

DESCRIPTION

A THERMOPHILIC ANAEROBIC BACILLUS AND A METHOD FOR PRODUCING ETHANOL BY USING THE SAME

FIELD OF THE INVENTION

A strain for producing ethanol and an acclimatizing method for improving its tolerance to high-concentration sugar, as well as a method for producing ethanol by using this strain. The present invention relates to a method for producing ethanol by fermenting a mutant strain of the thermophilic anaerobic bacillus (thermoanaerobacterium aotearoense SCUT27/ Δ ldh) using glucose and xylose as main raw materials.

BACKGROUND OF THE INVENTION

Ethanol is a new type of clean fuel, and is an important direction for the development and utilization of renewable energy. Development of bio-ethanol is a general trend. The second-generation bio-ethanol is made from plant fibers through pretreatment, hydrolysis of inorganic acid or cellulase, and then biological fermentation. Hydrogen energy is attracting the attention of researchers because of its cleanness, high energy density, a variety of production methods and other characteristics.

The main obstacle to the production of ethanol from cellulose substances as raw

materials is the high cost. Fermentation of the thermophilic anaerobic bacterium thermoanaerobacterium can well solve the problem. Producing chemicals with the above-mentioned bacterium as the fermentation strains has the following advantages (O-Thong, S. et al., International Journal of Hydrogen Energy, 2008. 33(4): 1204-1214): (1) Reducing the amount of cellulase added in the fermentation process of the cellulose raw materials. This is because the strain itself is a kind of hemicellulolytic microorganisms, and can hydrolyze xylan at a high temperature to ferment and produce a variety of chemicals, thereby reducing the demand for cellulase. (2) The high-temperature continuous fermentation production process is conducive to the separation and purification of ethanol. (3) Ethanol can be produced by fermentation of crude materials. As purebred fermentation has higher requirements for sterilization of the equipment in the industrial-scale fermentation production process of ethanol, there is a substantial increase in economic costs. However, for the fermentation production with thermophilic bacteria, as most of the room-temperature microorganisms are difficult to be produced at high temperatures, the requirements for sterilization of the fermentation process are not high, thereby further reducing the production cost. (4) High-temperature organic wastewater can be used as fermentation raw materials, which reduces the production cost on one hand and reduces the environmental pressure on the other hand.

In the applicant's previous study, a thermophilic anaerobic bacillus was screened (Li, S., et al., Bioresource Technology, 2010,101(22): 8718-8724, Cai, Y., et al., Enzyme and Microbial Technology, 2011. 48(2): p. 155-161), which could effectively

utilize glucose, xylose and other substrates to accumulate ethanol, hydrogen and so on by fermentation. The lactate dehydrogenase gene was knocked out of it through genetic modification, and the metabolic pathway was transformed, such that the yields of the mutant H₂ and ethanol were increased by 2.7 and 2.4 times, respectively. However, the strain was affected by the high sugar osmotic stress in the presence of a high-concentration sugar substrate, such that the growth lag period is too long, the fermentation cycle is too long, and the production cost is substantially increased. The high-concentration sugar substrate tolerance is necessary for producing ethanol by the simultaneous saccharification and fermentation of the cellulosic biomass.

From an industrial point of view, it is preferred that a thermophilic anaerobic bacillus strain tolerant to high sugar is screened, tolerance of the thermophilic anaerobic bacillus to the sugar substrate concentration is improved and the properties are stable, and the thermophilic anaerobic bacillus can be applied to the high-concentration sugar substrate fermentation, shorten the fermentation lag period and reduce the process amplification cost, such that the thermophilic anaerobic bacillus strain is more suitable for industrialized production.

CONTENTS OF THE INVENTION

A purpose of the present invention is to provide a strain for producing ethanol and a method for producing ethanol by using the same.

The starting strain of the thermophilic anaerobic bacillus thermoanaerobacterium aotearoense SCUT27/ Δ *ldh* P8G0 (erythromycin resistance)

was cultured in such a manner that the concentration of the sugar substrate was gradually increased, and this process was repeated for several generations, until the bacterium had an OD₆₀₀ value up to 0.6 with stable properties in 6 h after inoculation at this concentration. Adding 1 mL of the bacterial solution to an isolation medium, culturing in an anaerobic incubator for 2-3 days, picking out a single colony, and culturing in a fermentation medium for 240 h, with the final concentration of ethanol up to 28.11 g/L and the conversion rate up to 1.22 mol/mol, and finally obtaining a mutant strain P8G3#4 (erythromycin resistance) which could grow normally and go down to posterity in the presence of a high-concentration substrate.

