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Fed-Batch Fermentation of L- Threonine by Escherichia Coli Supplemented with B-Vitamins

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Abstract. B-vitamins can be used as cofactors in the synthesis process of L-threonine. Concentration of the cofactor can effectively change the activity of specific enzymes and reallocate the distribution of carbon substances in the cells. Theeffects of B-vitamins, including calcium pantothenate (VB_5), cobalamin (VB_{12}), choline chloride (VB_4) and nicotinamide (VB_3), on L-threoninefermentation by fed-batch culture of *Escherichiacoli* JLTHRin a 5L fermentorwere investigated. The results showed that the production of L-threonine was increased to 133.4 g/L, which was increased by 7.8% when supplemented with 1 g/L VB_4 .The production of L-threonine was increased to 130.6 g/L, which was increased by 5.6% when supplemented with10mg/L VB_3 . At last, when a combination of 1g/L VB_4 and 10mg/L VB_3 was added to the glucose solutionwith1.5g/L betainehydrochloride, 138.4g/LL-threonine was accumulated, which was increased by 11.9%.

Introduction

L-threonine is one of the eight essential amino acids of human and animals. It has important physiological functions and significance and can promote human growth and restore human fatigue [1]. Threonine is the fourth most important amino acid following lysine, methionine and tryptophan, and widely used in medical, food, feed and other industries. Thus, the microbial fermentation of L-threonine has been the focus of many researches and developments through strain improvement and fermentation process optimization. [1, 2]A mutant *E. coli* HS3 strains was isolated in which aspartokinase was released from end-product inhibition. The concentrations of the carbon source and the nitrogen source were optimized to improve the production of L-threonine.[3] The fed-batch culture system by adding biotin and oxygen-enriched air to basal minimal salt medium was applied in L-threonine production using the *E. coli* MT201 strain and a production of threonine (80.2 g/L) was achieved.[4] Okamoto established an process for L-threonine fermentation with addition of D, L-methionine and iron by an L-methionine autotrophic *E. coli* mutant and achieved a final titer of 101g/L of threonine. [3] Various fermentation substrates and conditions were investigated by *E. coli* TRFC strain. Sucrose was found to be the optimal initial carbon source and glucose was selected as the optimal feeding medium for L-threonine production. [5]

L-Threonine is biosynthesized through the glycolysis pathway, tricarboxylic acid cycle, and the threonine synthesis pathway from glucose. In this metabolic process, a

number of key enzymes play crucial roleson L-threonine synthesis.Vitamins are a group of trace organic substances, which are essential for microorganism growth and metabolism. They are needed as enzyme cofactors involved in reactions essential for cellular functions. Concentration of the coenzyme factor can effectively change the activity of specific enzymes and reallocate the distribution of carbon substances in the cells. Therefore, cofactor regulation is a highly efficient mean in the microbial metabolic regulation.B-vitamins, choline chloride, etc. can be used as cofactors in the synthesis process of L-threonine. The effects of B-vitamins on L-threonine production by *E. coli* have not yet been systematically investigated so far. In this study, the effects ofVB_5, VB_{12}, $VB_3$and VB_4 on the fermentation of L-threonine were investigated.

Materials and Methods

Strains and Materials

Escherichiacoli JLTHR, Jilin University Laboratory for preservation of bacteria.

Medium

The seed medium contained the following components(per liter of deionized water): glucose 35g, corn syrup 30mL, yeast extract 3g, $MgSO_4 \cdot 7H_2O$ 0.5g, $MnSO_4$ 10mg, $FeSO_4 \cdot 7H_2O$ 10mg, KH_2PO_4 1.5g, citric acid 2.0g, ammonium sulfate 2.0g, pH 7.0, 115℃, 15 min.

