Kor. J. Appl. Microbiol. Bioeng
Vol. 11, No. 3, 175~180(1983)

Lysine Production by Thialysine Resistant Mutant of *Candida utilis* (I)

— Isolation of High Lysine Excreting Mutant of *Candida utilis* —

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(Received May 21, 1983)

*Candida utilis*의 Thialysine 耐性変異株에 依한 Lysine生産(I)

—*Candida utilis*의 Lysine을 生産하는 Thialysine耐性変異株의 分離—

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(1983년 5월21일수리)

Thialysine significantly inhibited the growth of wild type strain *Gandida utilis* NCYC-359. In the absence of thialysine, the culture reached stationary phase after 24hr, however, in the presence of 0.5% thialysine, the culture reached stationary phase after 40hr, respectively. Effect of amino acid or vitamin was investigated on recovery of the growth of wild type strain from thialysine inhibition. Glycine, methionine, arginine and tryptophan recovered growth inhibition by thialysine to some extent. However, vitamins were inert. Especially, lysine at one eighth concentration of thialysine recovered almost fully the growth inhibition.

Thialysine resistant mutants were induced from the parent strain of *Candida utilis* NCYC-359 by NTG treatment. Colonies of thialysine resistant mutants were obtained on agar minimal medium supplemented with 0.1-0.5% thialysine. The frequency of thialysine resistant mutants induced by the first mutation was the highest at 0.1%. The wild strain produced no appreciable lysine extracellularly. However, almost thialysine resistant mutants excreted appreciably. Lysine excretion increased after repeated mutation. Finally, of the thialysine resistant mutants induced by NTG, *Candida utilis* TRN-4006 was obtained. This strain excreted lysine (400μg/ml) into the medium with a concomitant decrease of lysine in the intracellular pool.

Lysine is one of the essential amino acid needed for balanced nutrition and development of human beings[1]. Because of the importance of lysine in nutrition and the lack of adequate lysine in cereal foods, attention has been given to the production of lysine from independent sources for use as supplement to animal and human goods[2].

Two distinct pathways exist in nature for the biosynthesis of lysine. Bacteria, higher plants, most algae and certain lower fungi utilize the diaminopimelic acid pathways[3] while yeast, other higher fungi and blue green algae utilize the aminoadipic acid pathways[4]. Lysine production in bacteria has been extensively investigated[5-9], and *Corynebacterium glutamicum* is used for the industrial production of lysine[8].

Relatively few investigations have been carried out using yeasts[10-12] for the production of lysine. We report here more detailed studies of the effect of thialysine on the growth of the parent strain and induction of thialysine resistant mutant of the parent strain producing a high concentration of lysine extracellularly.

Materials and Methods

Microorganisms and media

Candida utilis NCYC-359 and thialysine resistant mutant, TRN-4006, of *Candida utilis* were used in this study. The composition of minimal medium, fermentation medium and complete medium were described in previous

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paper[12].

Induction of thialysine resistant mutants

Mutation experiments were carried out as described previously[12].

Analytical methods

After cells were removed by centrifugation at 3,000rpm for 10min, the supernatant solution was used for assay of extracellular lysine. Free intracellular lysine was extracted essentially according to the method of Kimura[13], as follows. The cells were rapidly washed twice with cold saline and suspended into 4ml of distilled water followed by heating in boiling water for 10min. The cellular extracts obtained by centrifugation were used to determine lysine. Intracellular and extracellular lysine were determined by a modification[12] of the acid ninhydrin method[14] or by paper chromatography[12].

Growth experiments

The fermentation medium (4ml) in a test tube was inoculated with a loopful of cells grown on a stock medium, and incubated at 29°C for 24hr with reciprocal shaking. The cells harvested by centrifugation were washed twice with sterilized saline and suspended in the same saline to give 1.0 of turbidity at 550nm. A 0.1ml aliquot of the suspension was transferred into various media placed in test tubes, and incubated at 29°C on a reciprocal shaker. The cell growth was measured turbidimetrically at 550nm with Shimadzu spectronic 20 photometer.

