Taurine Production in Engineered *Halomonas elongata*

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ABSTRACT

Taurine (Tau) is a conditionally essential amino acid in fish diet. Dietary supplementation of Tau in fish feed improved their survival rate, growth rate, protein and energy retention, metabolism, antioxidation response, stress response, disease resistance, muscle texture, and reproductive performance. Therefore, Tau is added to plant-protein-based fish feed due to the absence of Tau in plant protein. *Halomonas elongata* OUT30018 is a moderately halophilic bacterium with the ability to utilize a wide range of sugars and amino acids, including the putrefactive non-volatile amines such as histamine and tyramine derived from biomass waste as carbon (C) and nitrogen (N) sources for cell growth. The work presented here aims to generate a Tau-producing *H. elongata* cell factory to be used as a single-cell aquaculture feed additive. In bacteria, Tau is synthesized via two pathways: the L-cysteine (L-Cys) sulfinic acid pathway, mediated by the L-Cys dioxygenase (CDO) and the Cys sulfinic acid decarboxylase (CSAD) enzymes and the L-Cys sulfonic acid pathway, mediated by the L-Cysteate synthase (CS) and the CSAD enzymes. To compare the efficiency of these pathways, the expression cassettes encoding the codon-optimized artificial genes encoding these enzymes were introduced into the genome of *H. elongata* OUT30018 to generate *H. elongata* CSAD-CDO, CSAD-CS, and CSAD-CDO-CS strains. Subsequently, the amount of Tau accumulated in the three recombinant strains cultured in the M63 minimal media containing 4% glucose, 3% w/v NaCl, and 5 mM Cys were compared by HPLC analysis. Our result shows that *H. elongata* CSAD-CDO, expressing the enzymes of L-Cys sulfinic acid pathway, was the only strain that successfully accumulated Tau in the cells. Currently, we are conducting tests to determine if the *H. elongata* CSAD-CDO can grow and produce Tau when cultivated in a medium made from Cys-rich biomass waste, such as wool and chicken feathers. The use of *H. elongata* CSAD-CDO cells grown in Cys-rich biomass waste medium as a single-cell Tau-rich feed additive could decrease costs and improve the sustainability of the aquaculture feed industry.

Keyword: Taurine/ Feed additives/ Essential amino acids/ Cysteine/ *Halomonas elongata*

1. INTRODUCTION

A moderately halophilic *Halomonas elongata* is a gram-negative eubacterium well adapted to high-salinity environments by producing and accumulating ectoine as a compatible solute [1]. *H. elongata* OUT30018, isolated from a high-salinity agricultural field in Khon Kaen, Thailand [2], is unique among the members of the *Halomonas* genus in its ability to utilize biomass-waste derived sugars [3] and amino acids, including histamine and tyramine, which are the major putrefactive non-volatile amines derived from biomass waste and composted fertilizer as carbon (C) and nitrogen (N) sources for growth [4, 5, 6]. Therefore, we have selected *H. elongata* OUT30018 as a host strain to develop cell factories to produce amino-acid-derived chemicals, including ectoine (Ect), L-proline (Pro), and γ-aminobutyric acid (GABA) using media derived from nitrogen-rich waste biomass [3, 7, 8, 9, 10, 11].

A sulfur-containing amino acid, Taurine (Tau), is a conditionally essential amino acid in the fish diet [12, 13]. Dietary supplementation of Tau has been shown to improve survival, growth, feed utilization, protein and energy retention, intermediate metabolism, antioxidation, stress reduction, disease resistance, muscle texture, and reproductive performance in fish [13, 14, 15, 16]. As a result, Tau supplementation is recommended in plant-protein-based fish feeds due to the absence of Tau in plant proteins [13, 16]. Here, we report the generation of *H. elongata* strain that biosynthesizes and accumulates Tau instead of Ect. The prospect of using medium derived from nitrogen and sulfur-rich

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waste biomass for the cultivation of this strain to be used as a Tau-rich feed additive for the sustainable aquaculture industry is discussed.

2. METHODOLOGY

2.1 Bacterial strains and plasmids

Bacterial strains used in this study are listed in Table 1. The *Escherichia coli* DH5α strain was used as a cloning host, while *E. coli* HB101 strain, which harbored a pRK2013 plasmid was used as a helper strain in the triparental conjugative transformation of *H. elongata*. Plasmids used in this study are listed in Tables 2.