Fermentation medium contained the following components(per liter of deionized water) :glucose 20g, $MnSO_4$ 10mg,$MgSO_4 \cdot 7H_2O$ 1.0g, $FeSO_4 \cdot 7H_2O$ 30mg,KH_2PO_4 2.0g, citric acid 1.0g, corn syrup 12mL, betainehydrochloride 0.5g , pH 7.0, 115℃, 15min.

Culture Method

Sterile deionized water of 200mL was introduced into the slant medium, and the bacteria on the slant were washed off and inoculated into a 5L fermentor with a liquid working volume of 2.8 Lat 37℃. The pH was adjusted to 7.0 with 25% (v/v) ammonia. The dissolved oxygen (DO) level was maintained at approximately 20% -35%saturation by adjusting the agitation and aeration rates. The culture grown in the seed fermenter was inoculated aseptically [15%, (v/v)] into 3 L of production medium in a 5 L fermenter. The temperature and dissolved oxygen (DO) level were maintained at 37 ℃ and 20%~35%. The pH was adjusted to7.0.Once the initial 20 g/L of glucose was consumed, 800g/L of glucosesolution with1.5g/Lbetainehydrochloride and different B-vitamins was continuously fed into the fermenter.0.2mg/L or 10mg/LVB_5, 0.2mg/L or 50mg/L VB_{12}, 10mg/L VB_3, 1g/LVB_4, or the combined B-vitaminssolution was added in 800g/L of glucose solutionrespectively with1.5g/Lbetainehydrochloride depending on the experiment.

Analytical Techniques

The DO, pH and temperature were measured automatically with electrodes attached to the fermenters. The culture samples were diluted with 0.15 MNaCl, and the optical density was measured at 600 nm with a spectrophotometer. The concentration of glucose was monitored by an SBA-40 E biosensor analyzer (Biology Institute of Shandong Academy of Sciences, China).The concentration of L-threonine in fermentation broth was determined according to the method of Wang et al.[6]

Results and Discussion

Effects of Calcium Pantothenate(VB_5) on L-threonine Fed-Batch Fermentation

Calcium pantothenate is the component of NAD, $NADH_2$, NADP and$NADPH_2$ and the most important hydrogen carrier of biological oxidation, which plays the role of hydrogen transfer.VB_5 of 0.2mg/L and 10 mg/L were added to the glucose solution with1.5g/Lbetainehydrochloride respectively to test the effects of VB_5on L- threonine fermentation by *E.coli*JLTHR.

According to the Fig.1, OD was increased to 58.7 and 63.4, feeding with 0.2mg/L and 10 mg/L VB_5, respectively. It indicated that$VB_5$was conducive to cell growth. However, the productions of L-threonine feeding with different concentration of VB_5were similar. It proved that VB_5 had little effect on L-threonine synthesis pathway.

