Scientific Research Publishing

Advances in Biological Chemistry, 2020, 10, 157-171
https://www.scirp.org/journal/abc
ISSN Online: 2162-2191
ISSN Print: 2162-2183

Efficient Production of L-Theanine Using Immobilized Recombinant *Escherichia coli* Cells Expressing a Modified γ-Glutamyltranspeptidase Gene from *Pseudomonas nitroreducens*

Putthapong Phumsombat[1], Chiharu Sano[1], Hiroki Ikezoe[1], Junji Hayashi[2], Takafumi Itoh[3], Takao Hibi[3], Mamoru Wakayama[1*]

[1]Department of Biotechnology, College of Life Sciences, Ritsumeikan University, Shiga, Japan
[2]Graduate School of Technology, Industrial and Social Sciences, Tokushima University, Tokushima, Japan
[3]Department of Bioscience and Biotechnology, Fukui Prefectural University, Fukui, Japan
Email: *wakayama@sk.ritsumei.ac.jp

How to cite this paper: Phumsombat, P., Sano, C., Ikezoe, H., Hayashi, J., Itoh, T., Hibi, T. and Wakayama, M. (2020) Efficient Production of L-Theanine Using Immobilized Recombinant *Escherichia coli* Cells Expressing a Modified γ-Glutamyltranspeptidase Gene from *Pseudomonas nitroreducens*. *Advances in Biological Chemistry*, **10**, 157-171.
https://doi.org/10.4236/abc.2020.106012

Received: October 17, 2020
Accepted: December 5, 2020
Published: December 8, 2020

Abstract

L-Theanine (γ-glutamylethylamide) is a naturally occurring amino acid derivative known to have several beneficial physiological effects as a diet supplement, and to give an umami taste when used as a food additive. The compound is industrially produced by γ-glutamyltranspeptidase from *Pseudomonas nitroreducens* (PnGGT). Using recombinant PnGGT, we have shown previously that Trp385, Phe417, and Trp525 are key amino acid residues for recognition of acceptor substrates at the PnGGT active site. Here, we demonstrate that a recombinant W525D mutant of PnGGT produces L-theanine from ethylamine and L-glutamine more efficiently than wild-type PnGGT, attributable to an increased ratio of transfer activity to hydrolysis activity. An efficient production of L-theanine was achieved by immobilizing *Escherichia coli* cells expressing the W525D PnGGT mutant (*E. coli*-W525D) using 2% alginate as the supporting material. The highest L-theanine production using immobilized *E. coli*-W525D, representing a conversion rate of 90%, was achieved in optimal reaction conditions of pH 10, 40˚C, and a substrate molar ratio of L-glutamine to ethylamine of 1:10. The immobilized *E. coli*-W525D retains 85% and 78% relative activity after storage for a month at 4˚C and room temperature, respectively. Immobilized *E. coli*-W525D thus has strong potential for use in the future commercial production of L-theanine on a large scale.

Keywords

L-Theanine, *γ*-Glutamyltranspeptidase, *Pseudomonas nitroreducens*, *Escherichia coli*, Immobilization

1. Introduction

L-Theanine (*γ*-glutamylethylamide) is an amino acid derivative approved by the Food and Drug Administration, USA, as a food additive to stimulate the Umami taste. L-Theanine has various additional reported benefits on human health, including improvement of concentration and learning ability [1], reduction of blood pressure [2], improvement of immune responses [3], and promotion of relaxation [4]. L-Theanine is naturally present in specific organisms such as *Camellia sinensis* (green tea) and the mushroom, *Xerocomus badius* [5]. It can be obtained by chemical synthesis or extraction from green tea, but both procedures include time-consuming, cost-ineffective, and complicated operational processes [6]. Hence, a biotechnological alternative for the production of L-theanine, using microbial enzymes such as *γ*-glutamyltranspeptidase (GGT) and glutamine synthetase [7] [8], has attracted significant attention. GGT is a ubiquitous enzyme that catalyzes the hydrolysis of the *γ*-glutamyl linkages of *γ*-glutamyl compounds and the transfer of their *γ*-glutamyl moieties to acceptor substrates [9]. It exists in a wide range of organisms from mammals to bacteria [10]. Bacterial GGTs have been studied in *Escherichia coli* (EcGGT) [11] [12], *Bacillus licheniformis* (BlGGT) [13], and *Pseudomonas nitroreducens* (PnGGT) [14]. The activity of GGTs produced by such cultured bacteria can be exploited to produce L-theanine when provided with L-glutamine and ethylamine as substrates.

We have reported previously that GGT from *P. nitroreducens* IFO12694 (PnGGT) has a high hydrolysis: transfer activity ratio (H/T ratio; with hydrolysis being the greater activity than transfer) and that this ratio is higher than that of other characterized GGTs [14]. Three-dimensional structural analysis of recombinant PnGGT explored the role of amino acid residues, W385, F417, and W525 in substrate recognition [15]. Mutations made at these positions based on the 3D structural analysis brought about dramatically decreased hydrolysis and increased transfer activities. In this study, we selected the W525D mutant, showing high transfer activity with a lowest H/T ratio among mutant enzymes which were examined and we evaluated high usability of immobilized recombinant *E. coli*-W525D for L-theanine production.

2. Material and Methods

2.1. Chemicals and Reagents

All analytical grade chemicals for culture media and GGT activity assay were purchased from FUJIFILM Wako Pure Chemical Corporation Ltd. (Osaka, Japan), unless otherwise stated.

2.2. Site-Directed Mutagenesis

The plasmid for the expression of wild-type (pET22b-PnGGT) was constructed previously [14]. PCR was performed using KOD-Plus-Neo DNA polymerase (Toyobo Co., Osaka, Japan), pET22b-PnGGT (template) with W525D primers, the forward primer 5'-GAAGATCAGGGACCGAACATGGGTGACATC-3' and the reverse primer 5'-GATGTCACCCATGTTGTCGCCCTGATCTTC-3', to generate the site-directed mutation. The PCR product was treated with DpnI at 37˚C for 1 h and transformed into *E. coli* XL-10 gold. Upon confirmation of their DNA sequence, the resulting plasmid was designated as pET22b-W525D.

