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L-Theanine Goes Greener: A Highly Efficient Bioprocess Catalyzed by the Immobilized γ-Glutamyl Transferase from *Bacillus subtilis*

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L-Theanine (L-Th) was synthesized by simply mixing the reactants (L-glutamine and ethylamine in water) at 25 °C and *Bacillus subtilis* γ-glutamyl transferase (*Bs*GGT) covalently immobilized on glyoxyl-agarose according to a methodology previously reported by our research group; neither buffers, nor other additives were needed. Ratio of L-glutamine (donor) to ethylamine (acceptor), pH, enzymatic units (IU), and reaction time were optimized (molar ratio of donor/acceptor = 1:8, pH 11.6, 1 IU mL^{-1}, 6 h), furnishing L-Th in 93% isolated yield (485 mg, 32.3 g L^{-1}) and high purity (99%), after a simple filtration of the immobilized biocatalyst, distillation of the volatiles (unreacted ethylamine) and direct lyophilization. Immobilized *Bs*GGT was re-used (four reaction cycles) with 100% activity retention. This enzymatic synthesis represents a straightforward, fast, high-yielding, and easily scalable approach to L-Th preparation, besides having a favorable green chemistry metrics.

Introduction

L-Theanine (L-Th; γ-glutamylethylamide), a unique amino acid found in green tea, is a valuable FDA-approved nutraceutical product with recognized umami taste properties and health benefits.[1] L-Th is used in food industry as flavour enhancer owing to its ability to boost the umami intensity of L-glutamate and inosine 5′-monophosphate (5′-IMP).[2] Moreover, it can easily cross the blood brain barrier acting at the brain level to reduce mental and physical stress,[3] thus improving mood and cognitive performance. From recent reports, L-Th seems to be involved also in cancer[4] and vascular disease prevention, enhancement of immune response,[5–7] and promotion of weight loss.[8]

L-Th was certified as a Generally Recognized as Safe (GRAS) ingredient by FDA and as such it is contained in a wide range of nutraceutical formulations on the market. For instance, Suntheanine by Taiyo Kagaku Ltd (Japan) is a nutraceutical product used for relaxation and to improve learning ability.[9] Several beverages such as Relarian (MiniChill),[10] Proloftin[11] and NeuroBliss[12] are used to increase mental focus, while providing relaxation during task performance. The chewing gum Neurogum (60 mg L-Th per serve) is also available on the market. This product has been claimed to reduce anxiety, to exert blood pressure benefits as well as to potentiate clinical effects of cancer drug therapy.[13]

Due to its taste-enhancing properties and claimed health benefits, the demand for L-Th in the next years is expected to grow significantly. To meet the increasing demand of L-Th from pharmaceutical and food industries, several methods for its production have been developed (i.e., extraction from green tea leaves, chemical or enzymatic synthesis). Extraction is wearisome, low yielding, time-consuming, and non-sustainable,[14] whereas chemical routes are made troublesome by both the extensive use of protective groups for the careful control of stereochemistry[15] and long reaction times[16] (Scheme 1).

Enzymes, on the other hand, are inherently selective, besides being biodegradable, not toxic to humans and environment, versatile, and active under mild conditions. Biocatalysis is thus recognized nowadays as a pivotal tool to address a greener and more sustainable chemistry.[17] Nevertheless, the use of enzymes in chemical reactions does not translate into eco-friendly and sustainable processes by definition: "greenness" assessment is a mandatory step to attest whether an enzyme-catalyzed synthetic route is truly superior to the conventional non-enzymatic path.[18] Environmentally-friendly practices are increasingly perceived as an added-value by

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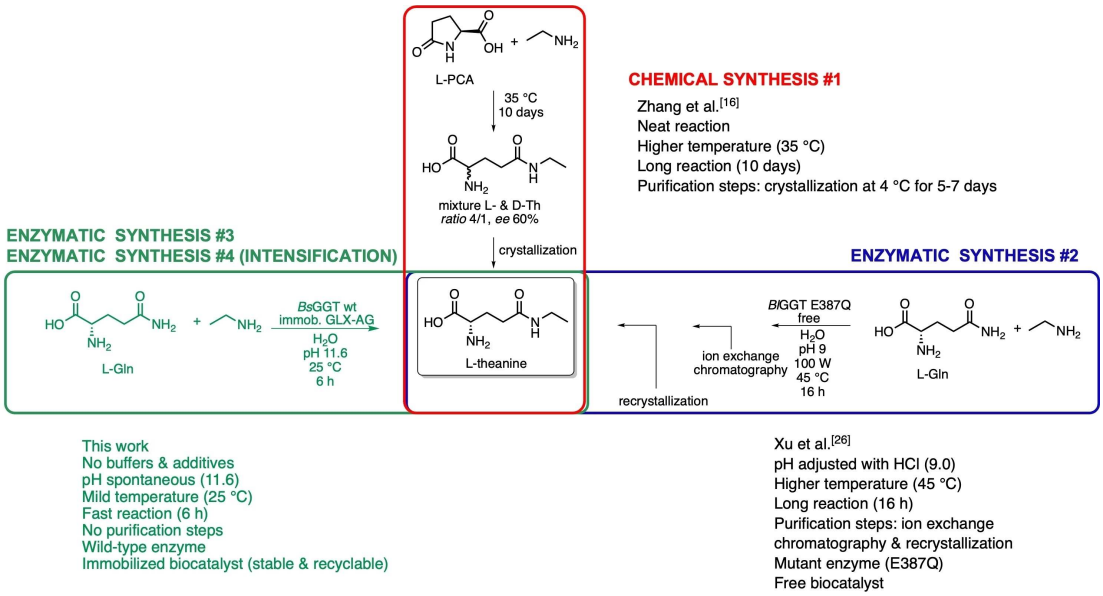