The above mutant strain is *thermoanaerobacterium aotearoense* P8G3#4, which has been preserved in China General Microbiological Culture Collection Center (referred to as CGMCC) under an accession number of CGMCC No. 9000 since the date of deposit of April 3, 2014, with the preservation address being Institute of Microbiology, Chinese Academy of Sciences, No. 3, 1 Beichen West Road, Chaoyang District, Beijing, P.R. China.

The P8G3#4 strain is a gram-positive bacterium in the shape of a long rod, having a diameter of about 0.6-1.0 μm and a length of about 2-15 μm , and making a tumbling motion observed under a microscope. It is a strict anaerobic bacterium, has an optimal growth temperature of 55°C with pH 6.3, and can growth by using a variety of sugar substrates, such as glucose, xylose, mannose, fructose, galactose, arabinose, trehalose, xylan, and dextran. This strain has the following genetic characteristics: It is a lactic acid metabolism-blocking mutant strain with

erythromycin resistance, with the erythromycin resistance concentration at 20-100 ng/ μ l. It can withstand the sugar substrate of a concentration of 120-150 g/L, with the intracellular UDP-Gal (UDP- α -D-galactose) and 1-(5-phosphate-D-ribose)-5-amino-4-imidazolecarboxylic acid (CAIR) accumulated and increased; and the expression of mtGDP in the metabolic pathway of glycerophosphatide was downregulated.

A method for producing ethanol by using the strain according to claim 1: First preparing a seed liquid of the thermophilic anaerobic bacillus CGMCC9000, then transferring this seed liquid to a fermentation medium in an inoculation amount of 10%-15% w/w, and agitating and culturing under an anaerobic condition at a culture temperature of 45°C-60°C for 50-250 h, and finally separating ethanol from the fermentation liquor.

The total sugar concentration of the fermentation medium is 80-150 g/L.

The initial pH of the fermentation medium is 5.5-6.5, and the stirring speed is 120-200 rpm.

The initial pH of the fermentation medium is 6.3.

The fermentation medium contains the following components in g/L: 80-100 of glucose, 40-60 of xylose, 1-10 of urea, 1-5 of a yeast extract, and 0.5-3 of ammonium chloride.

The fermentation medium also contains the following components in g/L: 1-4 of tripotassium citrate, 0.5-3 of citric acid monohydrate, 0.5-3 of sodium sulfate, 0.5-3 of potassium dihydrogen phosphate, 1-4 of sodium bicarbonate, 0.5-3 of magnesium

chloride hexahydrate, 0.05-0.5 of ferrous chloride tetrahydrate, 0.05-0.4 of calcium chloride dihydrate, 0.5-3 of cysteine hydrochloride monohydrate, 0-0.1 of pyridoxamine dihydrochloride, 0-0.01 of p-aminobenzoic acid, 0-0.01 of D-biotin, 0-0.01 of vitamin B12, and 0-0.01 of vitamin B1.

The seed liquid was prepared as follows: Putting the thermophilic anaerobic bacillus CGMCC9000 in a seed medium, and culturing at 45°C-60°C at a rotational speed of 100-250 rpm for 8-24 h.

The seed medium contains the following components in g/L: 2-4 of glucose, 2-4 of xylose, with the other components the same as those in the fermentation medium. The components are mainly 1-10 of urea, 1-5 of a yeast extract, and 0.5-3 of ammonium chloride. Preferably, it also contains the following components in g/L: 1-4 of tripotassium citrate, 0.5-3 of citric acid monohydrate, 0.5-3 of sodium sulfate, 0.5-3 of potassium dihydrogen phosphate, 1-4 of sodium bicarbonate, 0.5-3 of magnesium chloride hexahydrate, 0.05-0.5 of ferrous chloride tetrahydrate, 0.05-0.4 of calcium chloride dihydrate, 0.5-3 of cysteine hydrochloride monohydrate, 0-0.1 of pyridoxamine dihydrochloride, 0-0.01 of p-aminobenzoic acid, 0-0.01 of D-biotin, 0-0.01 of vitamin B12, and 0-0.01 of vitamin B1.