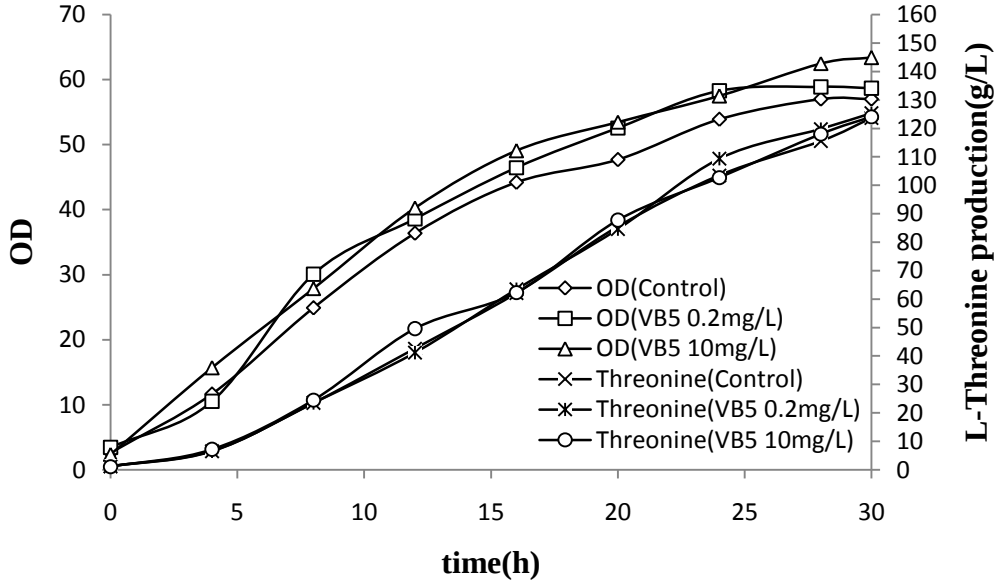

Figure 1. Effects of VB_5 on L-threonine fed-batchfermentation

Effects of Cobalamin(VB_{12})on L-threonine Fed-Batch Fermentation

Cobalamin is a structurally complex cofactor, consisting of a modified tetrapyrrolewitha centrally chelated cobalt. Cobalamin is usually found in one of two biologically active forms: methylcobalamin and adenosylcobalamin. Most prokaryoteshave cobalamin-dependent enzymes. Inbacteria , these enzymes include methioninesynthase, ribonucleotide glutamate and methylmalonyl-CoAmutases, ethanolamine ammonia-lyase, and diol dehydratase.[7]VB_{12} can transfer methyl to provide a common unit of carbon for the biotransformation, in order to facilitate the synthesis of a variety of cell substances. High concentration of VB_{12} can effectively improvethe methyl-malonyl CoA isomerase activity, through the isomerization of succinyl-CoA, and enter the tricarboxylic acid cycle route, thus can effectively improve the efficiency of the citric acid cycle and improve the yield of oxaloacetate.

To test the effects of VB_{12}on L- threonine fermentation by *E.coli*JLTHR, VB_{12} of 50 mg/L and 0.2mg/Lwere added to the glucose solution with1.5g/Lbetainehydrochloride, respectively. As shown in Fig.2, the OD on supplementation of0.2mg/L $VB_{12}$was up to 62.6, which was higher than thatof feeding with 50 mg/LVB_{12}. It indicated that low concentration of VB_{12} was beneficial to the growth of cells. At low concentrations of VB_{12}, it could effectively participate in DNA

synthesis and metabolism of carbohydrates etc. to increase biomass. However, the productions of L-threonine feeding with different concentration of VB_{12}were similar. The supplementation of VB_{12}wasnot contributed to a higher concentration of L-threonine compared to that feeding with betainehydrochloride only.

