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Induction of mutation and development of a high L-tryptophan yielding *Bacillus subtilis*

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A regulatory mutant *Bacillus subtilis* GSX1159 was employed for the development of high-tryptophan yielding strain via random mutation using ethylelemine and UV irradiations. It followed selection and protoplast fusion between high L-threonine yielding strain and multiple L-threonine analog-resistant strain to develop such a high yielding strain which can prevent feedback inhibition. A high L-threonine yielding strain *Bacillus subtilis* GSX1794 was developed by induced mutation. It was then subjected to protoplast fusion with multiple L-threonine analog-resistant strain *Bacillus subtilis* GSX2170. It led to the development of a multiple analog-resistant high L-threonine yielding strain *Bacillus subtilis* GSX2480, which could accumulate up to 8.3 mg/ml L-threonine (64.3% greater accumulation than *Bacillus subtilis* GSX1159) in the fermentation broth on subsequent fermentation trials.

Keywords: *Bacillus subtilis*, ethylenemine, UV irradiations, L-threonine, analog.

Introduction

L-Tryptophan is an essential amino acid with an indole side chain. Due to such unique structural feature it is used as a precursor for a numerous neurotransmitters, such as serotonin, melatonin etc. and related to sleep, mood, perception of pain etc. It is thus used for the chemical synthesis of some antidepressant drugs in the treatment of schizophrenia[1–3]. The market for L-tryptophan is gradually expanding in which China seems to play a dominating role to meet the demand[4,5].

The chemical method of tryptophan synthesis was introduced first for its large scale production; however it produced racemic mixture of DL-tryptophan from which separation of stereospecific L-form was very difficult[6]. Then it followed by very expensive enzymatic synthesis[7]. Then after the isolation of L-glutamic acid producing microorganism *Micrococcus glutamicus* by Japanees scientist Kinoshita and his coworkers in 1957, the focus on stereo-specific fermentation of L-tryptophan was taken under consideration. Several microorganisms, such as *Bacillus subtilis*, *Pseudomonas aeruginisa*, *Escherichia coli* etc. were characterized and the presence of tryptophan synthase, tryptophanase or both was identified[8–10].

With the advancement of the biotechnological tools, it is much easier to develop new stretagies for microbial production of a desired metabolite. Among different methods, metabolic engineeringis mostly preferred. Random mutation followed by selection is commonly practiced in this regard. Zhao and his co-workers developed an L-tryptophan over producing *Escherichia coli* using a specific site directed mutagenesis[9]. A new strain of *Escherichia coli* FB-04 (*pta*1) was developed by gene manipulation technique for over production of L-tryptophan[11].

In this present study, we were intended to develop a high L-tryptophan yielding multiple analog-resistant strain of *Bacillus subtilis* which can accumulate high amount of L-tryp-

tophan in the fermentation broth as well as can also prevent the feed-back inhibition during its synthesis.

Materials and methods:

Selection of microorganism: Bacillus subtilis GSX1159, a regulatory mutant was employed for the development of high L-tryptophan yielding mutant strain by mutagenic treatments using ethylenemine and UV irradiations and subsequently protoplast fusion with multiple analog-resistant strain[12,13].

Mutagenic treatments: Cell suspension (1 ml containing 3×10^6 cells) was added to 9 ml ethylenemine solution of different concentrations (170.3 mmol/ml, 133.7 mmol/ml and 63.2 mmol/ml) prepared from a stalk solution (500 mmol/ml). After a certain period of incubation (Fig. 1), 1 ml of sample was diluted in water in each case. The diluted bacterial cells were then platted on CD agar medium and incubated for 48 h at 28°C for colony development. After proper growth, the colonies were transferred to yeast extract-agar slants and stored at 4°C[12].

2 ml of bacterial suspension (6×10^6 cells) were taken in a petridish (5 cm diameter) and exposed to UV irradiations from a distance of 12 cm for different periods of time (Table 2). The source of radiation was a Hanovia germicidal lamp (15 Watt). The treated bacterial cells were incubated for 48 h at 28°C and selected colonies were transferred to yeast extract-agar slants and stored at 4°C[12].

The cells selected from different stages of treatment with mutagens were finally tested for L-tryptophan production by surface culture fermentation process.