Scheme 1. Synthetic approaches to the synthesis of L-Th discussed in the text. The bioprocess described in this work is highlighted in green.

consumers and producers.[19] A biocatalyzed route to L-Th can thus answer the need of both a more efficient and greener synthesis.

γ-Glutamyl transferases (GGTs, EC 2.3.2.x) catalyze the cleavage of the γ-glutamyl bond of γ-glutamyl donor substrates, and the transfer of the γ-glutamyl moiety to an amine of an acceptor substrate by a transpeptidation reaction.[20] The application of GGTs to the synthesis of L-Th has been extensively studied.[21–23] However, the L-Th yield remains not satisfactory for its production on a large scale because the transpeptidation reaction is plagued by concurrent autotranspeptidation and hydrolysis of the donor substrate (Figure S1 in the Supporting Information).[20] In order to foster the transpeptidation activity of GGTs over the side-reactions and thus enhancing L-Th formation, many strategies have been investigated, which mainly rely either on reaction engineering (e.g., source of GGTs, type of γ-glutamyl donors, donor/acceptor ratio, time of reaction, pH, temperature), or on the development of genetically-engineered GGT-mutants.[24] Although reaction engineering has been widely applied to date, most of the studies with isolated (immobilized) enzymes were carried out without product isolation, as recently reviewed by Liu et al.[24] On the other hand, in recent years, protein engineering has been also exploited to maximize the transpeptidation activity, while suppressing the enzyme side-activities (i.e., donor hydrolysis and autotranspeptidation, poly-glutamylation).[25–27]

For example, from a collection of *Bacillus licheniformis* ER15 GGT (*Bl*GGT) variants, the Arg109Lys mutation was shown to increase the transpeptidation activity and catalytic efficiency in the synthesis of L-Th affording 80% conversion compared to the wild-type enzyme (60% conversion).[25] Furthermore, in a very recent paper, a mutant of *Bl*GGT with a significantly higher thermal stability than the wild-type enzyme was prepared and screened in the synthesis of L-Th by an ultrasound-assisted method at 45 °C using L-glutamine and ethylamine as substrates (Scheme 1).[26] However, as reported in several examples about the biocatalyzed synthesis of L-Th on a preparative scale, also in this case L-Th recovery required tedious purification steps (ion exchange chromatography and recrystallization).[21,26,28,29] Enzyme immobilization is an additional technique of the chemist's toolbox. Immobilized enzymes are characterized by a generally higher operational stability and an easier handling that allow their straightforward recovery and reuse.[30–34] Biocatalyst stability under reaction conditions can be a major issue for the success of the biotransformation, particularly when multimeric enzymes are used. Multimeric enzymes may be inactivated by either dissociation of the protein subunits, or their uncorrected assembly. Immobilization and post-immobilization techniques such as protein-carrier multipoint attachment can prevent these phenomena from occurring.[35] Furthermore, as immobilization in a confined space may alter the optimal enzyme conformation (therefore affecting activity, selectivity, specificity, and inhibition), only a reproducible and controlled immobilization-stabilization procedure allows to derive full benefits.[36] In this work, *Bacillus subtilis*-GGT (*Bs*GGT, a heterodimeric protein) has been immobilized on glyoxyl-agarose (GLX-AG)[37] for the preparative synthesis of L-Th. The reaction conditions have been extensively investigated and finally optimized, considering L-glutamine (L-Gln)/ethylamine ratio, pH, *Bs*GGT units, and reaction time. The product was recovered through an easy and simplified purification protocol based on removal of the immobilized biocatalyst by filtration, distillation of the unreacted ethylamine and direct lyophilization. Moreover, a set of chemometric parameters has been used to assess greenness and sustainability of this new biocatalyzed strategy (depicted in green in Scheme 1) in comparison with a recent biotransformation based on the same synthetic pattern (L-Gln and ethylamine were used as substrates).[26]