The present invention has the following beneficial effects:

(1) The thermophilic anaerobic bacillus P8G0, which was obtained from our laboratory by screening and constructing, was used as the parent strain, which was acclimatized to possess the high sugar substrate tolerance according to a conventional method, thus obtaining the mutant strain P8G3#4. The mutant strain has the following

genetic characteristics: It is of the L-lactate dehydrogenase deficiency type, has the erythromycin resistance, and has a sugar substrate concentration tolerance range of more than 120-150 g/L, with the intracellular UDP-Gal (UDP- α -D-galactose) and 1-(5-phosphate-D-ribose)-5-amino-4-imidazolecarboxylic acid (CAIR) accumulated and increased; the expression of mtGDP in the metabolic pathway of glycerophosphatide was downregulated.

(2) Fermenting in a 5 L fermentor with the mixed sugar of 120 g/L glucose and xylose as the substrate to produce ethanol, with the lag period shorten to about 1/4 of the original, the final concentration of ethanol up to 28 g/L and the conversion rate up to 1.2 mol/mol. The present invention provides screened mutants, which can rapidly ferment and accumulate the target product ethanol by using the high sugar substrate, producing less by-products and making the process amplification relatively easy, suitable for industrialization production.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the growth curves of the strain P8G3#4 with high sugar tolerance after acclimation and the starting strain P8G0. a) The growth of P8G0 at different carbon source concentrations, -■- 30 g/L, -●- 81 g/L, -▲- 120 g/L; and b) the growth of P8G3#4 at different carbon source concentrations, -□- 30 g/L, -○- 81 g/L, -△- 120 g/L; wherein the sugar substrate is glucose and xylose (2:1, g:g).

Fig.2 is a schematic view showing the significant enrichment of the cell metabolic pathway function of P8G3#4 and P8G0. A) An ABC transporter system,

wherein the right figure shows the lactose/L-arabinose transporter in the oligosaccharide and polyol transport system, and the left figure shows the branched chain amino acid transporter in the phosphate and amino acid transport system; B) two-component regulation system (two-component system), GlnL regulation system; C) the Lac family in the phosphotransferase system (PTS), wherein the right figure shows the transport of lactose, and the left figure shows the transport of cellobiose; and D) DNA replication related elements. The gray boxes represent cumulative metabolites.

DETAILED DESCRIPTION OF THE EMBODIMENTS

Example 1

Acclimation and Isolation of strain

The high sugar-tolerant thermophilic anaerobic bacillus strain of the present invention can be obtained as follows:

In the present invention, the starting strain used for screening is the laboratory-screened, mutated and conserved thermophilic anaerobic bacillus (*thermoanaerobacterium aotearoense* SCUT27/ Δldh), which is here labeled P8G0.

Screening medium: Glucose: 80-100 g·L⁻¹; xylose: 40-60 g·L⁻¹; (NH₄)₂SO₄: 1-5 g·L⁻¹; MgCl₂·6H₂O: 1-5 g·L⁻¹; KH₂PO₄: 0.5-5 g·L⁻¹; K₂HPO₄: 0.5-5 g·L⁻¹; CaCl₂·2H₂O: 0.1-2.0 g·L⁻¹; Na-β-glycerophosphate: 2-10 g·L⁻¹; FeSO₄·7H₂O: 0.0001-0.001 g·L⁻¹; a yeast extract: 2-10 g·L⁻¹; cysteine hydrochloride monohydrate (C₃H₇NO₂S·HCl·H₂O): 0.1-2.0 g·L⁻¹; resazurin: 0.001-0.01 g·L⁻¹; agar powder: 5-20

g·L⁻¹; and erythromycin: 20-100 ng/μl.

The starting strain P8G0 was first cultured in an anaerobic shake flask with 81 g/L screening medium (glucose and xylose at a mass ratio of 2:1); after the growth absorbance value OD₆₀₀ of the strain was 2.0 or more, transferring to a new anaerobic shake flask medium, and repeating this process for several generations, until the strain P8G1, having OD₆₀₀ up to 0.6 and stable properties in 6 h after inoculation at this concentration, was obtained;

by the same method as in step (1), the strain P8G2 was obtained under the condition of 102 g/L glucose and xylose (2:1, g:g);

by the same method as in step (1), the strain P8G3 was obtained under the condition of 120 g/L glucose and xylose (2:1, g:g); and

a single colony P8G3#4 was obtained by plate culture.