Lysine production

For screening, a loopful of mutant cells grown on minimal medium plus 0.1% thialysine was inoculated into 4ml of fermentation medium in a test tube and incubated at 29°C for 96hr with reciprocal shaking. Growth of TRN-4006 strain to accumulate lysine was also caried out as the same culture condition as above with fermentation medium.

Chemicals

Thialysine (S-2-Aminoethyl-L-Cysteine, Thiosine or AEC) and N-methyl-N'-nitro-N-nitrosoguanidine (NTG) were purchased from Sigma Chemical Co. Ninhydrin from E. Merck Co. All other chemicals were analytical grade reagents and commercially available.

Results and Discussion

Effect of thialysine on the growth of wild type of *Candida utilis*

Thialysine has been reported to inhibit growth of yeast[10-12,15]. In order to investigate thialysine effect on the growth of the parent strain of *Candida utilis*, a 0.1ml aliquot of the suspension was inoculated into the minimal medium containing various concentration of thialysine in the range of 0.01-0.5% and incubated at 29°C with a reciprocal shaker. At 8hr intervals, optical density of each culture was measured at 550nm. The result was shown in Fig. 1. The growth of wild type strain NCYC-359 was significantly inhibited by thialysine. The initiation of growth was delayed with increasing concentrations of thialysine. Growth inhibition was proportional to concentrations of thialysine in the range of 0.01-0.5%. The organism could not grown within about 24hr when 0.5% thialysine was added.

The growth inhibitory of thialysine is explained by the feedback inhibition and repression of homocitrate synthase, the first enzyme of the aminoadipic acid pathway for the biosynthesis of lysine, by thialysine[16,17]. This regulation of homocitrate synthase (and possibly other enzymes of lysine biosynthesis) by thialysine causes the wild type cells to limit the synthesis of lysine and a subsequent inhibition of growth occurs due to the lysine limiting conditions.

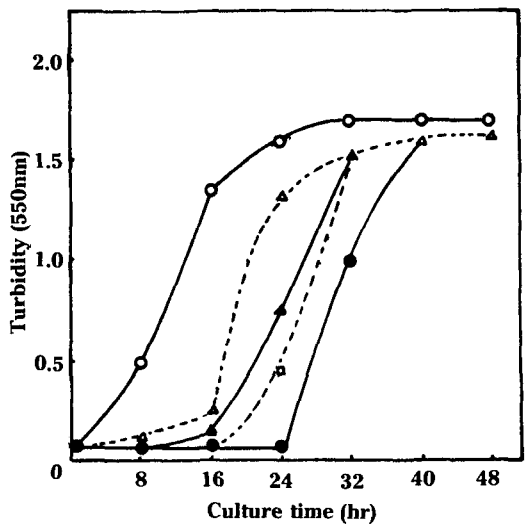

Fig. 1. Effect of Thialysine on the Growth of Wild type strain of *Candia utilis* NCYC-359

A portion of the cell suspension was inoculated into the minimal medium supplemented with various concentrations of thialysine and incubated at 29°C with a reciprocal shaker. The turibidity was measured at 550nm. The concentrations of thialysine in the medium are as follows: o—o, none; ▲·····▲, 0.01% ■—■, 0.05%: □·····□, 0.1%: ●—●, 0.5%.