Table 1. List of Bacterial strains

Strains	Genotypes and Phenotypes	References
Escherichia coli		
DH5α	F$^-$ ΦH5α*lacZDM15*Δ*(lacZYA-argF)U169 recA1 hsdR17(*r_K^- m_K^+*) supE44* Δ$^-$*thi-1 gyrA relA1* Used as cloning host and donor host for triparental mating.	[17]
HB101	F$^-$, *hsd S*20(rB-, mB-), *rec*A13, *ara*-14, *proA*2, *lacY*1, *galK*2, *rpsL*20 (str), *xyl*-5, *mtl*-1, *supE*44, *leuB*6, *thi*-1, Δ(*mcrC-mrr*), Used as a host for pRK2013 plasmid.	[18]
Halomonas elongata		
OUT30018	Wild type strain (Osaka University Type Culture, formerly designated as KS3). Salt-tolerant phenotype (0.3 to 21% w/v NaCl) due to ability to produce and accumulate ectoine as a major osmolyte.	[2]
KA1	Δ*ectABC* Ectoine-deficient salt-sensitive mutant of *H. elongata* OUT30018 with genomic deletion of *ectABC* gene cluster.	[8]
CSAD-CDO	Δ*ectABC*::*mCherry-CSAD-CDO* Engineered strain, in which the *ectABC* gene cluster on the genome of *H. elongata* OUT30018 was replaced with the salt-inducible Tau-synthetic *mCherry-CSAD-CDO* operon. Produce and accumulated Tau in the cell.	This study
CSAD-CS	Δ*ectABC*::*mCherry-CSAD-CS* Engineered strain, in which the *ectABC* gene cluster on the genome of *H. elongata* OUT30018 was replaced with the salt-inducible Tau-synthetic *mCherry-CSAD-CS* operon.	This study
CSAD-CDO-CS	Δ*ectABC*::*mCherry-CSAD-CDO-CS* Engineered strain, in which the *ectABC* gene cluster on the genome of *H. elongata* OUT30018 was replaced with the salt-inducible Tau-synthetic *mCherry-CSAD-CDO-CS* operon.	This study

Table 2. List of Plasmids

Plasmids	Descriptions	References
pK18N*mobsacB*-U_{ectA}-*mCherry-CSAD-CDO*-D_{ectC}	pK18N*mobsacB* containing an artificial Tau biosynthetic *mCherry-CSAD-CDO* gene cluster, which was put under the control of the salt-inducible *ectA* promoter (U_{ectA}) and *ectC* terminator (D_{ectC}); Kan^r.	This study
pK18N*mobsacB*-U_{ectA}-*mCherry-CSAD-CS*-D_{ectC}	pK18N*mobsacB* containing an artificial Tau biosynthetic *mCherry-CSAD-CS* gene cluster, which was put under the control of the salt-inducible *ectA* promoter (U_{ectA}) and *ectC* terminator (D_{ectC}); Kan^r	This study
pK18N*mobsacB*-U_{ectA}-*mCherry-CSAD-CDO-CS*-D_{ectC}	pK18N*mobsacB* containing an artificial Tau biosynthetic *mCherry-CSAD-CDO-CS* gene cluster, which was put under the control of the salt-inducible *ectA* promoter (U_{ectA}) and *ectC* terminator (D_{ectC}); Kan^r.	This study

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2.2 Generation of recombinant H. elongata strains

To generate Tau-producing *H. elongata* strains, pK18N*mobsacB*-based plasmids harboring different artificial Tau biosynthesis operons (Table 2) were introduced into *H. elongata* OUT30018 strain by *E. coli* HB101/pRK2013-mediated triparental mating method followed by the integration of the introduced Tau biosynthesis operons into the genome of *H. elongata* OUT30018 by homologous recombination [8, 19]. Briefly, three bacterial strains: the donor *E. coli* DH5α strains harboring the plasmid, the helper *E. coli* HB101 harboring pRK2013 conjugative plasmid, and the recipient *H. elongata* OUT30018 were co-cultured for triparental conjugation on Omnipore™ membrane (Merck Millipore, Darmstadt, Germany), which was placed on a solid Luria-Bertani (LB) medium containing 2% w/v NaCl. After triparental conjugation, cells were cultured on a solid LB medium containing 6% NaCl and 100 mg/L Kanamycin (Kan) to select the first crossover strains, which were transferred to an LB solid medium supplemented with 6% NaCl and 15% sucrose for selection of the second crossover strains with sucrose-tolerant phenotype.