2.3. Bacterial Strains and Growth Conditions

P. nitroreducens IFO12694 was cultured in the medium described previously [16]. *E. coli* Rosetta-gami B (RGB) was cultured in a medium containing 0.5% w/v yeast extract, 1% w/v polypeptone, 0.33% w/v $(NH_4)_2SO_4$, 0.68% w/v KH_2PO_4, 0.71% w/v Na_2HPO_4, 0.05% w/v glucose, 0.2% w/v lactose, and 0.015% w/v $MgSO_4$. These strains grew overnight at 37˚C with an agitation of 100 rpm. Antibiotics were used, when required, at the following concentrations: 0.1% v/v ampicillin, 0.1% v/v chloramphenicol, and 0.1% v/v kanamycin.

2.4. Determination of the Best Strain for L-Theanine Production

P. nitroreducens IFO12694 (the original strain), *E. coli*-PnGGT, *E. coli*-W525D, and *E. coli*-pET22b (harboring empty vector) were cultured in optimal conditions to evaluate their GGT activities and L-theanine production. Bacterial cells were harvested by centrifugation at 16,890 g for 10 min at 4˚C. Cell pellet was then suspended in 10-fold (w/v) 10 mM Tris-HCl buffer (pH 8). To precisely analyze their GGT activities and L-theanine production abilities, cell suspension of each strain was adjusted to equal cell density at 600 nm with suspension buffer. To evaluate hydrolysis and transfer activities of each cell-free extract, cell suspension was disrupted with sonication and separated from debris by centrifugation. Prepared cell-free extract from each strain was used for its hydrolysis and transfer activities according to the method described below. For evaluation of L-theanine production, reaction mixture containing 25 mM L-Gln, 250 mM ethylamine, and 100 mM borate buffer (pH 10) was mixed with 100 µL of cell-free extract or 100 µL of cell suspension (free cells) or 200 mg of immobilized cells. L-Theanine formed for 1 h reaction was determined using a high-performance liquid chromatography (HPLC; Shimadzu, Kyoto, Japan) system equipped with a COSMOSIL $5C_{18}$-MS-II (Nacalai, Kyoto, Japan) according to the procedure described previously [14].

2.5. Enzyme Assay

The GGT activity was determined according to the procedure of Imaoka *et al.* [14]. For the hydrolytic activity, the reaction mixture containing 2.5 mM *γ*-L-glutamyl-*p*-nitroanilide (G*p*NA), 100 mM imidazole buffer (pH 9.0), and the

enzyme was incubated at 30˚C for 5 - 15 min. The reaction was stopped by adding 10% acetic acid, and the released *p*-nitroaniline was estimated by measuring absorbance at 410 nm. One unit of the GGT hydrolysis activity was defined as the amount of the enzyme required to catalyze the formation of 1 μmol of *p*-nitroaniline per min. For the transfer activity, a reaction mixture containing 40 mM L-glutamine (L-Gln), 20 mM hydroxylamine, 100 mM imidazole buffer (pH 7.5), and the enzyme was incubated at 30˚C for 5 - 15 min. The reaction was stopped by adding a solution containing 200 mM $FeCl_3 \cdot 6H_2O$, 120 mM trichloroacetic acid, 250 mM HCl, and water at a ratio of 8:2:1:13. The formation of *γ*-L-glutamylhydroxamate was evaluated by measuring the absorbance at 540 nm. One unit of the GGT transfer activity was defined as the amount of the enzyme required to catalyze the formation of 1 μmol of *γ*-L-glutamylhydroxamate per min.

2.6. Immobilization of the Bacterial Cells

Cell immobilization was performed by the method reported by Lishen *et al.* [17] with some modifications. The bacterial cells were grown and harvested as described above, and the cell pellet was resuspended in 10 mM Tris-HCl buffer (pH 7.5). The cell suspension was homogeneously mixed with supporting materials such as 4% (w/v) sodium alginate solution at the ratio of 1:1. The mixture was applied to a syringe and dropped into 2% (w/v) $CaCl_2$ solution. The resulting immobilized cell beads were left to harden at 4˚C overnight. Two hundred mg of immobilized beads were used for the optimization of the reaction conditions as described below. Immobilized cells prepared by all methods [18]-[23] were subjected to evaluation of L-theanine production at 40˚C in 100 mM borate buffer (pH 10) for 1 h. L-theanine production (% yield) was analyzed using HPLC according to the method described above.

2.7. Optimization of pH, Temperature, and Molar Ratio of Substrates for L-Theanine Production

Optimization of pH for L-theanine production by immobilized cells expressing W525D PnGGT was examined using 100 mM borate buffer at pH values of 8 to 11. The optimal temperature for L-theanine production was determined using the range 25˚C - 50˚C in conditions of optimal pH. To determine the optimal molar ratio of substrates for L-theanine production, the ratio of L-Gln to ethylamine was varied from 1:0.5 to 1:10. For all optimization experiments, L-theanine formation in the various substrate molar ratios was analyzed by HPLC as described earlier. All assays were performed in triplicate, and average values as well as the standard deviations are presented.

2.8. Analysis of the Residual Activity of Immobilized Cells after Treatment with Disinfectants

According to the method previously described by Bindal and Gupta [24], 200 mg

of immobilized cells were treated with sodium hypochlorite (0.05% w/v) and sodium azide (0.05% v/v) for 10 min at room temperature. Immobilized beads were separated from the solution and washed twice with sterilized water prior to analysis of residual activity using the hydrolysis activity assay described earlier. The water washes were spread onto LB-agar plates, and resulting colonies were counted to determine the number of colony-forming units.

2.9. Analysis of the Residual Activity of the Immobilized Cells after Treatment with $CaCl_2$

The degree of stability to which cells are immobilized after treatment with buffer containing $CaCl_2$ was examined according to a method by [25], with modification. Immobilized beads, 200 mg, were incubated in 20 mL of 100 mM imidazole buffer (pH 7.5) containing $CaCl_2$ at concentrations of 2, 10, 50, and 200 mM at room temperature for 1 h. Leakage of cells from the beads was determined by measuring PnGGT hydrolysis activity in the wash buffer.