Table 1. Recovery of the Growth of ***Candida utilis*** **NCYC-359 from Thialysine Inhibition by Amino Acid and Vitamin**

Amino Acid/ vitamin (0.1%)	Recovery of growth (%)	Amino Acid (0.1%) Vitamin	Recovery of growth (%)
Glycine	65	Glutamine	35
Alanine	0	Lysine	100
Aspartate	0	Arginine	85
Aspargine	0	Histidine	65
Valine	0	Phenylalanine	0
Leucine	0	Tyrosine	0
Isoleucine	0	Tryptophan	68
Serine	0	Proline	0
Methionine	65	Cystine	0
Threonine	0	Ornithine	0
Glutamate	0	Cysteine	0
Inositol	0	Biotin	0
Pyridoxine-HCl	0	Nicotinic acid	7
Ca-pantothenic acid	6	P-aminobenzoic acid	15
Folic acid	0	Asscorbic acid	8
Thiamine-HCl	0	Riboflavin	0

An aliquot of the seed suspension was inoculated into the minimal medium containing 0.1% thialysine and 0.1% each amino acid or 0.1% thialysine and 0.1% each vitamin. Relative growth was represented as % to the growth in the absence of thialysine and amino acid or vitamin. The turbidity was measured at 550nm after cultivation at 29°C for 18hr.

Reversal of thialysine inhibition by amino acids and vitamins

The effect of amino acids and vitamins was examined to eluciate whether inhibition by thialysine was related to amino acid or vitamin metabolites. A 0.1ml aliquot of the suspension was inoculated into the minimal medium containing 0.1% thialysine and 0.1% each amino acid or vitamin, and cultivated at 29°C for 18hr with a reciprocal shaker. Relative growth was represented as % to the growth in the absence of thialysine and each amino acid or vitamin. The turbidity was measured at 550nm. Table 1 showed that the growth inhibition by thialysine was recovered to some extent by addition of glycine, methionine, glutamine, arginine, histidine and tryptophan but vitamins were not at all or only slightly effective. Especially, the protective effect of lysine was much higher than of other amino acids and lysine of one eighth concentration of thialysine restored almost fully the growth inhibition by 0.1% thialysine (Fig. 2).

The specific recovery of thialysine inhibition by lysine suggests that thialysine interfers directly with lysine biosynthesis. Therefore, a regulatory mutant which is resis-

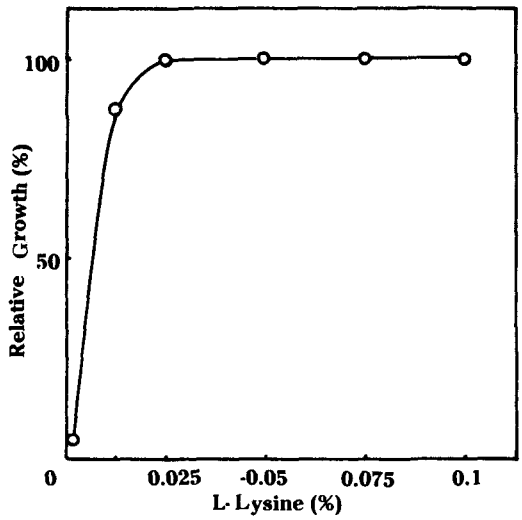

Fig. 2. Reversal of Thialysine Inhibition by L-Lysine.

A portion of the seed suspension was inoculated into the minimal medium containing 0.1% thialysine and 0.125, 0.025, 0.05 and 0.1% lysine. Relative growth was represented as % to the growth in the absence of thialysine and lysine. The turbidity was measured at 550nm after 18hr incubation.

tant to thialysine, a potent antimetabolite of lysine, is probably suitable for lysine overproduction.

Induction of thialysine resistant mutants from wild type strain and their lysine productivity

In isolating the high lysine excreting mutant from wild type, an attempt was made to derive thialysine resistant mutants. The parent strain was treated with NTG, mutagenic treatment was performed according to procedures described in previous paper[12]. As shown in Table 2, thialysine resistant mutants grown within 18 hr incubation at 29°C decreased with increasing concentrations of thialysine in the range of 0.1 to 0.5%. The frequency of mutants induced by the first mutation at 0.1% thialysine was 4-fold times as compared with 0.03% at 0.5% thialysine.

The mutants were cultured to examine the productivity of lysine with fermentation medium. The results are shown in Table 3, and although thialysine was in high concentration in medium, good excretors were not induced. No clear relationship was found between lysine excreting ability of mutants and their resistibility to thialysine.