Development of analog-resistant strain: Different concentrations (10–50 mg/ml) of multiple L-tryptophan analogous compounds (7-azatryptophan, fluorotryptophan, 5-hydroxytryptophan, 5-aminotryptophan, 5-bromotryptophan, 5-methoxytryptophan, α-methyltryptophan and methyltryptophan) were used to develop multiple-analog-resistant strain.

Protoplast fusion: Bacillus subtilis GSX1794 (high L-tryptophan yielding strain) were subjected to protoplast fusion with *Bacillus subtilis* GSX2170 (multiple analog-resistant strain). The cells were harvested in 100 ml growth medium composed of: glucose, 15 g/L; peptone, 8 g/L; yeast extract, 8 g/L; NaCl, 3 g/L; $MgSO_4.7H_2O$, 0.3 g/L; K_2HPO_4, 1 g/L; KH_2PO_4, 1 g/L; $MnSO_4.4H_2O$, 0.1 g/L and biotin, 2 μg/ml in 250 ml Erlenmeyer conical flask at 28°C for 24 h. Then the cell suspensions were centrifuged separately at 10,000 rpm for 10 min. The pellets were aseptically collected and transferred to a protoplasting medium composed of sucrose, 1.0 g/L; maleate buffer (pH 7.0), 0.02 g/L; $MgCl_2.H_2O$, 20 mg/L and lysozyme, 100 μg/ml. After protoplast fusion (observed under phase contrast microscope), protoplasts were fused in a medium containing the same composition similar to the protoplasting medium along with PEG (30%), dimethyl sulfide (15%) and $CaCl_2$, 10 mg/L. The suspension was shaken at 50 rpm on a rotary shaker with incubator at 30°C for 10 min and then it was diluted 10 fold with protoplast medium buffer (pH 7.0). The suspension was then centrifuged for 5 min at 25,000 rpm at 5°C using a cold centrifuge apparatus (EPLX3491). The palette was collected and plated for colony formation for 48 h at 30°C. The colonies were transferred to 2% agar slant medium and kept it at 4°C[13].

Viable counting of protoplast (Reversion if protoplast): Protoplasts were diluted with 10 ml of dilution fluid and plated into petridish (diameter 5 cm) containing agar medium and allowing to grow at 30°C for 48 h and subjected to subsequent fermentation trials[13].

Physical conditions for growth: The fermentation was carried out using medium volume, 25 ml; initial pH, 7.0; shaker speed, 200 rpm; age of inoculum, 48 h; cell density, 2×10^6 cells/ml and temperature, 30°C[13].

Composition of basal salt medium: L-Tryptophan fermentation was carried out using the basal salt medium composed of: glucose, 100 g/L; urea, 0.8 g/L; K_2HPO_4, 1 g/L; KH_2PO_4, 1 g/L; $MgSO_4.7H_2O$, 0.025 g/L; $FeSO_4.7H_2O$, 0.1 g/L; biotin, 80 μg/L; H_2O, 1 L and pH was adjusted to 7.0[12,13].

Analysis of L-tryptophan: Descending paper chromatography was employed for detection of L-tryptophan in culture broth and was run for 18 h on Whatman No. 1 Chromatographic paper. The solvent system used include:n-butanol: acetic acid:water (2:1:1). The spots were visualized by spraying with a solution of 0.2% ninhydrin in acetone and quantitative estimation of L-tryptophan was done using colorimetric method[12].

All chemical reagents used in this study were analytical grade (AR grade) and Borosil glass goods used were throughout the study.

Statistical analysis: Values were expressed as mean ±SEM, where n = 6. Data were analyzed using One way

ANOVA followed by Dunett's post-hoc multiple comparison test using a soft-ware Prism 4,0[13].

Reagents and glass goods: All the reagents used in this study were analytical (AR) grade and purchased from Merck. Double distilled deionizerd water and Borosil glass goods were used throughout the study.

at different time intervals is given in Fig. 1. Fig. 2 shows the pattern of productive variants developed with the treatment of different concentrations of ethylenemine at different time intervals.