Example 2

Strain identification of P8G3#4 after acclimation

In order to identify the difference of strains before and after acclimatization, the difference of metabolic genes between P8G0 and P8G3#4 was analyzed by the transcriptomics method, and differential genes were subjected to cluster analysis, so as to identify the reasons on the gene expression level that lead to the growth and metabolism differences between the two strains P8G0 and P8G3#4.

The transcriptome sequencing analysis was performed on the starting strain thermophilic anaerobic bacillus (*thermoanaerobacterium aotearoense* SCUT27/*Aldh*) P8G0 and the screened mutant strain P8G3#4 that was able to grow normally in the

presence of the high-concentration substrate. Analyzing differential expression genes in the two samples, with the screening criteria being $|\log FC| \geq 1$ (i.e., a double difference); analyzing differential expression genes in the two samples, with P8G0 and P8G3#4 having altogether 69 genes that have significant differences in the expression abundance; compared with the starting strain P8G0, there were respectively 35 and 24 genes that were up-regulated and down-regulated in the early stage of logarithmic growth of the strain, and P8G3#4 was different from P8G0 in transcriptome.

By the KEGG metabolic pathway analysis (Fig. 2), a total of 27 genes were annotated to 20 signaling pathways, with more genes annotated to the following pathways or systems: a purine metabolic pathway (Purine metabolism), an ABC transporter system (ABC transporters), a galactose metabolic pathway (Galactose metabolism), amino sugar and nucleotide sugar metabolic pathways (Amino sugar and nucleotide sugar metabolism), a nitrogen metabolism pathway (Nitrogen metabolism), a phosphotransferase system (Phosphotransferase system), chloroalkane and chloroalkene degradation pathways (Chloroalkane and chloroalkene degradation), alanine, aspartate and glutamate metabolic pathways (Alanine, aspartate and glutamate metabolism), and a glycerophospholipid metabolic pathway (Glycerophospholipid metabolism), at an amount of 5, 4, 4, 4, 4, 3, 3 and 2, respectively.

There are the following findings according to the differential gene expression analysis: (1) Compared with the starting strain before acclimation, the intracellular

UDP-Gal (UDP- α -D-galactose) and 1-(5-phosphate-D-ribose)-5-amino-4-imidazolecarboxylic acid (CAIR) of the mutant strain P8G3#4 were accumulated and increased (Fig. 2B); (2) the expression of mtGDP in the intracellular glycerophospholipid metabolic pathway of P8G3#4 was down-regulated (Fig. 2C), leading to the increased intracellular glycerol content (3) and up-regulated ABC transporter (Fig. 2A), resulting in increased content of several intracellular small molecule solutes, such as lactose and branched chain amino acids; (4) the DNA replication capability was decreased (Fig. 2D), and the cellobiose transport system in the Lac family of the phosphotransferase system (PTS) was down-regulated. The physiological basis of upregulation or downregulation of these differential gene expressions is the stress response made to maintain the balance of osmotic pressure inside and outside the cell, the cell volume and the turgor being made to reach normal levels by increasing the intracellular water activity so as to maintain the normal physiological metabolism of cells.

Example 3

Shake flask fermentation and culture, and comparing the growth difference between the high sugar-tolerant strain and the starting strain after acclimation

Preparation of the seed liquid:

An MTC modified medium was used as the seed medium, wherein the various combinations could be divided into A, B, C, D, E liquids that, after respective high-temperature sterilization, were mixed at a ratio of 45:2:1:1:1 before being used.

The specific composition of each liquid is shown in Table 1.