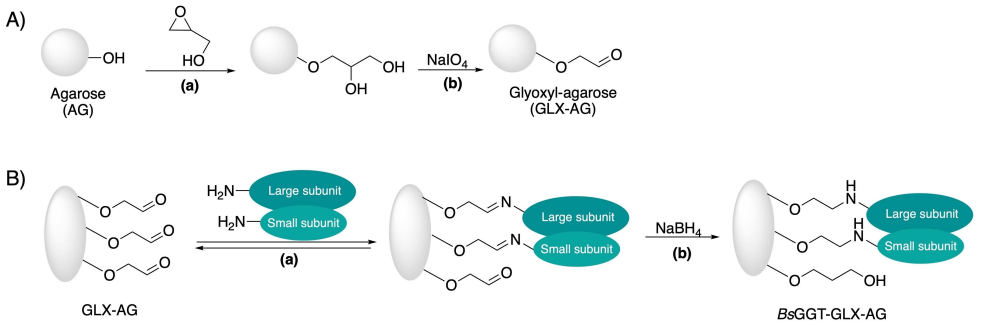


Figure 1. Immobilization of *Bs*GGT on GLX-AG. Experimental conditions: A) agarose activation: (a) AG (15 g) was suspended in a solution of $NaBH_4$ (14.25 mg mL^{-1})/NaOH 1.7 M (7.1 mL), H_2O (3.6 mL) and glycidol (5.1 mL), RT, mechanical stirring, 18 h; (b) 100 mM $NaIO_4$ (102 mL), RT, mechanical stirring, 2 h and B) enzyme immobilization: (a) *Bs*GGT (5.7 mg mL^{-1}) and GLX-AG (5 g) were suspended in 50 mM $NaHCO_3$ pH 10.0 (63.5 mL). Immobilization loading 1 $mg_{protein}$ per $g_{carrier}$, RT, mechanical stirring, 3 h, (b) $NaBH_4$ (1 mg mL^{-1}), RT, mechanical stirring, 30 min.

Results and Discussion

Immobilization of *Bs*GGT

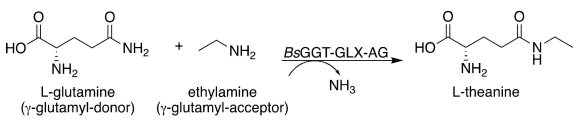
With the aim to develop a robust and recyclable biocatalyst for preparative applications, *Bs*GGT was covalently immobilized on GLX-AG[38,39] upon an extensive screening[37] of carriers and immobilization conditions, resulting in excellent immobilization yields (97% immobilized protein and 95% immobilized activity) and high activity (12.7 IU g^{-1}) (Figure 1). As we recently reported,[37] this immobilized biocatalyst was highly stable also under storage, retaining 90% activity after 10 months at 4 °C.

Synthesis of L-Th: study of the reaction parameters (analytical scale)

The immobilized-*Bs*GGT was used to catalyze the synthesis of L-Th by a transpeptidation reaction starting from L-Gln (γ-glutamyl donor) and ethylamine (γ-glutamyl-acceptor) in pure water (without the addition of buffers or other additives) at 25 °C (Scheme 2).

The reaction was first run on an analytical scale (2 mL) to screen a set of selected reaction parameters (i.e., donor/acceptor molar ratio, pH and reaction time) and define the conditions allowing for the highest conversion of L-Th, while limiting the formation of byproducts (due to hydrolysis and autotranspeptidation of the γ-glutamyl-donor or L-Th) (Figure S1). The results are reported in Table 1.

First, the reaction was performed at pH 10.0, following protocols previously reported,[28,40] and exploring different donor/acceptor molar ratios (i.e., 1:1, 1:4 and 1:8) (Table 1; entries 1–3).



Scheme 2. Synthesis of L-Th by *Bs*GGT-GLX-AG-catalyzed transpeptidation.

Table 1. Synthesis of L-Th catalyzed by *Bs*GGT-GLX-AG: study of the reaction parameters (analytical scale).[a]

Entry	[L-Gln] [mM]	[Ethylamine] [mM]	pH	*Bs*GGT-GLX-AG [IU mL^{-1}]	Conversion [%]
1	100	100	10.0	0.5	18
2	100	400	10.0	0.5	69
3	100	800	10.0	0.5	89
4	100	800	11.6	0.5	>99
5	200	1600	11.6	1.0	>99
6	300	2400	11.6	1.5	73

[a] Experimental conditions: 2 mL total volume, 25 °C, 6 h, mechanical stirring.