2.10. Analysis of the Long-Term Stability and Reusability of the Immobilized Cells

The long-term stability and reusability of immobilized cells was investigated according to the method described by [24]. To test reusability, immobilized beads subjected to the enzyme hydrolysis activity assay were separated from the reaction buffer and reused in a repeated assay, performing this cycle 13 times. To assess stability upon storage, the residual hydrolysis activity of the immobilized beads was determined at intervals between 0 and 4 weeks after storage at 4˚C or room temperature.

3. Results and Discussion

3.1. Selection of the Best Strain to Be Used for Immobilization

To evaluate the GGT activities and L-theanine production of *P. nitroreducens*, *E. coli*-PnGGT, and *E. coli*-W525D, we measured the hydrolysis and transfer activities of their cell-free extracts and L-theanine formed using their cell-free extracts, free cells, and immobilized cells as well as *E. coli*-pET22b as a control. As shown in **Table 1**, *E. coli*-W525D showed 9.05 U/mg of transfer activity (U_T) in the cell-free extract which was higher than those of *P. nitroreducens* and *E. coli*-PnGGT.

Moreover, it also showed 21.42 of U_T/U_H ratio 100-times higher than those of *P. nitroreducens* (0.21) and *E. coli*-PnGGT (0.22). Hydrolysis and transfer activities of *E. coli*-pET22b were negligible, and it did not show significant effects on GGT activities and L-theanine production of other recombinant strains, *E. coli*-PnGGT and *E. coli*-W525D. Consequently, *E. coli*-W525D exhibited the highest L-theanine production in all cases using cell-free extract, free cells, and immobilized cells among strains examined. Based on the results, *E. coli*-W525D was selected as a best strain for immobilization study for L-theanine production.

Table 1. GGT activities and L-theanine production of the strains used in this study.

Strains	Specific activity of cell-free extract			Theanine production (mM)		
	Hydrolysis (U_H/mg)	Transfer activity (U_T/mg)	U_T/U_H	Cell-free extract	Free cells	Immobilized cells
P. nitroreducens IFO12694	0.28	0.06	0.21	0.29	0.27	0.29
E. coli-PnGGT	13.9	3.02	0.22	0.29	0.30	0.29
E. coli-W525D	0.42	9.05	21.42	20.6	19.8	20.3
E. coli-pET22b	0.01	<0.01	0.08	0.07	0.09	0.08

Protein concentration of the cell-free extract of each strain was adjusted in the range of 3 - 13 mg/mL. Amounts of cells contained in cell suspension and immobilized cells were adjusted to be approximately 100 mg/mL and 50 mg/g immobilized gel, respectively.

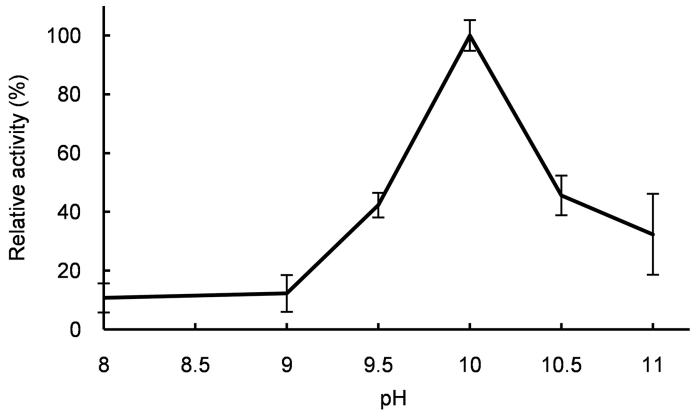
3.2. Optimal Reaction Conditions for L-Theanine Synthesis by Immobilized *E. coli*-W525D

Efficient synthesis of L-theanine by immobilized *P. nitroreducens* cells has previously been demonstrated [26]. It is clearly more economical to use immobilized cells, obtained from a suspension of harvested enzyme-expressing *E. coli* cultures, than to use purified enzyme in any biotechnological application. We thus selected *E. coli*-W525D showing the highest L-theanine-producing ability for such cell immobilization and for the subsequent optimization of reaction conditions for L-theanine production. Upon varying the pH (pH 8 - 11) and temperature (25˚C - 50˚C), immobilized *E. coli*-W525D demonstrated a pH optimum of 10, as shown in **Figure 1(a)**. The result was consistent with most previous studies on L-theanine production (**Table 2**), which indicated optimal activity at high alkaline pH [13]. The optimal temperature for L-theanine production was 40˚C, as shown in **Figure 1(b)**, which is slightly higher than most previous data (**Table 2**). However, there are some method-dependent differences between reports of temperature effects. For example, some reports showed a decrease in the transfer activity at temperatures higher than 37˚C, that might be attributed to a low boiling point of ethylamine [27], whereas work with immobilized cells [28] has indicated an optimal temperature of 50˚C.

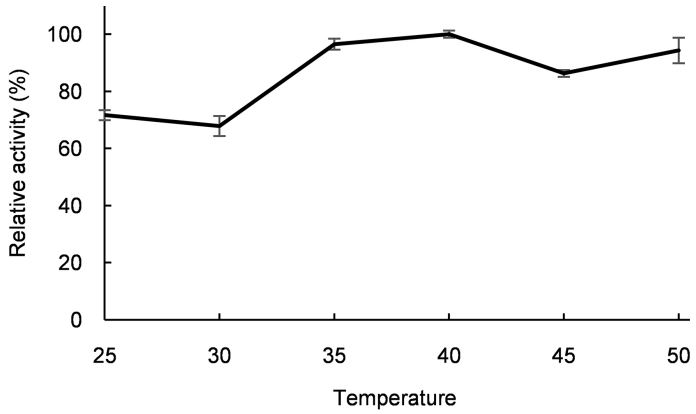
3.3. Effect of Substrate Molar Ratio on L-Theanine Production

To further increase the yield of L-theanine produced by *E. coli*-W525D, we investigated the optimal molar ratio of substrates in the reaction. When the concentration of L-Gln was fixed, the highest amount of L-theanine was obtained by the reaction of L-Gln and ethylamine at a 1:10 molar ratio (**Figure 1(c)**). In previous studies, the ratio of substrates for optimal L-theanine production showed various values ranging from 1:1.6 to 1:11 [28] [29]. The highest L-theanine production level in these studies was obtained at a ratio of 1:2.5, giving a 94% conversion rate [30], but the ratio is highly dependent on the individual characteris-