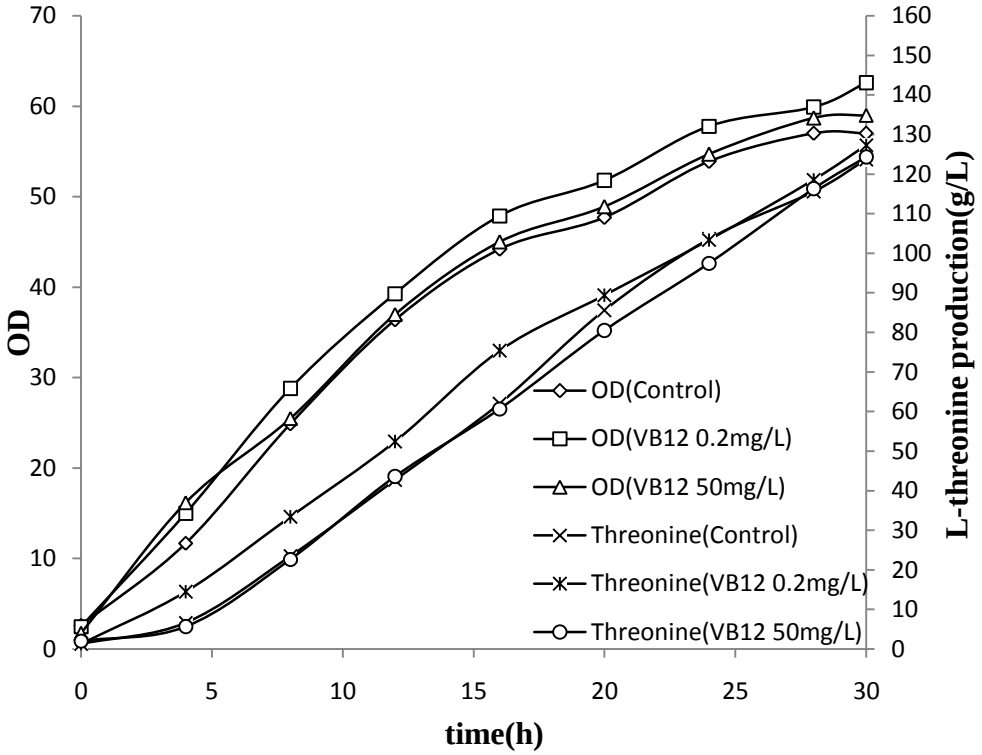

Figure 2. Effects of VB_{12} on L-threonine fermentation

Effects of Nicotinamide (VB_3)on L-threonine Fed-batch Fermentation

To test the effects of nicotinamideon L- threonine fermentation by *E.coli*JLTHR, VB_3 of 50 mg/L and 0.2mg/L were added to the glucose solution with1.5g/L betainehydrochloride, respectively.

According to Fig. 3, not only OD but also the production of L- threonine was increased feeding with VB_3.The titer of L- threonine reached 130.6g/L feeding with 10mg/L VB_3, which was 5.6% higher than that of feeding with betainehydrochloride only. The titer of L- threonine reached 126.4g/L feeding with 2mg/L VB_3, which was 2.2% higher than that of feeding with betaine hydrochloride only.VB_3 is an important component of coenzyme I and coenzyme II. It plays an important role in the glycolysis pathway and participates in the intracellular oxidation process. Therefore, VB_3 can effectively improve the growth of bacteria, and increase the production of L-threonine.