Effect of UV irradiations:

In this way maximum productive variant *Bacillus subtilis*

Table 1. Mutational induction with ethylenemine and development of different mutants, their nutritional requirements and pattern of extracellular accumulation of different L-amino acids

Concentration(s) of ethylenemine (mmol/ml)	Exposure time (min)	Total number of auxotrophs	Extracellular L-amino acid pattern						Requirements of vitamins	*r*
			L-Tryptophan			Others				
			Similar to parents	More than parents	Less than parents	L-Methionine	L-Glutamic acid	L-Lysine		
170.3	10	21	07	02	02	03	02	05	17	02
	20	33	11	06	03	04	03	06	24	03
	30	11	01	04	–	02	04	–	11	02
	40	17	03	07	02	–	05	–	09	01
	50	22	07	05	03	03	01	03	17	02
	60	37	11	07	03	06	06	04	31	03
133.7	10	33	07	13	07	02	03	01	30	02
	20	31	14	06	07	02	–	02	31	06
	30	21	07	02	03	02	06	01	19	02
	40	24	06	08	03	04	02	01	17	03
	50	29	13	04	05	02	02	03	21	07
	60	19	07	04	03	02	–	01	13	02
63.2	10	06	02	03	–	01	–	–	06	01
	20	18	03	07	03	02	01	02	11	02
	30	29	13	05	02	03	02	04	22	06
	40	21	14	03	–	05	–	–	21	03
	50	20	05	07	–	03	04	02	21	02
	60	27	13	03	01	07	02	01	22	03

r is the frequency of spontaneous reversion $\times 10^4$.

Results

Effect of ethylenemine:

The regulatory mutant *Bacillus subtilis* GSX1159 accumulated very little amount of L-tryptophan (0.4 mg/ml) in the fermentation broth after 72 h of fermentation and then the productivity dropped further. So, it is not economically viable. In addition, its production also underwent product-mediated feed-back inhibition. The abilities of different mutants developed from mutagenic treatment of *Bacillus subtilis* GSX1159 with ethylelemine has been shown in Table 1. The % of survivors with different concentrations of ethylenemine

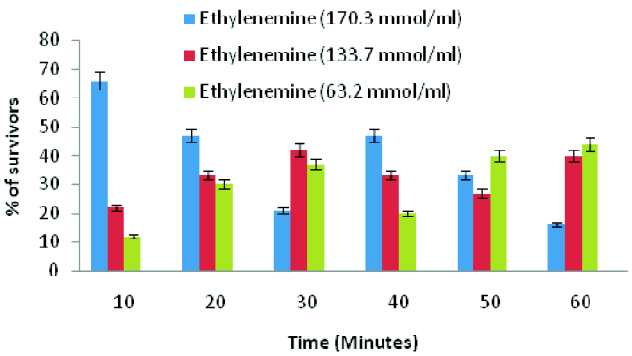

Fig. 1. Percentage of survivors with different concentrations of ethylenemine (Values were expressed as mean ±SEM, where n = 6).

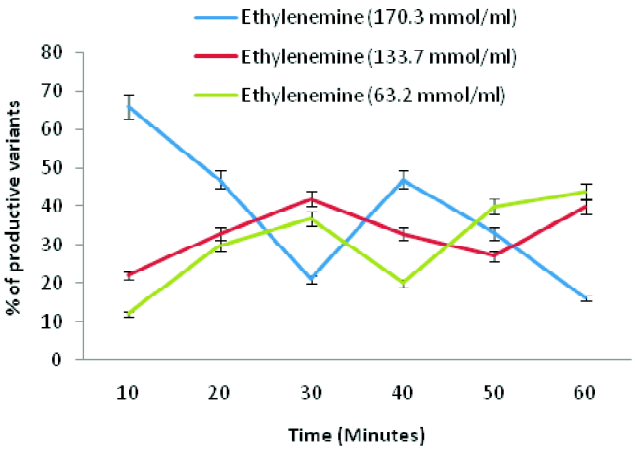

Fig. 2. Distribution of productive variants with different concentrations of ethylenemine at different time intervals (Values were expressed as mean ±SEM, where n = 6).

GSX1670 was developed which accumulated 3.3 mg/ml L-tryptophan in the fermentation broth which underwent further mutational study using UV irradiations for different time intervals to develop further improved strain. Different mutant strains developed from UV irradiations, their nutritional requirements and their pattern of amino acid secretion has been depicted in Table 2.

Maximum productive variants were obtained on 30 min exposure with UV irradiations, among which *Bacillus subtilis* GSX1794 accumulate 7.3 mg/ml L-tryptophan.