Table 2. Induction of Thialysine Resistant Mutants from Parent Strain.

Conc. of AEC in the medium (%)	Surviving cell* (cells/ml)	Resistant colonies** counted (cells/ml)	Frequency (%)
0.1	9,000	10	0.11
0.3	2,000	0.55	0.027
0.5	450	0.15	0.033

* Surviving cells in the Suspension treated with NTG except auxotrophs, ** Colonies were counted after incubation at 29/C for 18hr.

Table 3. Lysine Productivity of Thialysine Resistant Mutants induced the first Mutation.

Conc. of AEC in the medium (%)	Total number of mutants*	L-lysine produced (mg/ml) and number of lysine producing mutants**		
		0.01	0.1-0.2	0.2
0.1	200	155	45	0
0.3	9	7	2	0
0.5	10	7	3	0

* The resistant mutants were those obtained in the first mutation, ** The mutants were cultured in fermentation medium in a test bube at 29°C for 4 days.

Genealogy of lysine producers from wild type strain and extracellular amino acids accumulated by mutant strain TRN-4006

The lysine productivity of thialysine resistant mutant (TRN-13) obtained from first mutation was increased by further mutation. Among many resistant mutants induced by four fold mutations, TRN-4006, the most potent lysine producer excreted 0.4mg of lysine per ml (in fermentation medium) when the mutant cells were cultured in 4ml of fermentation medium in a test tube with a reciprocal shaker at 29°C for 4 days, whereas the wild type strain produced no appreciable amount of lysine extracellularly (Fig. 3).

Free amino acids accumulated by TRN-4006 were analyzed by an automatic amino acid analyzer JLC-6AH. Lysine was predominantly found in culture broth. Other amino acids were not produced or only negligibly as shown in Table 4.

Growth response of the wild type NCYC-359 and mutant TRN-4006 strains on thialysine

Growth inhibition of the parent and the mutant by thialysine was investigated. A 0.1ml aliquot of the suspension was inoculated into the minimal medium containing 0.1 to 0.3% thialysine and cultivated at 29°C for 16hr with a reciprocal shaker. Relative growth was represented as % to the growth in the absence of thialysine. The turbidity was measured at 550nm. In the case of the parent strain, the growth was inhibited by 0.3% thialysine about 90%

		Lysine Produced (μg/ml)
C. utilis NCYC-359		
TRN-13	0.1%-AEC^r	100
TRN-103	0.1%-AEC^r	250
TRN-310	0.3%-AEC^r	340
TRN-4006	0.3%-AEC^r	450

Fig. 3. Genealogy of L-Lysine Producers from *Candida utilis* NCYC-359

very strongly. In contrast, high lysine excreting mutant TRN-4006 was slightly inhibited by 0.3% thialysine about 25% (Fig. 4). It is believed that the feedback insensitivity and the depression in lysine excreting thialysine resistant mutant would account for the production and excretion of excess lysine[16,17].

Time course of lysine accumulation by strain TRN-4006

An example of the time course of lysine production by mutant TRN-4006 was presented in Fig. 5. During the course of fermentation in fermentation medium, the amount of extracellular lysine increased with increased cell growth and reached maximum in the last stationary phase. From 10% of glucose, 450 μg/ml of extracellular lysine was finally obtained at 72hr of cultivation. Intracellular lysine was accumulated by about 20mg per g of cells (dry wt.) in

Table 4. Extracellular Amino Acids Accumulated by strain TRN-4006

Anino acids produced	Amount of products (μg/ml)
Lysine	388
Histidine	14
Arginine	trace
Aspartate	trace
Threonine	trace
Serine	33
Glutamate	34
Glycine	trace
Alanine	75
Valine	trace
Methionine	32
Isoleucine	trace
Leucine	trace

The mutant was cultured in fermentation medium in a test tube with a reciprocal shaker at 29°C for 4 days. Amino acids accumulated were analyzed with an automatic amino acid analyzer JLC-6AH.