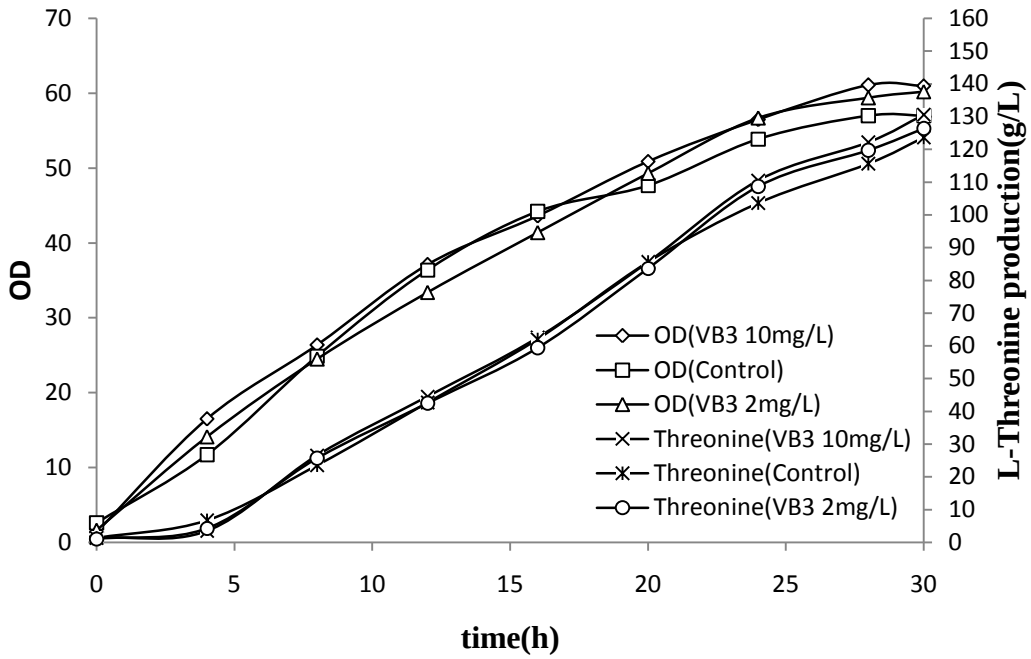

Figure 3. Effects of VB_3 on L-threonine fed-batchfermentation

Effects of Choline Chloride (VB_4)on L-threonine Fed-batch Fermentation

To test the effect of choline chlorideon L- threonine fermentation by *E.coli*JLTHR, choline chloride of 1g/ Lwere added to the glucose solutionwith1.5g/L betainehydrochloride.

As shown in Fig.4, addition of choline chloride not only increased OD, but also improved L-threonine production greatly. The production of L-threonine achieved 133.4g/L.Choline is an important component of lecithin, used to constitute the cell membrane and promote cell physiological metabolism, and thus can promote cell growth.*E.coli*is extensively used as a host for producing L-threonine. However, it is challenged by highsalt concentration in the fermentation broth.The osmotic stress triggered by high salt concentration results in both structural and physiological injury of cells. Choline chloride is a synthetic precursor of betaine. Itcan act as stress protectant or methyl donor for the biosynthesis of structurally complex compounds. Moreover, Choline chloride can protect the activity of enzymes associated with L-threonine synthesis.In addition, because choline chloride can increase the mobility of the cell membrane, it is conducive to the output of L-threonine.