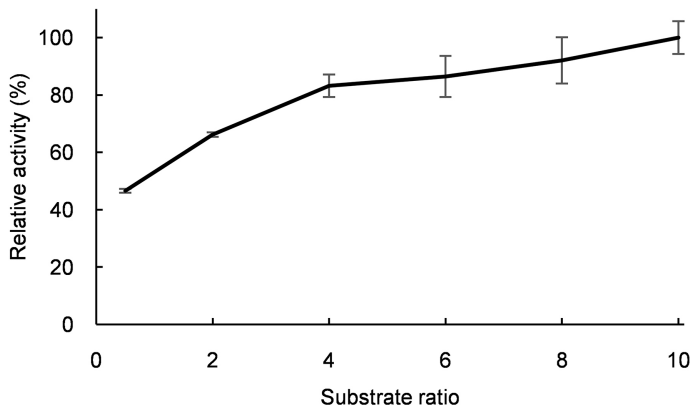
tics of each GGT and how it was used in the assays. In our experiments, using immobilized cells, a 1:10 molar ratio of substrates gave the optimum yield of product, with a 90% conversion rate, which is the highest ever reported rate using immobilized cell.



(a)



(b)



(c)

Figure 1. L-Theanine production in various conditions with immobilized cells of *E. coli*-W525D mutant. (a) pH-dependence of L-theanine production, pH 8 - 11, (b) Temperature-dependence of L-theanine production, 25˚C - 50˚C, and (c) Dependence of L-theanine production on the substrate molar ratio using L-Gln and ethylamine and ratios from 0.5 to 10. The values given in the figures represent the mean ± SD (n = 3).

Table 2. Summary of host strains, GGT source, production condition and yields reported in other researches about the process of L-theanine production with GGT.

Expression host	GGT source	Type	pH	Temp (˚C)	Substrate Ratio of donor (M): accepter (M)	Yield (%)	Ref.
B. subtilis SK11.004	Wild type	Free enzyme	10	37	0.02:0.05	94	[30]
B. subtilis 168	*B. pumilus* ML413	Batch system	10	30	0.58:5.8	63	[27]
C. glutamicum SYPA5-5	*B. subtilis*	Batch system	10	37	0.02:0.06	86.9	[32]
E. coli K-12	Wild type	Free enzyme	10	37	0.2:1.5	60	[11]
E. coli	Wild type	Immobilized GGT cell	10	50	0.3:3	87.2	[28]
E. coli	Wild type	Free enzyme	9	37	0.048:1.6	63.8	[33]
E. coli BL21	*B. subtilis* 168	Free enzyme	10	37	0.2:2.2	78	[34]
E. coli BL21	*B. licheniformis* ER-15	Immobilized enzyme	10	37	0.08:0.6	87	[13]
E. coli BL21	*E. coli* K-12	Free enzyme	10.5	37	0.267:2	80	[34]
E. coli M15	*E. coli* NovaBlue	Free enzyme	10	37	0.01:0.04	45	[12]
E. coli M15	*E. coli* NovaBlue	Immobilized enzyme	10	40	0.025:0.04	27	[29]
E. coli BL21	*P. nitroreducens* DSM 14399	Free enzyme	10	37	0.3:1.5	40	[35]
P. nitroreducens IFO12694	Wild type	Free enzyme	11	30	0.7:1.5	39	[31]
P. nitroreducens IFO12694	Wild Type	Immobilized cell	10	40	0.025:0.25	1.43	This study
E. coli RGB	*P. nitroreducens* IFO12694	Immobilized cell	10	40	0.025:0.25	1.43	This study
E. coli RGB	W525D PnGGT recombinant	Immobilized cell	10	40	0.025:0.25	90	This study

3.4. Effect of the Immobilizing Material on L-Theanine Production

We investigated the efficiency of various immobilizing materials for L-theanine production, utilizing materials referred to in previous studies: 4% κ-carrageenan [18]; 2% κ-carrageenan [19]; 4% Gelatin, 2% alginate and 16% glyceroli [23]; 2% Alginate in KPB and 1% gelatin [22]; 2.5% alginate and starch [20]; and 2% carrageenan with 1% locus bean gum [21]. High L-theanine conversion rate was obtained when using 2% κ-carrageenan with 1% locus bean gum, 4% κ-carrageenan, and 2% alginate as the immobilizer, as shown in **Figure 2**. Interestingly, additional supporting materials such as gelatin, glycerol and starch added in combination with alginate led to lower L-theanine production. The observations were consistent with a more open matrix favoring substrate diffusion and accessibility to cellular enzymes. The yield of L-theanine production using 2% alginate was similar to that achieved in a previous study [28], which applied 3% alginate to immobilized cells and resulted in a conversion rate of 87%.

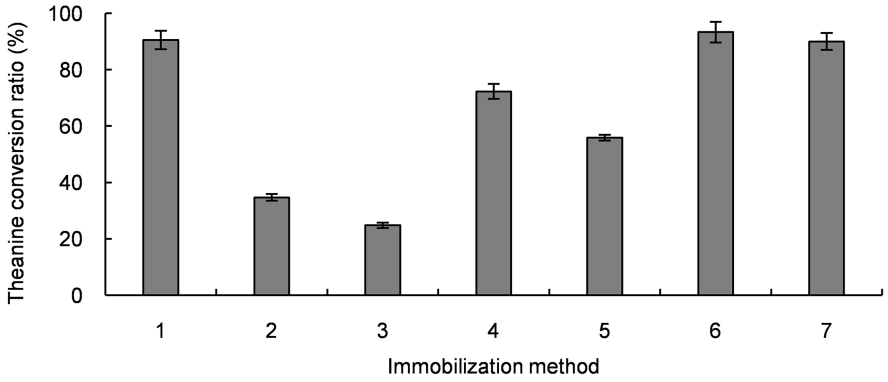


Figure 2. Effect of immobilization methods on L-theanine production. *E. coli*-W525D mutant cells were used for immobilization. The numbers indicate the immobilization material used: 1, 4% κ-carrageenan; 2, 2% κ-carrageenan; 3, 4% Gelatin, 2% alginate and 16% glycerol; 4, 2% Alginate in KPB and 1% gelatin; 5, 2.5% Alginate and starch; 6, 2% Carrageenan and 1% locus bean gum; 7, 2% alginate. The values for L-theanine yield reported are representative of the mean ± SD (n = 3).