2.3 Media and growth conditions

LB medium, consisting of 5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl, was used for routine culture of *E. coli* strains. A high-salinity LB medium containing 6% NaCl was used for the routine culture of *H. elongata* strains. LB media were sterilized by autoclave at 121 °C for 15 minutes. Antibiotics were added to the culture medium to select transformant *E. coli* and *H. elongata* harboring plasmids with antibiotic-resistant genes. The concentration of Ampicillin in the medium was 100 μg/mL for *E. coli*, and the concentration of Kan in the medium was 50 μg/mL for *E. coli* and 100 μg/mL for *H. elongata*. For solid medium, 15 g/L agar was added. To select a counter-selectable marker gene (*sacB*) used in *H. elongata* transformation, 150 g/L sucrose was added to the M63 minimal media [20]. For Tau production analysis, *H. elongata* strains were cultured in 5 mL of M63 minimal media, which contained 100 mM KH_2PO_4, 15 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4$, 3.9 μM $FeSO_4$, 4% glucose, 3% NaCl, and 5 mM cysteine (Cys). The pH of M63 media was adjusted to 7.2 with KOH solution, and the media were filter-sterilized using a bottle top filter with 0.22 μm pore-size polyethersulfone (PES) membrane (Sartorius, Germany). For routine culture of *E. coli* and *H. elongata* strains, the cultures were incubated at 37°C in a water bath shaker with an agitation rate of 120 rpm.

2.4 Extraction of major osmolytes from H. elongata cells

Intracellular free amino acids of *H. elongata* cells were extracted by a hypo-osmotic extraction method [1, 8, 21]. Briefly, *H. elongata* cells were harvested from the culture medium by centrifugation at 10,000 *g* for 3 min, and the weight of the cell pellet was recorded as cell fresh weight (CFW). The cell pellets were then suspended in pure water (20 μL per 1 mg CFW). After centrifugation at 10,000 *g* for 3 min, the supernatant containing free amino acids was collected as a major osmolyte sample.

2.5 High-Performance Liquid Chromatography (HPLC) analysis of dabsyl amino acids

Dabsylation of amino acids was performed based on a previously described method [22] with a slight modification [8, 10]. The dabsyl amino acids solution of each sample was collected and filtered through a filter vial with 0.2 μm pore-size Polytetrafluoroethylene (PTFE) membrane (SEPARA® Syringeless filter, GVS Japan K.K., Tokyo, Japan) before being used as an HPLC sample. The quantification of dabsyl amino acids was carried out using an HPLC system (Shimadzu, Kyoto, Japan) equipped with a UV/VIS detector (SPD-10 A VP), an autosampler (SIL-10 AD VP), two pumps (LC-10 AD VP), degasser (DGU-14A), system controller (SCL-10A Vp), and column oven (CTO-10AC VP). The LabSolutions LC software (Shimadzu, Kyoto, Japan) was used to control the system and collect data. Dabsyl amino acids were separated through an analytical C18 column (Poroshell 120, 2.7 μm, EC-C18, 4.6 × 75 mm, Agilent Technologies Inc.) equipped with C18 guard column (Poroshell 120, 2.7 μm Fast Guard, EC-C18, 4.6 × 5 mm, Agilent Technologies Inc.) using a mobile phase gradient system consisting of 15% acetonitrile in 20 mM sodium acetate (pH 6.0) (mobile phase A) and 100%

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acetonitrile (mobile phase B). Dabsyl amino acids were detected by the UV/VIS detector at 468 nm. The injection volume was 10 µL, the flow rate was 0.5 mL/min, and the column temperature was maintained at 25°C. The eluent gradient was set as previously described [8].

3. RESULTS AND DISCUSSION

3.1 Metabolic pathway engineering for Tau biosynthesis in H. elongata

In this study, two metabolic pathways for Tau biosynthesis, the L-cysteine sulfinic acid pathway mediated by the L-cysteine dioxygenase (CDO) and the L-cysteine sulfinic acid decarboxylase (CSAD) enzymes and the L-cysteine sulfonic acid pathway mediated by the L-cysteine sulfonic acid synthase (CS) and the CSAD enzymes (Figure 1), were engineered in *H. elongata*.