As expected, at the defined endpoint (6 h), the highest conversion (86%) was achieved with the 1:8 molar ratio (Table 1, entry 3). The excess of ethylamine shifts the reaction equilibrium toward the product and suppresses byproducts formation, in agreement also with literature.[28,29,41] In the following runs, the 1:8 donor/acceptor molar ratio was maintained, and the reaction was performed at the spontaneous pH (11.6) of the aqueous solution of the reagents, thus avoiding the addition of 1 M HCl for pH adjustment to 10.0. We decided to eliminate the addition of HCl for pH adjustment as we observed the formation of ethyl ammonium chloride in the preparative reactions performed at pH 10.0 (see the next paragraph), which required an additional ion exchange chromatography step for L-Th downstream, therefore increasing the cost and complexity and diminishing the sustainability of the overall process. By using a molar ratio of donor/acceptor = 1:8 and pH 11.6, *Bs*GGT-GLX-AG catalyzed the synthesis of L-Th with an excellent conversion (>99%) after 6 h (Table 1, entry 4) (Figure S2). It is worth mentioning that the free, non-immobilized *Bs*GGT was completely inactive (1% conversion) under the same reaction conditions (data not shown), whereas at pH 10.0 it gave a conversion comparable to that of the immobilized biocatalyst (data not shown). These data corroborate the advantage of using the immobilized *Bs*GGT, which couples robustness and stability under operational conditions with recyclability and reuse of the biocatalyst, as well as a simplified product downstream (see next paragraphs).

To sum up, the reaction conditions for the synthesis of L-Th were: an aqueous solution of L-Gln (100 mM) and ethylamine (800 mM) at pH 11.6 (spontaneous), 0.5 IU mL^{-1} *Bs*GGT-GLX-AG (12 IU g^{-1}), 25 °C, and 6 h.

Synthesis of L-Th: semi-preparative scale

Once the best conditions on the analytical scale were defined, we moved to a semi-preparative scale (15 mL) in order to isolate L-Th.

As mentioned in the previous paragraph, at pH 10.0 the formation of ethyl ammonium chloride was detected, as confirmed by ^{1}H NMR spectroscopy (Figure S3) and silver nitrate precipitation assay (Figure S4A). The high amount of this salt made the purification of L-Th wearisome and poorly efficient, besides generating extra waste. The first approach to the purification of L-Th was attempted by ion exchange chromatography, as reported by Bindal and Gupta.[29] However, the process required a very large amount of water/ammonia solution for the elution that had to be removed at the end with energy consumption. Thus, in order to get rid of this tedious procedure, the reaction was carried out at the spontaneous pH (11.6) generated upon the solubilization of the reagents in water. Under these conditions, L-Th was obtained in 93% isolated yield (248 mg) and 97% HPLC purity (3% unreacted L-Gln) (Figure S5). The workflow for the downstream of L-Th was, indeed, smooth and straightforward: filtration of the reaction mixture under reduced pressure to remove and recover the immobilized biocatalyst, removal of the excess ethylamine under reduced pressure, and direct lyophilization of the remaining aqueous solution. The resulting product was characterized by ^{1}H NMR spectroscopy (Figure S6) and silver nitrate precipitation assay (Figure S4B), which confirmed the absence of ethyl ammonium chloride, and by electrospray ionization mass spectroscopy (ESI-MS) (Figure S7).

Synthesis of L-Th: process intensification

In order to intensify the process, two additional reactions were performed on the analytical scale (2 mL) by using a 1:8 donor/acceptor molar ratio, but increasing the concentration of the substrates (Table 1, entries 5 and 6). When twice of the substrate concentration (200 mM L-Gln and 1600 mM ethylamine) was used (Table 1, entry 5), the conversion of L-Th was >99% as in the reference reaction (entry 4). At a higher concentration (300 mM L-Gln and 2400 mM ethylamine), the conversion reached only 73% (Table 1, entry 6). Under these conditions the reaction mixture was not homogeneous, thus preventing the immobilized biocatalyst from being evenly dispersed in the system and leading to its accumulation in the aqueous phase. However, after 24 h the conversion reached 93.4%. Reaction scaling-up (200 mM L-Gln and 1600 mM ethylamine) to the final volume of 15 mL resulted in 93% isolated yield of L-Th (485 mg) and 99% HPLC purity (1% unreacted L-Gln).

Recycling of *Bs*GGT-GLX-AG

Recycling of immobilized *Bs*GGT was performed by evaluating both the conversion and the isolated yield of L-Th under the reaction conditions of the reference reaction (Table 1, entry 4): L-Gln (100 mM) and ethylamine (800 mM) pH 11.6 (spontaneous), 0.5 IU mL^{-1} *Bs*GGT-GLX-AG (12 IU g^{-1}), 25 °C, 6 h. After each reaction cycle (6 h), a sample of the reaction mixture was taken out to quantify the conversion (HPLC), while the biocatalyst was filtered under reduced pressure, washed with water (5 mL), and re-used for the following reaction runs. The supernatant was recovered, ethylamine was removed under reduced pressure, and the remaining aqueous solution was lyophilized to calculate the reaction yield. The product isolated after each reaction cycle was analyzed by HPLC to assess its purity. As shown in Table 2, the immobilized *Bs*GGT was successfully re-used for 4 cycles with excellent and reproducible conversion (> 99%), isolated yields (>90%) and purity (93–97%), with the unreacted L-Gln as the sole impurity.