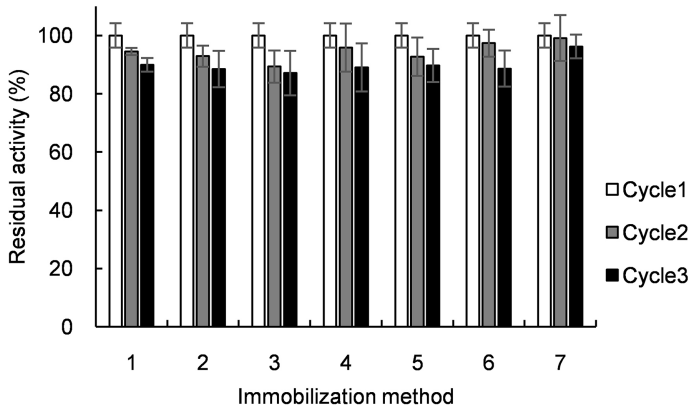
Stability of immobilized cells and its dependence on the immobilizer materials were evaluated by measuring residual activity for L-theanine production after three-time usages of immobilized cells at different temperatures, 30˚C and 40˚C (**Figure 3**). Immobilized cells with both 4% and 2% κ-carrageenan demonstrated high stability at 30˚C, but at 40˚C the activity massively decreased after three-time usages. Conversely, cells immobilized by alginate exhibited high stability at both 30˚C and 40˚C. Taken together, the results demonstrated that 2% alginate, with no additional matrix materials, provided the best support for active and stable immobilized cells in the production of L-theanine.

3.5. Effect of $CaCl_2$ on Stability of the Immobilized Cells

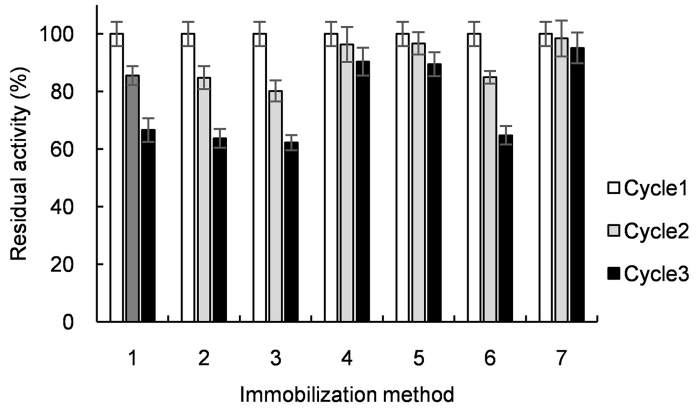
Calcium chloride solution is proposed to assist the stability of immobilized cells made using alginate and to prevent loss, or leakage, of active enzyme [25]. Hence, immobilized cells made by alginate with *E. coli*-W525D were washed with different concentrations of $CaCl_2$ solution and enzyme activity in the wash solution was assayed. **Figure 4** shows the relative enzyme activity detected in the $CaCl_2$ washes. For wash solutions of 50 and 200 mM $CaCl_2$, negligible enzyme activity was detected. The result showed that treatment of the immobilized cells with buffer containing $CaCl_2$ at 10 mM or higher was effective for stabilization of the immobilized cells.

3.6. Effect of Disinfectants on the Storage of the Immobilized Cells

After use, immobilized cells were treated with disinfectant to prevent them from microbial contamination and degradation and thus to increase their shelf life. It was, however, also important to assess whether the disinfectants chosen, NaN_3 and NaClO, had a negative effect on cellular enzyme activity. Residual GGT activity of immobilized cells decreased to 91% and 95%, respectively, after treatment with wash solutions containing NaN_3 and NaClO, thus demonstrating very



(a)



(b)

Figure 3. Effect of immobilization methods on the stability of immobilized cells. The residual activity of each sample of immobilized cells was measured after having repeated their use three times for 1 h at each temperature (1. 4% κ-carrageenan, 2. 2% κ-carrageenan, 3. 4% gelatin 2% alginate and 16% glycerol, 4. 2% Alginate in KPB and 1% gelatin, 5. 2.5% Alginate and starch, 6. 2% Carrageenan and 1% locus bean gum and 7. 2% alginate). (a) stability recorded as residual activity at 30˚C and (b) stability recorded as residual activity at 40˚C, Values for L-theanine production represent the mean ± SD (n = 3).

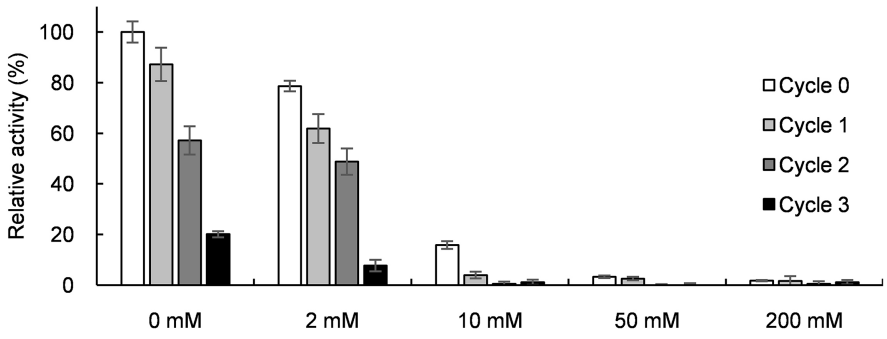
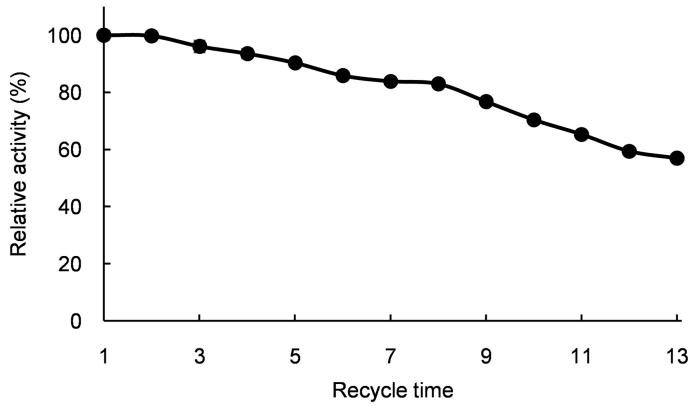


Figure 4. Effect of $CaCl_2$ concentration on the stability of immobilized cells. Immobilized cells (*E. coli*-W525D) were washed for 1 h with 100 mM imidazole buffer (pH 7.5) containing $CaCl_2$ at concentrations ranging from 0 to 200 mM. After the treatment, enzyme activity present in the wash solution was measured. The values reported for enzyme activity represent the mean ± SD (n = 3).