Development of multiple analog-resistant mutants of Bacillus subtilis GSX:

Bacillus subtilis GSX1794 was then subjected to develop multiple analog-resistant strain which was intended to employ for protoplast fusion with *Bacillus subtilis* GSX1794 to develop a multiple analog-resistant mutant of *Bacillus subtilis* for L-tryptophan production in large scale.

Different culture conditions for protoplast fusion were examined in this study. Development of spheroblasts and their disruption in hypotonic solution were used as indicators

Table 2. Nutritional requirements and the pattern of amino acid secretion by different mutants developed from *Bacillus subtilis* GSX1670 on UV irradiations at different time intervals

Periods of exposure (min)	Total number of auxotrophs	Extracellular amino acid pattern										*r*
		L-Tryptophan			Others				Nutrients required			
		Similar to parents	More than parents	Less than parents	L-Glutamic acid	L-Threonine	L-Lysine	L-Methionine	Yeast extract	Amino acids	Vitamins	
10	36	11	02	07	09	02	02	03	17	11	08	03
20	29	09	05	04	03	02	04	02	11	07	11	01
30	41	21	09	07	–	02	01	01	17	04	20	01
40	39	17	11	06	03	–	02	–	09	07	23	03
50	23	08	11	02	–	–	–	02	–	04	19	01
60	12	02	03	01	–	04	02	–	01	04	07	01

r is the frequency of spontaneous reversion $\times 10^4$.

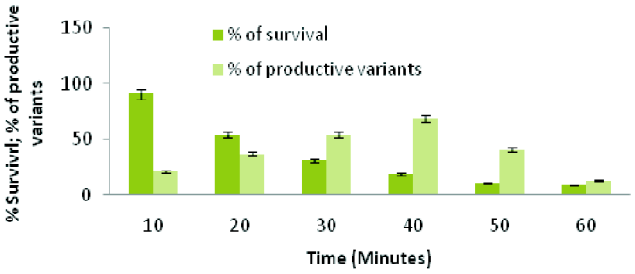

Fig. 3. The scoring of survivor and % of productive variants obtained by the treatment of UV irradiations (Values were expressed as mean ±SEM, where n = 6).

in protoplast fusion study. Cells were plasmolysed in hypotonic solution (0.01% NaCl) after 3 h suspension. Maximum stimulatory effect was obtained with 0.8% soft agar medium (Fig. 4). Colonies appeared after 2 days of incubation at 27°C and reaches optimum level after 7 days of incubation. Different concentrations of polyethylene glycerol or PEG (10–100%) were examined to study its effect on protoplast fusion (Fig. 4). Maximum fusion frequency was obtained with 40% PEG (Table 4). During incubation (8–48 h), maximum fusion frequency was resulted with 24 h of incubation (Fig. 5). The

Table 3. Development of multiple analog-resistant strain of *Bacillus subtilis* for L-tryptophan production

Analogs	Strain	Concentration(s) of analog(s) (mg/ml)				
		10	20	30	40	50
7-Azatryptophan	*Bacillus subtilis* GSX1794	+	–	–	–	+
Fluorotryptophan	*Bacillus subtilis* GSX1794	–	+	+	–	–
5-Hydroxytryptophan	*Bacillus subtilis* GSX1794	–	+	–	+	–
5-Aminotryptophan	*Bacillus subtilis* GSX1794	+	–	–	+	+
5-Bromotryptophan	*Bacillus subtilis* GSX1794	+	–	+	–	+
5-Methoxytryptophan	*Bacillus subtilis* GSX1794	+	+	+	+	
α-Methyltryptophan	*Bacillus subtilis* GSX1794	+	+	–	–	+
Methyltryptophan	*Bacillus subtilis* GSX1794	+	+	–	–	–

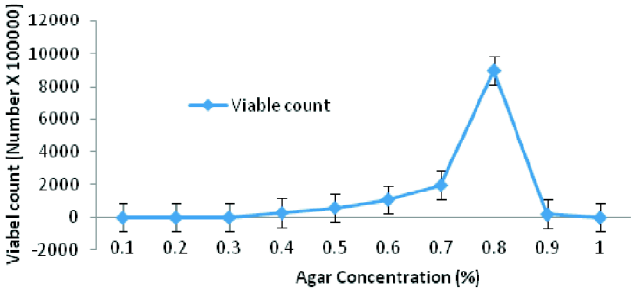

Fig. 4. Effect of soft agar concentration on viable count of bacterial cells (Values were expressed as mean ±SEM, where n = 6).