To produce Tau in *H. elongata*, we designed *H. elongata*'s codon-optimized genes encoding the combinations of CSAD, CDO, and CS, and then assembled them with a *mCherry* reporter gene encoding a red fluorescent protein to generate three types of artificial Tau biosynthesis operons (Figure 2). Each artificial Tau biosynthesis operon is introduced into the genome of *H. elongata* OUT30018 at the *ectABC* locus by homologous recombination using 1.0 kb 5'-flanking region of the *ectA* gene (U_{ectA}) and 1.0 kb 3'-flanking region of the *ectC* gene (D_{ectC}). The expression of mCherry red-florescent reporter protein allowed us to quickly select recombinant *H. elongata* cells directly on the selection medium (Figure 3A). The correct integrations of the transgenes into the genome of the recombinant *H. elongata* strains were also confirmed by colony PCR using specific primers to amplify the Tau biosynthesis operons (Figure 3B).

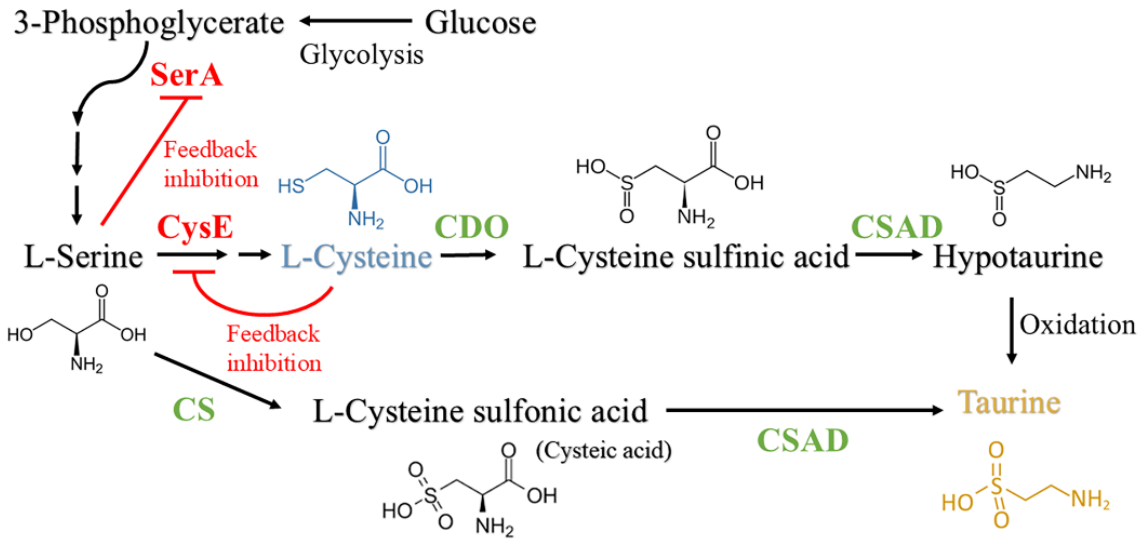

Figure 1. The two biosynthesis pathways of taurine: 1. the L-cysteine sulfinic acid pathway mediated by the L-cysteine dioxygenase (CDO) and the L-cysteine sulfinic acid decarboxylase (CSAD) enzymes and 2. the L-cysteine sulfonic acid pathway mediated by the L-cysteine sulfonic acid synthase (CS) and the CSAD enzymes. In these pathways, 3-Phosphoglycerate dehydrogenase (SerA) is feedback-regulated by L-Serine, and Serine-O-acetyltransferase (CysE) is feedback-regulated by L-Cysteine.