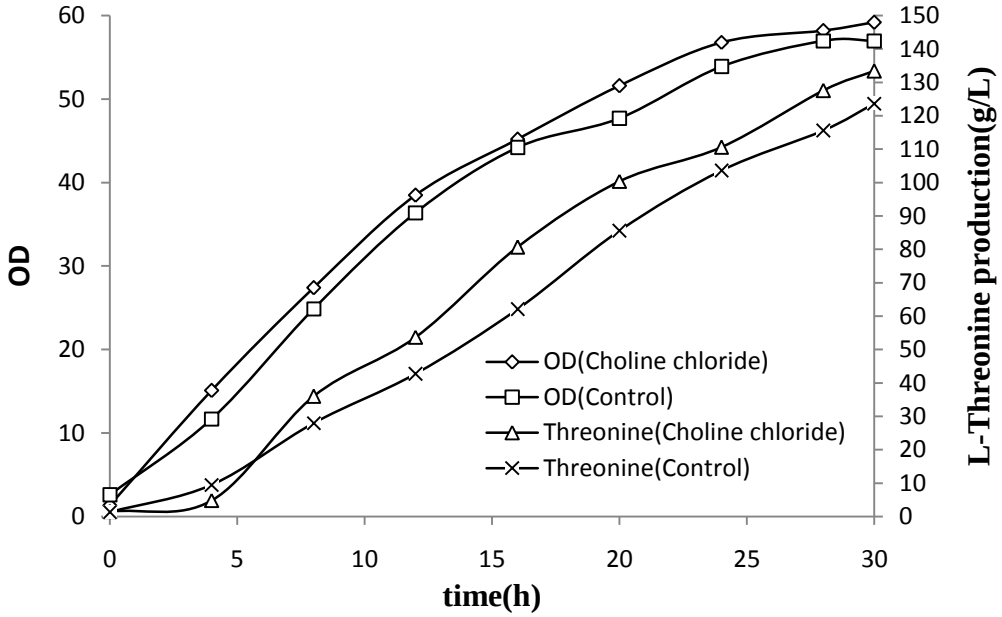

Figure 4. Effects of choline chloride on L-threonine fed-batchfermentation

Effects of Combined B-vitamins on L-threonine Fed-batch Fermentation

Since the supplementation of VB_3 and VB_4 had contributed to a higher concentrationof L- threonine, the combined B-vitamins solution of10mg/L VB_3 and 1g/L$VB_4$was added to the glucose solutionwith1.5g/L betainehydrochloride. As shown in Fig.5,in the early stage of fermentation, the growth of *E.coli*JLTHRwas not much different with the control. But with the rapid growth of *E.coli*JLTHR, it began to produce more metabolites, thus the growth difference was gradually shown up. OD supplemented with VB_3 and $VB_4$was increased to 61.5. The addition of VB_3 and VB_4compound has an11.9% increase in production of L-threonine(138.4g/L)compared with feeding with betaine hydrochloride only.

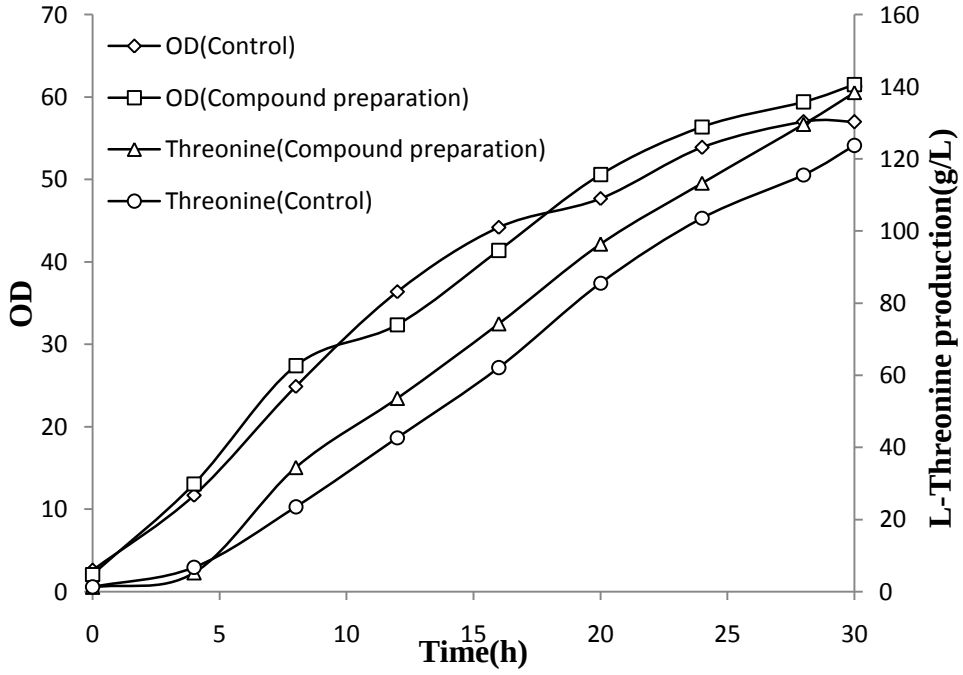

Figure 5. Effects of Combined B-vitamins on L-threonine fed-batchfermentation

Conclusions

Since vitamins are essential for microorganism growth and metabolism, the effects of supplying B-vitamins on L-threonine fed-batchfermentation were intensivelystudied in this work. The L-threonine production of adding1g/LVB_4in glucose solution with1.5g/Lbetainehydrochloridereached133.4g / L, which was the highest among that of supplying B-vitamins. The L-threonine production of adding10mg/L VB_3in glucose solution with1.5g/L betainehydrochloride was increased to 130.6g/L, which was higher than that of supplyingVB_{12} and VB_5. At last, the highest L-threonine production of 138.4g/L was obtained by fed with glucose solution containing 1.5g/L betaine hydrochloride,10mg/L $VB_3$and 1g/LVB_4.

To additionally improve L-threonine production by *E. coli*JLTHR, further studies are needed by optimization of the fermentation process and construction of new strains.

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