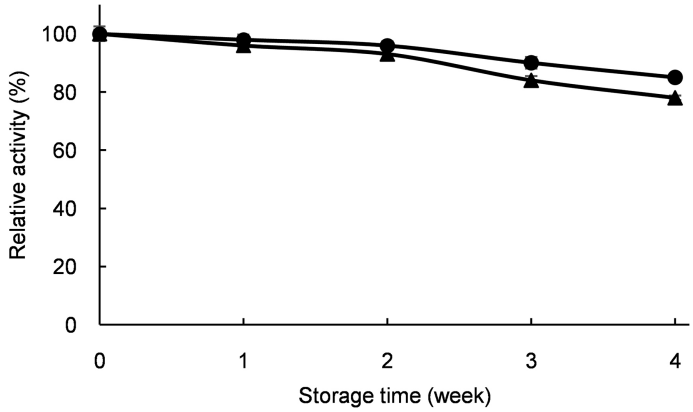
good retention of activity. The wash solutions generated no bacterial colonies on agar plates in comparison to a wash solution with no added disinfectant (>200 colonies formed). This result was similar to a previous study [24] which showed 100% residual activity, and supports use of such disinfectants to prolong the shelf life of immobilized cells.

3.7. Reusability and Storage Stability of the Immobilized Cells

After 13 rounds of use, the activity of immobilized cells decreased to 57% with respect to hydrolysis activity (**Figure 5(a)**). There are some previous reports, where immobilized cells on 3% alginate retained 12.8% activity after ten-time usage [28] and immobilized enzyme on Ca-alginate-κ-carrageenan showed 55% activity after six repeated uses [29]. The shelf life of immobilized cells was investigated at both 4˚C and 25˚C. At 4˚C, enzyme activity of immobilized cells decreased slightly to 98% and 85% after the storage for one week and one month, respectively (**Figure 5(b)**). At 25˚C, the activity also decreased slightly to 96% and 78% after storage for one week and one month, respectively. Similarly, Bindal and



(a)



(b)

Figure 5. Assessment of the reusability and stability of immobilized cells. A) Reusability of immobilized cells; B) Stability of immobilized cells (-●-) after storage at 4˚C and room temperature (-▲-), The values of activity are hydrolysis activity yields and represent the mean ± SD (n = 3).

Gupta [24] observed activity decreases to 82% and 75% after one month at 4˚C and room temperature, respectively. Small decrease in activity observed during the storage might be partially due to microbial contamination, which would be prevented by regular treatment with disinfectant to maintain an aseptic environment.

3.8. L-Theanine Production by W525D

Immobilized *E. coli*-W525D cells with 2% alginate exhibited 23 mM of L-theanine production from 25 mM L-Gln and 250 M ethylamine, giving a 90% yield. This yield was much higher than that of immobilized *E. coli* RGB cells expressing PnGGT (wild-type), which showed a 1.43% conversion rate. L-Theanine production by wild-type *P. nitroreducens* immobilized cells was significantly low under the same condition. The reaction conditions such as L-Gln and ethylamine concentrations were different to those for the enzyme reaction reported previously by Tachiki *et al.* [31]. As shown in **Table 1**, however, the expression level of GGT, indicated by the difference of specific activities between the original strain and the recombinant *E. coli* might be considered to mainly affect the yield of L-theanine production using immobilized cells in this study. Currently, the highest L-theanine conversion rate reported thus far is 94%, for the synthesis using free enzyme from *B. subtilis* SK11.004 [30]. However, the conversion rate reported for *E. coli*-W525D in our study are the highest achieved thus far for the synthesis using immobilized cells. The proven benefits of immobilization of *E. coli*-W525D on alginate, together with the reusability of the immobilized cells and their tolerance to various conditions, demonstrates a high potential for its application to L-theanine production for the food industries, critically avoiding the complex and expensive enzyme purification process.

4. Conclusion

The objective of this research was to increase L-theanine production utilizing the *E. coli* RGB expression system for production of a W525D mutant of PnGGT, named *E.* coli-W525D, which displayed increased transfer activity. The immobilized cells expressing *E. coli*-W525D demonstrated an increased L-theanine production rate compared with that of the other immobilized cells, including wild-type (*E. coli*-PnGGT). Optimal production of L-theanine was observed in borate buffer, pH 10, at 40˚C and in the presence of a 1:10 molar ratio of substrates L-Gln and ethylamine. The most suitable cell immobilization material, 2% alginate, retained 57% residual activity after reuse 13 times. Furthermore, 85% and 78% residual activity was observed after storage of immobilized cells for a month at 4˚C and room temperature, respectively. These results show that immobilized *E. coli*-W525D cells achieve one of the best reported conversion rates for biosynthesis of L-theanine and the best yield thus far from immobilized cells rather than enzyme, avoiding any purification steps. The immobilized *E. coli*-W525D cells thus have potential application in biosynthesis of L-theanine

for the food and supplement industries.