Component label	Composition	Concentration (g/L)
A	Glucose	2
	Xylose	2
B (25×)	Tripotassium citrate	50
	Citric acid monohydrate	31.25
	Sodium sulfate	25
	Potassium dihydrogen phosphate	25
	Sodium bicarbonate	62.5
C (50×)	Urea	250
	Ammonium chloride	75
	Yeast extract	50
D (50×)	Magnesium chloride hexahydrate	50
	Ferrous chloride tetrahydrate	5
	Calcium chloride dihydrate	10
	Monohydrate cysteine hydrochloride	50

E (50×)	Pyridoxamine	
	dihydrochloride	1
	P-aminobenzoic acid	0.2
	D-biotin	0.1
	Vitamin B12	0.1
	Vitamin B1	0.2

Specifically, the preparation method of the seed medium was as follows: Respectively preparing the A, B, C, D liquids and putting them in a serum bottle, vacuuming, filling with nitrogen, sterilizing at 115°C for 20 min, and storing on standby; filtering and sterilizing the E liquid, directly injecting it into the serum bottle that had been filled with nitrogen and sterilized, and storing on standby; injecting the corresponding volume of the above prepared B, C, D, E liquids using a syringe into the serum bottle that had been filled with the A liquid, until the working concentration was reached, thereby obtaining the seed medium.

Preparation of the fermentation medium:

In the composition of the fermentation medium, the concentration of the carbon source in the A liquid was 30 g/L, 81 g/L and 120 g/L, respectively, wherein the mass ratio of glucose to xylose was 2:1, and the rest of the components were consistent with the components of the seed medium. The specific preparation process was consistent with the preparation, sterilization and other handling methods of the seed medium.

The overnight cultured P8G0 and P8G3#4 were inoculated into a serum bottle

containing 50 mL of a freshly prepared fermentation medium at a ratio of 1:10. Culturing the fermentation liquor in a cradle for 84 h at a temperature of 55°C and a rotational speed of 150 rpm, sampling every 6-12 h to determine OD₆₀₀, and calculating the dry cell weight (Fig. 1):

$$Y = 0.421X - 0.006 \quad (R^2 = 0.99)$$

Wherein Y is dry cell weight (DCW), g/L; and X is OD₆₀₀.

It can be seen from Fig. 1a that at the normal medium concentration, the time required for DCW of the starting strain P8G0 to reach 0.5 was 8 h, and the DCW could reach 1.5 after being cultured for 24 h and could reach a maximum of 2.3 at 60 h. When the substrate concentration was raised to 81 g/L, the time required for DCW of the strain to reach 0.5 was prolonged to 42 h, and likewise a longer time of 82 h was required at 120 g/L. Comparatively, the growth of the high sugar-tolerant strain P8G3#4 at three concentrations (Fig. 1b) was consistent with that of the starting strain at the normal medium concentration, indicating that the high sugar-tolerant strain P8G3#4 had overcome the shortcomings that growth lag period was long in the presence of the high-concentration substrate.

Example 4

Fermentor culture and comparison of differences in metabolites of strains before and after acclimation

Fermentation conditions: Putting the seed liquid at an inoculum amount of 10% into a 5 L automatic reactor of NBS Company filled with 3 L of the medium (120 g/L

mixed sugar substrate). The reactor was purged with nitrogen for 30 min before inoculation, and again purged with nitrogen for 30 min after inoculation, so as to ensure that the fermentation environment was anaerobic.

The metabolites ethanol, acetic acid and lactic acid as well as the sugar consumption were determined with Waters 2695 high-pressure liquid chromatography (HPLC).

1. Determination of consumption of glucose and xylose

Chromatographic column: Aminex HPX-87P (Biorad)

Mobile phase: Ultrapure water

Flow rate: 0.6 ml/min

Column temperature: 60°C

Detector temperature: 40°C

Sample preparation: Adding 1 g of CaCO_3 to 2 ml of the fermentation liquor, oscillating for 1 min, centrifuging at 16,000 g for 5 min, and filtering the supernatant through a 0.22 μm membrane. Used to detect the residual sugar.

Sample amount: 10 μl

Detector: A differential detector

2. Detection of metabolites ethanol, acetic acid and lactic acid

Chromatographic column: Aminex HPX-87H (Biorad)

Mobile phase: 5 mM H_2SO_4

Flow rate: 0.6 ml/min

Column temperature: 60°C

Detector temperature: 40 °C

Sample preparation: Adding 100 µl of 10% H₂SO₄ to 1.9 ml of the fermentation liquor, centrifuging at 16,000 g for 5 min, and filtering the supernatant through a 0.22 µm membrane. Used to detect acid and ethanol.

Sample amount: 10 µl

Detector: A differential detector.