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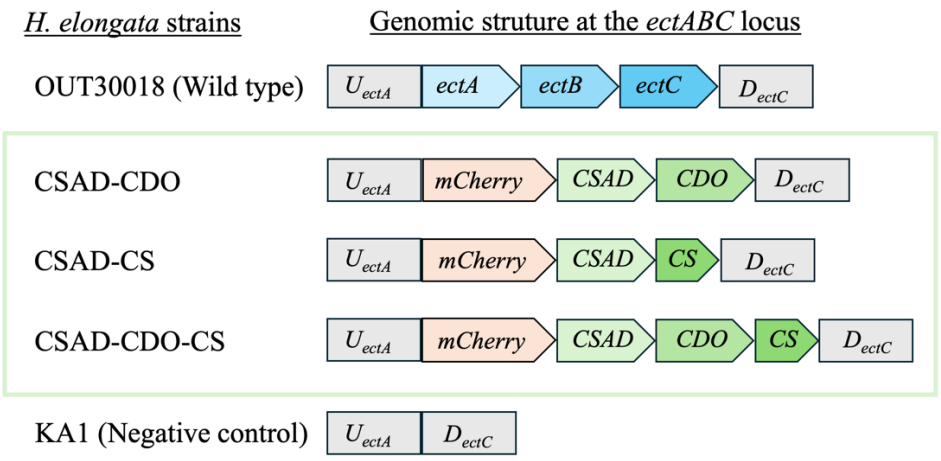

Figure 2. Schematic diagram showing genomic structure at the *ectABC* locus of the wild-type and recombinant *H. elongata***.**
U_{ectA}: a 1.2-kb 5'-upstream region of the *ectA* gene. Contains a salt-inducible *ectA* promoter region for homologous recombination at the *ectABC* locus.
D_{ectC}, a 1.2-kb 3'-downstream region of the *ectC* gene. Contains the *ectC* terminator region for homologous recombination at the *ectABC* locus.
ectA: a gene that encodes an L-2,4-diaminobutyric acid (DABA) acetyltransferase (DAA).
ectB: a gene that encodes a DABA transaminase (DAT).
ectC: a gene that encodes an ectoine synthase (ES).
mCherry: a gene that encodes a red fluorescent mCherry protein used as a visual aid to facilitate the selection of transformed *H. elongata*. Correct expression of the mCherry protein also indirectly verifies correct transgenes' expression.
CSAD: a gene that encodes L-cysteine sulfinic acid decarboxylase (CSAD).
CDO: a gene that encodes L-cysteine dioxygenase (CDO).
CS: a gene that encodes L-cysteine sulfonic acid synthase (CS).

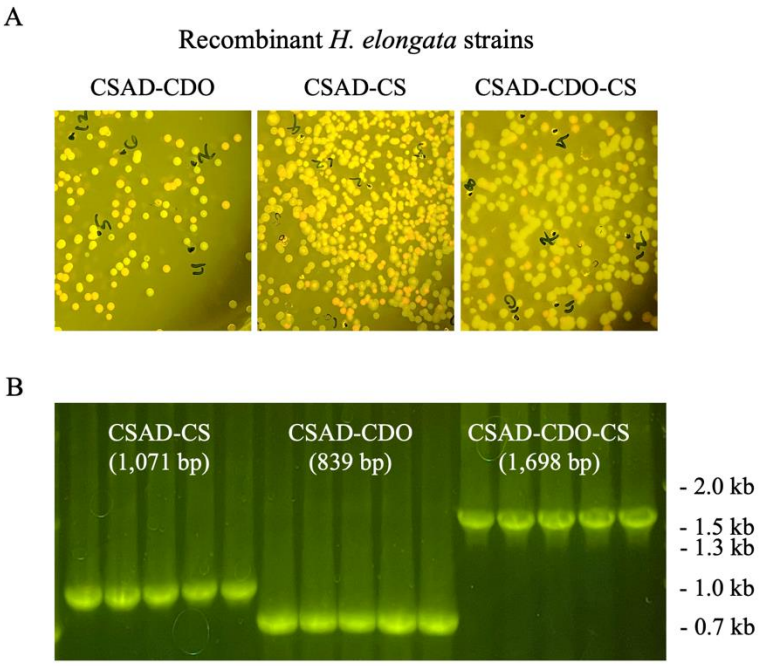

Figure 3. Verification of recombinant *H. elongata* CSAD-CDO, CDAD-CS, and CDAD-CDO-CS strains. A. Pink colonies of transformed *H. elongata*, as a result of the expression of the red fluorescent mCheery, facilitate the selection of the positive clones on the selection medium. B. Genomic PCR using specific primers to amplify the artificial Tau biosynthesis operons that were integrated into the genome of the positive clones. DNA size marker is shown on the right of the PCR products photo. bp, base pair; kb, kilobase.