Acknowledgements

This work was supported by JICA Innovative Asia Scholarship.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Haskell, C.F., Kennedy, D.O., Milne, A.L., Wesnes, K.A. and Scholey, A.B. (2008) The Effects of L-Theanine, Caffeine and Their Combination on Cognition and Mood. *Biological Psychology*, **77**, 113-122. https://doi.org/10.1016/j.biopsycho.2007.09.008

[2] Yokogoshi, H., Kato, Y., Sagesaka, Y.M., Takihara-Matsuura, T., Kakuda, T. and Takeuchi, N. (1995) Reduction Effect of Theanine on Blood Pressure and Brain 5-Hydroxyindoles in Spontaneously Hypertensive Rats. *Bioscience, Biotechnology, and Biochemistry*, **59**, 615-618. https://doi.org/10.1271/bbb.59.615

[3] Miyagawa, K., Hayashi, Y., Kurihara, S. and Maeda, A. (2008) Co-Administration of L-Cystine and L-Theanine Enhances Efficacy of Influenza Vaccination in Elderly Persons: Nutritional Status-Dependent Immunogenicity. *Geriatrics & Gerontology International*, **8**, 243-250. https://doi.org/10.1111/j.1447-0594.2008.00483.x

[4] Lu, K., Gray, M.A., Oliver, C., Liley, D.T., Harrison, B.J., Batholomeusz, C.F., Phan, K.L. and Nathan, P.J. (2004) The Acute Effects of L-Theanine in Comparison with Alprazolam on Anticipatory Anxiety in Humans. *Human Psychopharmacology: Clinical and Experimental*, **19**, 457-465. https://doi.org/10.1002/hup.611

[5] Casimir, J., Jadot, J. and Renard, M. (1960) Séparation et Caractérisation de la N-éthyl-γ-glutamine à partir de *Xerocomus badius*. *Biochimica et Biophysica Acta*, **39**, 462-468. https://doi.org/10.1016/0006-3002(60)90199-2

[6] Mu, W., Zhang, T. and Jiang, B. (2015) An Overview of Biological Production of L-Theanine. *Biotechnology Advances*, **33**, 335-342. https://doi.org/10.1016/j.biotechadv.2015.04.004

[7] Suzuki, H., Izuka, S., Miyakawa, N. and Kumagai, H. (2002) Enzymatic Production of Theanine, an "Umami" Component of Tea, from Glutamine and Ethylamine with Bacterial γ-Glutamyltranspeptidase. *Enzyme and Microbial Technology*, **31**, 884-889. https://doi.org/10.1016/S0141-0229(02)00213-2

[8] Yamamoto, S., Uchimura, K., Wakayama, M. and Tachiki, T. (2004) Purification and Characterization of Glutamine Synthetase of *Pseudomonas taetrolens* Y-30: An Enzyme Usable for Production of Theanine by Coupling with the Alcoholic Fermentation System of Baker's Yeast. *Bioscience, Biotechnology, and Biochemistry*, **68**, 1888-1897. https://doi.org/10.1271/bbb.68.1888

[9] Tate, S.S. and Meister, A. (1981) γ-Glutamyl Transpeptidase: Catalytic, Structural and Functional Aspects. In: Najjar, V.A., Ed., *The Biological Effects of Glutamic Acid and Its Derivatives*, Springer, Dordrecht, 357-368. https://doi.org/10.1007/978-94-009-8027-3_23

[10] Ikeda, Y. and Taniguchi, N. (2005) Gene Expression of γ-Glutamyltranspeptidase. *Methods in Enzymology*, **401**, 408-425.

https://doi.org/10.1016/S0076-6879(05)01025-6

[11] Suzuki, H., Kumagai, H. and Tochikura, T. (1986) γ-Glutamyltranspeptidase from *Escherichia coli* K-12: Purification and Properties. *Journal of Bacteriology*, **168**, 1325-1331. https://doi.org/10.1128/JB.168.3.1325-1331.1986

[12] Yao, Y.F., Weng, Y.M., Hu, H.Y., Ku, K.L. and Lin, L.L. (2006) Expression Optimization and Biochemical Characterization of a Recombinant γ-Glutamyltranspeptidase from *Escherichia coli* Novablue. *The Protein Journal volume*, **25**, Article No. 431. https://doi.org/10.1007/s10930-006-9037-0

[13] Bindal, S. and Gupta, R. (2014) L-Theanine Synthesis Using γ-Glutamyl Transpeptidase from *Bacillus licheniformis* ER-15. *Journal of Agricultural and Food Chemistry*, **62**, 9151-9159. https://doi.org/10.1021/jf5022913

[14] Imaoka, M., Yano, S., Okumura, M., Hibi, T. and Wakayama, M. (2010) Molecular Cloning and Characterization of γ-Glutamyltranspeptidase from *Pseudomonas nitroreducens* IFO12694. *Bioscience, Biotechnology, and Biochemistry*, **74**, 1936-1939. https://doi.org/10.1271/bbb.100199

[15] Hibi, T., Imaoka, M., Shimizu, Y., Itoh, T. and Wakayama, M. (2019) Crystal Structure Analysis and Enzymatic Characterization of γ-Glutamyltranspeptidase from *Pseudomonas nitroreducens*. *Bioscience, Biotechnology, and Biochemistry*, **83**, 262-269. https://doi.org/10.1080/09168451.2018.1547104

[16] Tachiki, T., Yamada, T., Ueda, M., Naemura, Y., Imamura, N. and Ju-ichi, S. (1996) Purification and Some Properties of Glutaminase from *Pseudomonas nitroreducens* IFO 12694. *Bioscience, Biotechnology, and Biochemistry*, **60**, 1160-1164. https://doi.org/10.1271/bbb.60.1160

[17] Xu, L.S., Gao, G.Z., Zhang, X.T. and Wang, M.T. (2018) Enzymatic Synthesis of γ-Glutamylmethylamide from L-Glutamylhydrazine and Methylamine Catalysed by Immobilized Recombinant γ-Glutamyltranspeptidase. *Biocatalysis and Biotransformation*, **37**, 86-91. https://doi.org/10.1080/10242422.2018.1459577

[18] Ellaiah, P., Prabhakar, T., Ramakrishna, B., Taleb, A. and Adinarayana, K. (2004) Production of Lipase by Immobilized Cells of *Aspergillus niger*. *Process Biochemistry*, **39**, 525-528. https://doi.org/10.1016/S0032-9592(01)00340-5

[19] Evageliou, V.I., Ryan, P.M. and Morris, E.R. (2019) Effect of Monovalent Cations on Calcium-Induced Assemblies of Kappa Carrageenan. *Food Hydrocolloids*, **86**, 141-145. https://doi.org/10.1016/j.foodhyd.2018.03.018