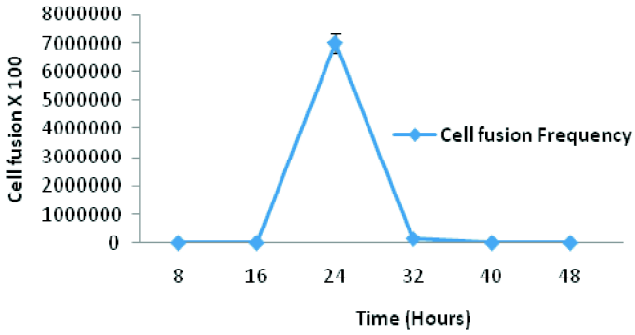

Fig. 5. Effect of incubation time on cell fusion frequency (Values were expressed as mean ±SEM, where n = 6).

Table 4. Effect of PEG on bacterial cell fusion frequency

PEG (%)	Cell fusion frequency
10	$11\times10^5 \pm 11\times10^2$
20	$7\times10^6 \pm 4\times10^2$
30	$4\times10^8 \pm 9\times10^3$
40	$17\times10^{11} \pm 3\times10^3$
50	$11\times10^6 \pm 4\times10^2$
60	$4\times10^6 \pm 7\times10^3$
70	$4\times10^5 \pm 11\times10^2$
80	$6\times10^3 \pm 3\times10^3$
90	$11\times10^3 \pm 11\times10^2$
100	$8\times10^2 \pm 8\times10^2$

Values were expressed as mean ±SEM, where n = 6.

Table 5. Effect of pH on bacterial cell fusion frequency

Time (h)	Cell fusion frequency
6.0	$6\times10^3 \pm 2\times10^2$
6.5	$11\times10^5 \pm 3\times10^3$
7.0	$17\times10^9 \pm 4\times10^3$
7.5	$11\times10^7 \pm 2\times10^2$
8.0	$7\times10^4 \pm 2\times10^2$

Values were expressed as mean ±SEM, where n = 6.

Table 6. Effect of sodium alginate on bacterial cell fusion frequency

Sodium alginate (%)	Cell fusion frequency
1.0	$11\times10^4 \pm 7\times10^3$
2.0	$7\times10^6 \pm 3\times10^3$
3.0	$17\times10^8 \pm 8\times10^3$
4.0	$21\times10^{11} \pm 2\times10^3$
5.0	$13\times10^7 \pm 6\times10^3$

effective fusion was resulted with pH 7.0 (Table 5). Sodium alginate, 4% (Table 6); sucrose, 2% (Table 7); $MgSO_4.7H_2O$, 0.05% (Table 8); EDTA, 0.4% (Table 9) and K_2HPO_4, 0.3% (Table 10) were proved to be the best for maximum protoplast fusion.

In this study a multiple analog-resistant high L-threonine yielding strain strain *Bacillus subtilis* GSX2480 was developed in this way which could accumulate 8.3 mg/ml L-tryptophan which was significantly higher ($p < 0.01$) compared to *Bacillus subtilis* GSX1159.

Table 7. Effect of sucrose on bacterial cell fusion frequency

Sucrose (%)	Cell fusion frequency
1.0	$3\times10^{9}\pm2\times10^{2}$
2.0	$18\times10^{12}\pm3\times10^{3}$
3.0	$17\times10^{11}\pm5\times10^{3}$
4.0	$11\times10^{8}\pm2\times10^{2}$
5.0	$7\times10^{6}\pm3\times10^{2}$

Values were expressed as mean ±SEM, where n = 6.

Table 8. Effect of $MgSO_4.7H_2O$ on bacterial cell fusion frequency

$MgSO_4.7H_2O$	Cell fusion frequency
0.01	$11\times10^{4}\pm3\times10^{2}$
0.02	$7\times10^{5}\pm2\times10^{3}$
0.03	$13\times10^{8}\pm4\times10^{3}$
0.04	$13\times10^{11}\pm2\times10^{2}$
0.05	$4\times10^{13}\pm2\times10^{2}$
0.06	$11\times10^{9}\pm4\times10^{2}$

Values were expressed as mean ±SEM, where n = 6.