3. Determination of lactic acid, acetic acid and ethanol in fermentation products (Table 1). The results show that, compared with the starting strain P8G0, the growth lag period of the acclimated strain P8G3#4 was shortened from the original 100 h to 24 h, and the dry cell weight at the end of fermentation was increased from 1.98 g/L to 4.26 g/L, increased by about 2 times. And the ethanol production and conversion rate also had a corresponding increase, with the final concentration of ethanol up to 28.11 g/L and the maximum conversion rate of ethanol up to 1.22 mol/mol.

Table 1 Comparison of top pot fermentation results of the strains before and after acclimation with 120 g/L mixed sugar as the substrate

Strain	Lag period (h)	DCW (g/L)	Ethanol		Acetic acid	Lactic acid	Hydrogen production (L)	Fermentation time (h)
			Concentration (g/L)	Conversion rate (mol/mol)				
P8G0	100	1.98	14.87	0.97	5.12	0	2.15	250
P8G3#4	24	4.26	28.11	1.22	5.02	0	3.09	200

CLAIMS

What is claimed is:

1. A thermophilic anaerobic bacillus, characterized in that: this strain is thermoanaerobacterium aotearoense P8G3#4, which has been preserved in China General Microbiological Culture Collection Center under an accession number of CGMCC No. 9000 since the date of deposit of April 3, 2014.

2. A method for producing ethanol by using the strain according to claim 1, characterized in that: first preparing a seed liquid of the thermophilic anaerobic bacillus CGMCC9000, then transferring this seed liquid to a fermentation medium in an inoculation amount of 10%-15% w/w, and agitating and culturing under an anaerobic condition at a culture temperature of 45°C-60°C for 50-250 h, and finally separating ethanol from the fermentation liquor.

3. The method according to claim 2, characterized in that: the total sugar concentration of the fermentation medium is 80-150 g/L.

4. The method according to claim 2, characterized in that: the initial pH of the fermentation medium is 5.5-6.5, and the stirring speed is 120-200 rpm.

5. The method according to claim 4, characterized in that: the initial pH of the fermentation medium is 6.3.

6. The method according to claim 2, 3, 4 or 5, characterized in that: the fermentation medium contains the following components in g/L: 80-100 of glucose, 40-60 of xylose, 1-10 of urea, 1-5 of a yeast extract, and 0.5-3 of ammonium

chloride.

7. The method according to claim 6, characterized in that: the fermentation medium also contains the following components in g/L: 1-4 of tripotassium citrate, 0.5-3 of citric acid monohydrate, 0.5-3 of sodium sulfate, 0.5-3 of potassium dihydrogen phosphate, 1-4 of sodium bicarbonate, 0.5-3 of magnesium chloride hexahydrate, 0.05-0.5 of ferrous chloride tetrahydrate, 0.05-0.4 of calcium chloride dihydrate, 0.5-3 of cysteine hydrochloride monohydrate, 0-0.1 of pyridoxamine dihydrochloride, 0-0.01 of p-aminobenzoic acid, 0-0.01 of D-biotin, 0-0.01 of vitamin B12, and 0-0.01 of vitamin B1.

8. The method according to claim 2, 3, 4 or 5, characterized in that: the seed liquid is prepared as follows: putting the thermophilic anaerobic bacillus CGMCC9000 in a seed medium, and culturing at 45°C-60°C at a rotational speed of 100-250 rpm for 8-24 h.

9. The method according to claim 8, characterized in that: the seed medium contains the following components in g/L: 2-4 of glucose, 2-4 of xylose, 1-10 of urea, 1-5 of a yeast extract, and 0.5-3 of ammonium chloride.

10. The method according to claim 9, characterized in that: the seed medium also contains the following components in g/L: 1-4 of tripotassium citrate, 0.5-3 of citric acid monohydrate, 0.5-3 of sodium sulfate, 0.5-3 of potassium dihydrogen phosphate, 1-4 of sodium bicarbonate, 0.5-3 of magnesium chloride hexahydrate, 0.05-0.5 of ferrous chloride tetrahydrate, 0.05-0.4 of calcium chloride dihydrate, 0.5-3 of cysteine hydrochloride monohydrate, 0-0.1 of pyridoxamine dihydrochloride,

0-0.01 of p-aminobenzoic acid, 0-0.01 of D-biotin, 0-0.01 of vitamin B12, and 0-0.01 of vitamin B1.