Besides confirming the high stability of the immobilized *Bs*GGT under reaction conditions, this study demonstrated that this biocatalyst can be recycled and reused, thus impacting positively on the overall productivity (Table 4) as well as on the economy of the overall process. Usually, the (bio)catalysts are the most expensive reagents, so the possibility to recover and reuse them can reduce the final cost of the product.

Green metrics

To calculate the usual chemometric parameters in order to quantify the "greenness" of the reactions, equations defined in literature were used for the enzymatic synthesis of L-Th, by using the Radial Polygons for a visual analysis of the parameters, as reported by Andraos et al.[42–44] These parameters are E-factor (E),[45–51] E-factor based on molecular weight (E_{MW}),[52] atom economy (AE),[53] mass intensity (MI),[54] material recovery parameter (MRP),[52] stoichiometric factor (SF),[52] and finally, reaction mass efficiency (RME),[52] which can be used as a threshold metric for assessing the true "greenness" of reactions, considering the "golden ratio" of a RME value $\geq$0.618.[55]

Undoubtedly, the most popular chemometric parameter is the E(nvironmental)-factor, defined as the kgs of "everything but the desired product" produced divided by kgs of product, including solvent losses and chemicals used in work-up.[47,49] In fact, its simplicity, perceptiveness, and broad applicability makes it

Table 2. Recycling of *Bs*GGT-GLX-AG.[a]

Cycle	Conversion [%][b]	Yield [%]	Purity [%][b]
1	>99	93	97
2	>99	90	93
3	>99	94	93
4	>99	96	97

[a] Experimental conditions: 100 mM L-Gln and 800 mM ethylamine in water (15 mL), pH 11.6, 0.5 IU mL^{-1} *Bs*GGT-GLX-AG, 25 °C, 6 h, mechanical stirring. [b] Determined by HPLC.

particularly useful to assess any organic reaction. Ideally, E-factor should be as close to zero as possible, as demanded by the first principle of Green Chemistry (preventing waste generation instead of removing it).[56] The real scenario must take into account the contribution of water and solvents, measured as cEF (complete E-factor).[49] This should be calculated, even if water and/or solvents are recycled after the reaction (see work by Dominguez de Maria[57] for a practical example on the effect of water on E calculation); on the other hand, simple E-factor (sEF) ignore water and solvents. Table 3 shows the E data calculated for both enzymatic syntheses using immobilized *Bs*GGT [preparative scale (Synthesis #3) and process intensification (Synthesis #4), compared to Synthesis #2 (enzymatic procedure reported by Xu et al.)[26] and the chemical synthesis (Synthesis #1) described by Zhang et al.,[16] as depicted in Scheme 1. The detailed calculations can be found in the Supporting Information (Tables S1–S5).

For Synthesis #3, an excellent yield (93%) and a very low E (sEF) factor (2.1) reflect the low amount of waste derived from this reaction. The cEF for Synthesis #3 is higher (85.3) although this value is somehow misleading because the only waste produced is water. Moving to intensified Synthesis #4, the yield is maintained at 93% while E-factor is lower (1.1, no water) and reduced to 44.9 including water consumption. It is worth noting that in the E-factor calculations ethylamine (removed s.v.) was reclaimed as waste. Nevertheless, it can be eventually recovered and recycled for additional reaction runs, thus reducing the environmental impact and further increasing the bioprocess sustainability.

On the other hand, for enzymatic Synthesis #2 [trans-peptidation catalyzed by a mutant enzyme (E387Q), scaled-up to grams, at pH 9.0 and using ultrasounds and a higher temperature (45 °C)], the yield reported was 89%, close to that obtained in #3, although #3 proceeded at lower reaction temperature without requiring any sonication. For #2, sEF is slightly lower than #3; anyhow, in #2 the enzyme is used as a soluble catalyst, and thus it cannot be recovered and reused at the end of the reaction, thus representing a waste. However, the exact amount of catalyst, although presumably low, could not be included in the green metric calculations reported in this work as it was not reported in the original paper. Remarkably, in #2 cEF is very high, because of the purification requirements (anion exchange resin chromatography with 0.5 mol L^{-1} hydrochloric acid as eluent, concentration by rotary evaporator, and recrystallization by alcohol precipitation); for this purification process in #2, as it was not fully specified in the paper, general assumptions established by Mercer et al.[58] were considered.