[20] Matto, M. and Husain, Q. (2009) Calcium Alginate-Starch Hybrid Support for Both Surface Immobilization and Entrapment of Bitter Gourd (*Momordica charantia*) Peroxidase. *Journal of Molecular Catalysis B: Enzymatic*, **57**, 164-170. https://doi.org/10.1016/j.molcatb.2008.08.011

[21] Nishida, Y., Sato, T., Tosa, T. and Chibata, I. (1979) Immobilization of *Escherichia coli* Cells Having Aspartase Activity with Carrageenan and Locust Bean Gum. *Enzyme and Microbial Technology*, **1**, 95-99. https://doi.org/10.1016/0141-0229(79)90105-4

[22] Shen, Q.Y., Yang, R.J., Hua, X., Ye, F.Y., Zhang, W.B. and Zhao, W. (2011) Gelatin-Templated Biomimetic Calcification for β-Galactosidase Immobilization. *Process Biochemistry*, **46**, 1565-1571. https://doi.org/10.1016/j.procbio.2011.04.010

[23] Tanriseven, A. and Doğan, Ş. (2002) A Novel Method for the Immobilization of β-Galactosidase. *Process Biochemistry*, **38**, 27-30. https://doi.org/10.1016/S0032-9592(02)00049-3

[24] Bindal, S. and Gupta, R. (2016) Thermo- and Salt-Tolerant Chitosan Cross-Linked

γ-Glutamyl Transpeptidase from *Bacillus licheniformis* ER15. *International Journal of Biological Macromolecules*, **91**, 544-553. https://doi.org/10.1016/j.ijbiomac.2016.05.106

[25] Birgisson, H., Wheat, J.O., Hreggvidsson, G.O., Kristjansson, J.K. and Mattiasson, B. (2007) Immobilization of a Recombinant *Escherichia coli* Producing a Thermostable *α*-L-Rhamnosidase: Creation of a Bioreactor for Hydrolyses of Naringin. *Enzyme and Microbial Technology*, **40**, 1181-1187. https://doi.org/10.1016/j.enzmictec.2006.08.026

[26] Abelian, V.H., Okubo, T., Mutoh, K., Chu, D.C., Kim, M. and Yamamoto, T. (1993) A Continuous Production Method for Theanine by Immobilized *Pseudomonas nitroreducens* Cells. *Journal of Fermentation and Bioengineering*, **76**, 195-198. https://doi.org/10.1016/0922-338X(93)90007-U

[27] Yang, T., Irene, K., Liu, H., Liu, S., Zhang, X., Xu, M. and Rao, Z. (2019) Enhanced Extracellular Gamma Glutamyl Transpeptidase Production by Overexpressing of PrsA Lipoproteins and Improving its mRNA Stability in *Bacillus subtilis* and Application in Biosynthesis of L-Theanine. *Journal of Biotechnology*, **302**, 85-91. https://doi.org/10.1016/j.jbiotec.2019.06.302

[28] Zhang, F., Zheng, Q., Jiao, Q., Liu, J. and Zhao, G. (2010) Enzymatic Synthesis of Theanine from Glutamic Acid *γ*-Methyl Ester and Ethylamine by Immobilized *Escherichia coli* Cells with *γ*-Glutamyltranspeptidase Activity. *Amino Acids*, **39**, 1177-1182. https://doi.org/10.1007/s00726-010-0553-z

[29] Hung, C., Lo, H., Hsu, W., Chen, S. and Lin, L. (2008) Immobilization of *Escherichia coli* Novablue *γ*-Glutamyltranspeptidase in Ca-alginate-*k*-carrageenan Beads. *Applied Biochemistry and Biotechnology*, **150**, Article No. 157. https://doi.org/10.1007/s12010-008-8244-x

[30] Shuai, Y., Zhang, T., Jiang, B. and Mu, W. (2010) Development of Efficient Enzymatic Production of Theanine by *γ*-Glutamyltranspeptidase from a Newly Isolated Strain of *Bacillus subtilis*, SK11.004. *Journal of the Science of Food and Agriculture*, **90**, 2563-2567. https://doi.org/10.1002/jsfa.4120

[31] Tachiki, T., Yamada, T., Mizuno, K., Ueda, M., Shiode, J.I. and Fukami, H. (1998) *γ*-Glutamyl Transfer Reactions by Glutaminase from *Pseudomonas nitroreducens* IFO12694 and Their Application for the Syntheses of Theanine and *γ*-Glutamylmethylamide. *Bioscience, Biotechnology, and Biochemistry*, **62**, 1279-1283. https://doi.org/10.1271/bbb.62.1279

[32] He, F., Yang, T., Xu, M., Zhang, X., Rao, Z. and Tang, L. (2016) Efficient Synthesis of L-Theanine by Recombinant Strain *Corynebacterium glutamicum* SYPA5-5. *Acta Microbiologica Sinica*, **56**, 1595-1605.

[33] Wang, H.Q., Yao, Z., Zhou, Z., Sun, Y., Wei, P. and Ouyang, P. (2012) Enzymatic Synthesis of Theanine with L-glutamine-Zn(II) Complexes. *Biotechnology and Bioprocess Engineering*, **17**, 1135-1139. https://doi.org/10.1007/s12257-012-0205-0

[34] Wang, Q., Min, C., Zhu, F., Xin, Y., Zhang, S., Luo, L. and Yin, Z. (2011) Production of Bioactive *γ*-Glutamyl Transpeptidase in *Escherichia coli* Using SUMO Fusion Partner and Application of the Recombinant Enzyme to L-Theanine Synthesis. *Current Microbiology*, **62**, 1535-1541. https://doi.org/10.1007/s00284-011-9891-7

[35] Pu, H.F., Wang, Q., Zhu, F.F., Cao, X., Xin, Y.Q., Luo, L. and Yin, Z.M. (2012) Cloning, Expression of Glutaminase from *Pseudomonas nitroreducens* and Application to Theanine Synthesis. *Biocatalysis and Biotransformation*, **31**, 1-7. https://doi.org/10.3109/10242422.2012.749462