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3.2 Verification of Tau production in the engineered H. elongata strains

To check the ability of the three engineered strains to produce and accumulate Tau, each strain was cultured in liquid M63 minimal medium containing 4% Glucose, 3% NaCl, and 5 mM Cys, and intracellular free-amino acids were extracted from their cells and analyzed by HPLC. As shown in Figure 4, the *H. elongata* CSAD-CDO was the only one that could efficiently produce Tau via the L-cysteine sulfinic acid pathway (Figure 4). Interestingly, Tau was not detected in the extracts from the *H. elongata* CSAD-CS and CSAD-CDO-CS (Figure 4).

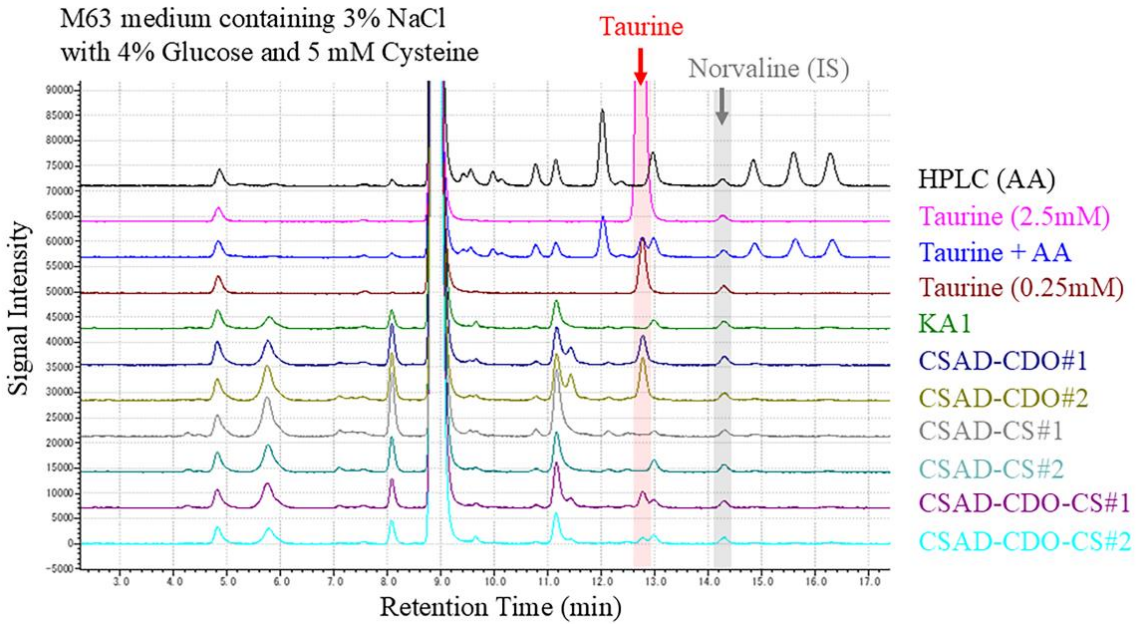

Figure 4. HPLC Chromatograms showing major osmolyte profiles of engineered *H. elongata* strains. *H. elongata* KA1 (negative control), CSAD-CDO, CSAD-CS, and CSAD-CDO-CS strains were cultured in M63 medium containing 3% NaCl, 4% glucose, and 5 mM Cys until the optical density at 600 nm (OD_{600}) reached more than 0.8, and major osmolytes inside the cells were extracted and analyzed by HPLC. AA, amino acid standards; IS, Internal standard Norvaline. Red and gray arrows indicate the peaks of Tau and Norvaline. #1 and #2 indicate independent recombinant clones.

4. CONCLUSIONS and PROSPECTS

In this study, we successfully engineered a Tau-producing *H. elongata* CSAD-CDO, which expresses the enzymes CSAD and CDO of the L-cysteine sulfinic acid pathway. As Cys is important as a precursor for Tau biosynthesis, we are developing a method to prepare a medium based on Cys-rich biomass waste, such as animal wool, human hair, and chicken feathers, to be used in the cultivation of *H. elongata* CSAD-CDO. The L-cysteine sulfinic acid pathway is constrained by feedback-inhibition control on SerA and CysE, which function upstream from the CDO and CSAD enzymes (Figure 1). Therefore, replacing these enzymes with their feedback-inhibition insensitive mutants could be next step to further enhance the ability of *H. elongata* CSAD-CDO to produce and accumulate Tau.

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