Finally, in the chemical synthesis reported by Zhang et al.[16] (Reaction #1) both E_{MW} and sEF are much smaller, as all the reagents' atoms are incorporated into the final product. On the other hand, this is a chemical reaction under solventless conditions, as L-pyroglutamic acid (L-PCA) is directly dissolved in ethylamine. Thus, cEF is intrinsically lower, as no solvent consumption is considered and also because this reaction is carried out at a higher scale (35 g of starting L-PCA).[16] Nevertheless, #1 is extremely slower than #3 and #4, as it requires 10 days heating the reaction mixture at 35 °C, and a subsequent recrystallization (cooling at low temperature) for 5–7 days. Reaction yields dramatically decreased after recrystallization, from 98.2% (crude) to 30%. Moreover, #1 did not proceed with retention of the stereochemistry, as mixtures of L- and D-theanine were finally detected (4:1 ratio, *ee* = 60%). Authors reported that this fact could be caused by the spontaneous hydrolysis of L-PCA, promoted by the water present in the ethylamine commercial solution. As a general conclusion of E values, Syntheses #3 and #4 produced less waste than #1 and #2.

As already mentioned, immobilized *Bs*GGT was efficiently employed in four consecutive reaction cycles without any decrease of the enzymatic performance. Therefore, this point should be considered in the productivity parameter (mass of product obtained divided by the enzyme amount). This parameter is illustrated in Table 4. Thus, we are quadruplicating the initial yield by a theoretical combination of 4 reaction cycles with the same amount of biocatalyst. This data reinforces the usefulness of employing immobilized biocatalysts, as mentioned in the Introduction.

Considering the other chemometric parameters (AE, MI, MRP, SF, and RME), Figure S8 shows the values obtained using Radial Pentagons as reported by Andraos et al.[50,51] Calculations are included in Tables S1–S5. The more regular representations are obtained in Synthesis #4 and in Synthesis #2 being the RME slightly higher in #4 (0.487 vs. 0.472).

From another point of view, energy consumption is an important component to be considered (especially in the preparation of bulk chemicals). In this sense, Christensen et al.[59] defined the climate factor (C-factor), to quantify the carbon footprint of chemicals, as the total mass of CO_2 emitted divided by the mass of product formed ($kg_{CO_2}/kg_{product}$). To join this C-factor with E-factor, the E^+ has been recently reported:[60] this metric considers the greenhouse gas emissions (as CO_2 emissions) generated from electricity used for processes (such

Table 3. E-factor calculations.

E-value	Synth. #1[16]	Synth. #2[26]	Synth. #3	Synth. #4
E_{MW}	$5.7x10^{-4}$	0.1	0.1	0.1
sEF[a]	4.54	1.1	2.1	1.1
cEF[b]	17.14	306.1	85.3	44.9
cEF-rec[c]	–	–	83.4	–

[a] Under reclaiming reaction solvents, catalysts, and byproducts, and all post-reaction materials. [b] Under committing all reaction solvents, catalysts, and byproducts, and post-reaction materials to waste. [c] Considering the use of 4 reaction cycles. For E-value acronyms, see text.

Table 4. Productivity using *Bs*GGT-GLX-AG for four consecutive reaction cycles.

Cycle	Yield [%]	Amount of product formed [mmol]	[mg]	accumulated [mg]	$mg_{product}/mg_{enzyme}$
1	93	1.395	243	243	0.39
2	90	1.35	235	478	0.76
3	94	1.41	246	724	1.16
4	96	1.44	251	975	1.56

as cooling, heating, stirring and pumping), and adds this value up to the classical E-factor. It can be considered a combination of E- and C-factors. Thus, Table 5 draws a comparison of the different methodologies (detailed calculations can be found in the Supporting Information).

As can be observed from data in Table 5, Synthesis #1 generates a much higher amount of CO_2 as derived from the higher temperature and reaction time required. On the other hand, Synthesis #2 also releases a higher amount of CO_2 compared to #3 and #4, although it is not clear in the paper from Xu et al.[26] whether the heating at 45 °C is produced only by ultrasounds (assumption used in Table 5), or rather another external heater is also required. In any case, the overall E^+ factor value, considering both the waste and energy consumption, clearly indicates how our enzymatic Synthesis #4 is an excellent sustainable and eco-friendly procedure which, undoubtedly, would be even better once the reaction could be scaled-up to higher substrate loadings.

Conclusion

The use of enzymes to produce bio-based chemicals is one of the strategies that are being actively pursued to enhance the transition toward more sustainable products, which is a priority of the EU Green Deal.[61] Nevertheless, efficiency and cost-competitiveness of biotransformations are still perceived as bottlenecks in translating bioprocesses from lab to large scale.

The enzymatic synthesis of L-theanine (L-Th) here described does possess most of the requirements both to meet the goals of the EU Green Deal and, for sure, to be further scaled-up beyond the gram scale.

