ال-گلوتامین و حجم محلول آنزیمی) بر غلظت تیانین تاثیر داشتند. افزایش حجم محلول آنزیمی اثر قابل توجهی بر غلظت تیانین داشت. بیشترین غلظت تیانین (43 mM) با اتیل آمین در غلظت 0/9 M، ال-گلوتامین در غلظت 0/3 M و حجم محلول آنزیمی 3 ml به دست آمد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ تعارض منافعی وجود ندارد.

تاریخچه مقاله

دریافت 10 ژانویه 2017
داوری 25 ژانویه 2017
پذیرش 7 مارچ 2017

واژگان کلیدی

- بیوسنتز آنزیمی
- ال-گلوتامیناز
- ال-تئانین
- روش سطح پاسخ
- *تریکودرماکونینجی*

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