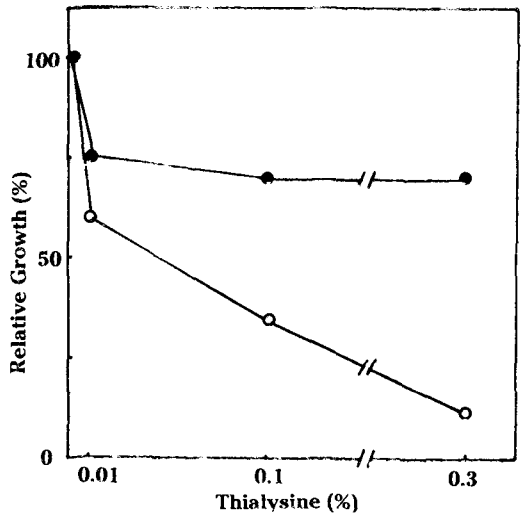

Fig. 4. Growth Response of the Wild type and the Mutant Strains on Thialysine

A portion of the seed suspension was inoculated into the minimal medium containing 0.01, 0.1 and 0.3% of thialysine. Relative growth was represented as % to the growth in the absence of thialysine. The turbidity was measured at 550nm after 16hr incubation.

●--●, TRN-4006; o——o, NCYC-359.

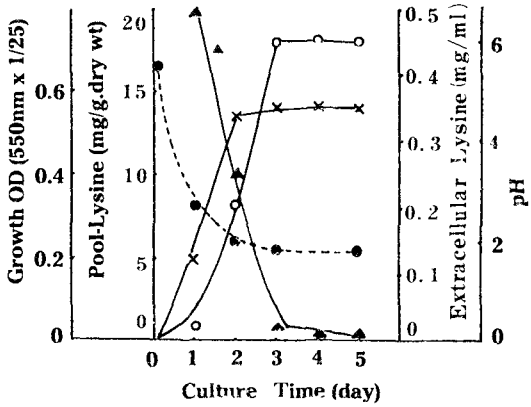

Fig. 5. Time Course of Lysine Accumulation by strain TRN-4006

The mutant cells were cultured in the fermentation medium in a test tube with a reciprocal shaker at 29°C. The determination of pool-lysine was performed after extraction for 10 min of 200mg wet weight yeast culture with 4ml hot water. Dry cell weight was determined by drying wet cells in a oven at 80°C.

▲—▲, pool-lysine; o—o extracellular lysine; x—x, growth OD; ●—●, culture pH.

early to middle logarithmic phase, and then rapidly decreased to about 50% of the highest concentration at the beginning of the stationary phase.

요 약

Lysine의 analogue인 thialysine은 野生株 *Candida utilis* NCYC-359의 生育을 強力하게 沮害했으며 0.1%에서는 18時間까지 9.5%에서는 24時間까지 本 酵母의 誘導期를 지연시켰다.thialysine에 의한 野生株의 生育沮害의 回復에 미치는 아미노산 및 비타민의 影響을 調査한 結果 glycine, methionine, arginine, histidine 그리고 tryptophan等은 어느程度 効果가 있었으며 비타민은 거의 효과가 없었다. 특히 최소배지에 L-lysine 을 thialysine의 1/8량만 添加해도 thialysine에 의한 生育沮害는 거의 일어나지 않았다. NTG 1 회 처리시 thialysine에 대한 耐性을 나타내는 頻度를 보면 최소배지에 thialysine 0.1% 添加時 그 頻度가 0.11%, 0.5%時 0.03%로 나타났으며 이 耐性変異株에 依한 lysine 生産能을 検討한 結果 최소배지에 添加된 thialysine의 濃度와 lysine 生産能과는 뚜렷한 関係가 成立되지 않았으며 NTG를 反復処理하여 菌体外로 lysine을 多量 分泌하는(450μg/ml) thialysine耐性変異株 *Candida utilis* TRN-4006을 최종적으로 分離選別하였다.

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