This synthetic scheme resulted in the generation of relatively low as well as "benign" waste (mostly water), being also characterized by a small C-footprint, as highlighted by complete E-factor and E^+-factor. It is worth noting that the latter metrics is far below the values calculated for the enzymatic route by Xu et al.[26] and the fully chemical route.[16]

On the other hand, this enzymatic synthesis of L-Th occurs in water at room temperature without the need for any additives but the reactants (L-glutamine and ethylamine) at the spontaneous pH (11.6), resulting in very high yield (93%) and purity (99%). The smooth product downstream, which avoids any purification steps and allows for the repeated recyclability of the immobilized biocatalyst, makes this bioprocess very attractive over the current established methods for a larger scale implementation which embraces the principles of sustainable chemistry.

Table 5. C-factor and E^+ calculations.

Metrics	Synth. #1[16]	Synth. #2[26]	Synth. #3	Synth. #4
cEF[a]	17.14	306.1	85.3	44.9
g_{CO_2} [b]	46.6×10^3	646.4[c]	24.2	24.2
C-factor	1.26×10^3	35	98	50
E^+ [d]	1.273×10^3	341.1	183.3	94.9

[a] From Table 3. [b] Calculated considering energy consumed and assuming 404 g_{CO_2} $KW^{-1} h^{-1}$, from 2015 Organisation for Economic Cooperation and Development (OECD) average.[60] [c] Assuming that the energy consumption is only provided by ultrasounds.[26] [d] cEF + C-factor.

Experimental Section

Materials and instruments

L-Glutamic acid (L-Glu), γ-(4-nitroanilide) (G*p*NA), glycylglycine (Gly-Gly), 4-nitroaniline, L-glutamine (L-Gln), ethylamine (66–72% purity in water), L-serine (L-Ser), L-theanine (L-Th), Bradford reagent, glycidol, sodium periodate ($NaIO_4$), sodium borohydride ($NaBH_4$), 1-fluoro-2,4-dinitrobenzene (Sanger's reagent), HPLC-grade solvents were purchased from Sigma–Aldrich (Milano, Italy).

Sepharose CL-6B (agarose) was from GE Healthcare (Uppsala, Sweden). Sodium bicarbonate ($NaHCO_3$) was from Carlo Erba (Cornaredo, Italy). Spectrophotometric assays were performed using a Shimadzu UV-1601 UV-Visible spectrophotometer equipped with magnetic stirring. HPLC analyses were carried out with a Merck Hitachi instrument equipped with UV/Vis detector, using a 250x4.6 mm Adamas RP C18-extreme column (SepaChrom, Rho, Milano, Italy). The N-terminal His-tagged GGT from *Bacillus subtilis* 168 (*Bs*GGT) was produced as previously described by Morelli et al.[40]

*Bs*GGT activity assay

The standard activity assay (2 mL) was performed at room temperature in Tris-HCl buffer (pH 8.5, 100 mM) containing G*p*NA (1 mM), Gly-Gly (100 mM) and an appropriate amount of enzyme (free *Bs*GGT: 1 μg; immobilized *Bs*GGT: 5–10 mg, under magnetic stirring). The reaction was monitored spectrophotometrically by measuring the formation of 4-nitroaniline at 410 nm in the kinetic mode.[62] The amount of 4-nitroaniline produced by the enzyme was quantified using a calibration curve and an extinction coefficient of 8300 $M^{-1} cm^{-1}$. One unit of *Bs*GGT was defined as the amount of enzyme that produces 1 μmole of 4-nitroaniline per minute from G*p*NA in the presence of the acceptor Gly-Gly.

Glyoxyl-agarose preparation

GLX-AG was prepared as previously reported.[63] Briefly, 5.0 g of agarose were suspended in a solution of 1.4 mL deionized water and 2.4 mL 1.7 M NaOH containing 14.2 mg mL^{-1} $NaBH_4$. Subsequently, 1.7 mL of glycidol were added dropwise keeping the vessel at 4 °C in an ice bath. After the addition of glycidol, the reaction was kept under stirring overnight at room temperature. The suspension was filtered, and the carrier was washed abundantly with deionized water. Oxidation was initiated by adding 34 mL of 100 mM $NaIO_4$. The reaction was carried out for 2 h at room temperature, then the carrier was filtered under reduced pressure and washed abundantly with deionized water and stored at 4 °C until use.

*Bs*GGT immobilization on glyoxyl-agarose

Immobilization of *Bs*GGT on GLX-AG was performed as reported in a previous work.[37] An enzyme loading of 1 mg was used per gram of carrier. A 10:1 ratio of volume of immobilization reaction:volume of the carrier was used. Briefly, GLX-AG was washed abundantly with $NaHCO_3$ buffer (pH 10.0, 50 mM) and then filtered under reduced pressure until dryness. 835 μL of *Bs*GGT (5.7 mg mL^{-1}) were

solubilized into 64 mL of $NaHCO_3$ buffer. Then, 5 g of carrier were added, and the suspension was allowed to stir for 3 h at room temperature. Finally, 71 mg of $NaBH_4$ were added to the mixture and incubated for 30 min for imines reduction. During immobilization, the supernatant was monitored by measuring the amount of protein in solution (Bradford assay), and the residual activity of the supernatant (10–100 μL) was checked by the standard activity assay described above. The immobilized enzyme was then filtered, washed with deionized water (20 mL) and stored at 4 °C.