Table 9. Effect of EDTA on bacterial cell fusion frequency

EDTA	Cell fusion frequency
0.1	$8\times10^{3}\pm2\times10^{2}$
0.2	$15\times10^{6}\pm3\times10^{3}$
0.3	$21\times10^{9}\pm7\times10^{3}$
0.4	$13\times10^{14}\pm2\times10^{2}$
0.5	$7\times10^{11}\pm6\times10^{2}$
0.6	$8\times10^{6}\pm5\times10^{2}$

Values were expressed as mean ±SEM, where n = 6.

Table 10. Effect of K_2HPO_4 on bacterial cell fusion frequency

$MgSO_4.7H_2O$	Cell fusion frequency
0.01	$3\times10^{3}\pm3\times10^{2}$
0.02	$11\times10^{6}\pm7\times10^{3}$
0.03	$21\times10^{15}\pm5\times10^{3}$
0.04	$13\times10^{11}\pm5\times10^{2}$
0.05	$9\times10^{13}\pm7\times10^{2}$
0.06	$6\times109\pm4\times10^{2}$

Values were expressed as mean ±SEM, where n = 6.

Discussion

With the increasing demands for L-amino acids in world market, L-amino acids hold the place among the most important products in industrial biotechnology. In recent years, a tremendous progress the development of tailor-made strains for amino acids, intensively driven from metabolic engineering holding up gradation of strain engineering into an optimization on a globe scale. This concept seems great for efficient L-amino acid fermentation, demanding for a global modification of metabolic fluxes – a challenge with regard to exceptionally complexity of underlying metabolic network, superimposed by different levels of metabolic and transcriptional control.

Over the last 70 years, the knowledge of microbial physiology, with the advancement of molecular biology, increased the efficiency of L-amino acid production by fermentation. The progress had a significant economic role in the field of fermentation biotechnology and L-amino acid production was commercialized using different mutant strains developed from wild or regulatory mutants by induction of mutagenic treatments followed by selection. The processes have been accelerated by combination between intracellular metabolite sensing and synthetic biological approaches[14]. The current practice of new strain development by mutagenic treatments followed by selection of high yielding strain is well established and these procedures can be optimized in terms of types and dosage of mutagens[15].

Riccardi *et al*. (1981) developed new multiple analog-resistant mutants of *Spirulina platenis* and subsequently tested for their potency in amino acid production. They observed one resistant to azetidine-2-carboxylic acid overproduced proline where as one resistant to fluorotryptophan and one resistant to ethionine did not over accumulate any of the tested L-amino acids[16]. Ikeda and his co-workers developed a new mutant strain of *Corynebacterium glutamicum* for L-lysine production[17]. A homoserine auxotroph was developed from *Corynebacterium glutamicum* by using NTG as mutagen[18]. Ganguly and Banik (2010) developed a new biotin-auxotrophic mutant *Micrococcus glutamicus* AB_{100} for L-glutamic acid production from a regulatory mutant *Micrococcus glutamicus* AB_{100} by induced mutation using ethylenemine and UV irradiation followed by selection[12]. Roy and Mukhopadhyay (2011) treated *Aureobacterium flavescens* by N-methyl-N-nitro-N-nitrosoguanidine to develop tyrosine plus phenylalanine double auxotrophic mutants among which 11A39 and 11A17 were selected for tryptophan production in minimal salt medium. These strains were then further be subjected to tryptophan analogues *p*-fluorotryptophan and 5-methyltryptophan treatments to develop multiple tryptophan analog-resistant strain[19]. Ganguly *et al*. (2014) developed another L-methionine mutant *Corynebacterium glutamicum* X300 from an L-glutamic acid produc-

ing mutant *Corynebacterium glutamicum* X1 using EMS and UV irradiations followed by development of multiple L-methionine analog-resistant strain and protoplast fusion[13].

Conclusion

In this present study, we developed a multiple analog-resistant, high L-tryptophan yielding strain *Bacillus subtilis* GSX2480 from *Bacillus subtilis* GSX1159. *Bacillus subtilis* GSX2480 accumulated up to 8.3 mg/ml L-tryptophan in the fermentation broth which was 64.3% higher than *Bacillus subtilis* GSX1159.

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