Analytical synthesis of L-theanine

L-Glutamine (100–300 mM, 29–87 mg) and ethylamine (100–800 mM, 0.017–0.399 mL) were suspended in deionized water (2 mL). The pH was either adjusted to 10.0 with HCl (6 M) or left spontaneous (pH 11.6). The reaction was initiated by addition of *Bs*GGT-GLX-AG (12 IU g^{-1}, 83–249 mg, 0.5–1.5 IU mL^{-1} reaction) and incubated at 25 °C under magnetic stirring. Samples (20 μL) were periodically withdrawn and derivatized with Sanger's reagent by following the standard protocol reported below and analyzed by HPLC (see below). Conversion was determined by using a calibration curve of an authentic sample of L-Th (Figures S9 and S10).

Preparative synthesis of L-theanine

L-Glutamine (100 mM, 219 mg) and ethylamine (800 mM, 1.0 mL) were suspended in deionized water (15 mL). The pH was either adjusted to 10.0 with HCl (6 M) or left spontaneous (pH 11.6). The reaction was initiated by addition of *Bs*GGT-GLX-AG (12 IU g^{-1}, 625 mg, 0.5 IU mL^{-1} reaction) and incubated at 25 °C under magnetic stirring for 6 h. The immobilized catalyst was removed by filtration under reduced pressure and washed with deionized water (5 mL). The filtrate was recovered, and the residual ethylamine was removed under reduced pressure. The aqueous solution was directly submitted to lyophilization. The purity was determined by HPLC (Figure S6). The identity of L-Th was confirmed by ^1H and ^{13}C NMR (Figure S5), and ESI-MS analyses (Figure S7).

Intensification synthesis of L-theanine

L-Glutamine (200 mM, 438 mg) and ethylamine (1600 mM, 2.0 mL) were suspended in deionized water (15 mL) pH 11.6. The reaction was initiated by adding *Bs*GGT-GLX-AG (12 IU g^{-1}, 1250 mg, 1 IU mL^{-1} reaction) and incubated at 25 °C under magnetic stirring for 6 h. The immobilized catalyst was removed by filtration under reduced pressure and washed with deionized water (5 mL). The filtrate was recovered, and the residual ethylamine was removed under reduced pressure. The aqueous solution was directly submitted to lyophilization.

Sample derivatization with Sanger's reagent and HPLC analysis

All samples were derivatized with Sanger's reagent following a standard protocol with slight modifications.[64] Briefly, samples were diluted 1/20 with milliQ water. An aliquot of the diluted samples (100 μL) was added to the derivatization mixture: 350 μL $NaHCO_3$ (pH 9, 100 mM), 50 μL serine (5 mM in water) (internal standard), 500 μL Sanger's reagent (20 mM in acetonitrile). The mixture was incubated in the dark in a thermomixer at 70 °C for 45 min under vigorous mixing (1000 rpm). Samples were cooled immediately in cold water (ca. 15 °C). Before HPLC analysis, the samples (100 μL) were diluted 1/1 in the mobile phase A.

HPLC analyses were carried out using a linear gradient of eluent A [water + 0.1 % *v/v* trifluoroacetic acid (TFA)] and eluent B (acetonitrile + eluent A 80:20): 0–10 min A:B 80:20 isocratic, 10–15 min A:B 70:30 linear gradient; 15–25 min A:B 70:30 isocratic; 25–30 min A:B 60:40 linear gradient; 30–35 min A:B 50:50 linear gradient; 35–40 min A:B 40:60 linear gradient; 40–60 min A:B 60:40 linear gradient; 60–70 min A:B 80:20 isocratic; flow rate: 1 mL min^{-1}; UV-detection: 356 nm; 25 °C.

Silver nitrate precipitation assay

A silver nitrate solution (0.1 M in water; 2 mL) was added to few mg of L-Th produced in house.

*Bs*GGT-GLX-AG recycling

The recycle of *Bs*GGT-GLX-AG was performed under the semi-preparative reaction conditions (see Table 1). Briefly, L-glutamine (100 mM, 219 mg) and ethylamine (800 mM, 1.0 mL) were suspended in deionized water (15 mL) at pH 11.6 (spontaneous). The reaction was initiated by adding *Bs*GGT-GLX-AG (12 IU g^{-1}, 625 mg, 0.5 IU mL^{-1} reaction) and incubated at 25 °C under magnetic stirring for 6 h. A sample was withdrawn at the end point and analyzed by HPLC as described above for the calculation of L-Th conversion. Afterwards, the reaction mixture was filtered s.v. on a glass filter to recover the immobilized biocatalyst. The residual activity of the biocatalyst after each cycle was determined by the standard activity assay described above.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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