

# Studies on Production of L-Tryptophan by Fermentative Cultivation

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## ABSTRACT

Abstract L-tryptophan is not abundant in the natural world. It mainly exists in the food of animal type such as meat, egg, and milk. For the food of plant type, except potatoes, the content of L-tryptophan in grains like rice and wheat is scarce. Four methods to produce L-tryptophan include hydrolysis of protein, chemical synthesis, enzymatic transformation and fermentation. In this study, fermentation is used to produce L-tryptophan. The yield of L-tryptophan may be affected by many factors such as the composition of media, carbon sources, organic and inorganic nitrogen sources, operating conditions such as temperature and pH, and batch or continuous. *Corynebacterium glutamicum* 21334 (CCRC 12511) was used to begin. Unfortunately, this strain produced no L-tryptophan. Mutant of this strain was then considered to improve the L-tryptophan yield. NTG (N-Methyl-N'-nitro-N-nitrosoguanidine) was then used to treat the original strain, and several potential strains were selected. Through a series of screening experiments, the mutant strains still showed no evidence to produce L-tryptophan. In other words, mutant didn't succeed in selecting a potential candidate for L-tryptophan production. Therefore, we decided to replace the strain with *Brevibacterium flavum* ATCC 21427 (CCRC 12509). The strain showed evidence to produce L-tryptophan after preliminary tests, so we used it to study optimal medium composition and culture conditions. The following three major components, carbon sources (glucose, fructose, and sucrose), organic nitrogen sources (yeast extract and peptone), and inorganic nitrogen sources ((NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and NH<sub>4</sub>Cl), were considered. The pH value during batch fermentation was also explored. Experimental results showed that carbon and organic nitrogen sources had significant effect to the yield of L-tryptophan, and inorganic nitrogen sources had not. The combination of glucose and yeast extract could have the highest yield of L-tryptophan. Under a medium volume of 30 mL (flask volume 250 mL) and 300 rpm, the concentration of L-tryptophan could reach 0.01 g/L after a cultivation of 96 h in a shaker of 150 rpm. In addition, this experiment also studied the effect of pH on the production of L-tryptophan. Three pH values (6.0, 7.0 and 8.0) were selected for testing. The results showed that the microbial growth and the L-tryptophan production were the best when the pH was set at 7.0. Furthermore, a fed-batch cultivation of *Brevibacterium flavum* ATCC 21427 was run with the following conditions: seed 2%, temperature 30°C, stirring rate 250 rpm, pH 7.0, working volume 2 L, and mass fraction of glucose around 4%. The concentration of L-tryptophan could reach 0.02 g/L under the above condition after a cultivation of 35 h. The concentration of glucose could almost remain constant if a proper control was imposed. Consequently, the microbial growth could sustain longer because a proper concentration of carbon source could maintain. Therefore, fed-batch cultivation may be a better way than a batch fermentation to produce L-tryptophan. Key words: L-tryptophan, mutant, fermentation, fed-batch cultivation

Keywords: L-tryptophan; discontinuous fed-batch cultivation; fermentation; mutant

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## REFERENCES

- 參考文獻 1.王文憲 (1991) 生物化學要論, p.299合記圖書出版社,台北. 2.李宛儒 (1996) 利用*Brevibacterium divaricatum* 營養要求性變異株發酵生產色胺酸之研究.國立台灣農業化學研究所碩士論文. 3.黃伯超, 游素玲 (1992) 營養學精要, p.148-152,p.720. 健康文化事業股份有限公司, 台北. 4.黃清龍 (1998) 生物技術的發展與應用, p.52-63,九州圖書文物有限公司, 台北. 5.郭坤地, 張天鴻 (1989) 胺基酸產業市場調查, 財團法人生物技術開發中心. 6.張為憲 (1984) 高等食品化學, p.67-103.華香園出版社, 台北. 7.劉英俊 (1996) 最新微生物應用工業, p.81-82.中央圖書出版社, 台北. 10.Gish, K., and C. Yanofsky (1993) Inhibition of expression of the tryptophanase operon on *Escherichia coli*. Byextrachromosomal copies of the *tna* leared region. *J. Bacteriol.* 175:623-627. 11.Hagino, H., and K. Nakaoyma (1975) L-tryptophan production by analog-resistant mutants derived from a phenylalanine and tyrosine double auxotroph of *Corynebacterium glutamicum*. *Agri. Bio. Chem.*, 39 (2) :343-349. 12.Hirahara, T., S. Suzuki, S. Horinouchi and T. Beppu (1992) Cloning, nucleotide sequences, and overexpression in *Escherichia coli* of tandem copies of a tryptophanase gene in an obligately symbiotic thermophile, *symbiobacterium thermophilum*. *Appl. Environ. Microbiol.*, 58 (8) :2633-2642. 13.Hwang, S. O., G. H. Gil, Y. J. Choi, K. R. Ksng, J. K. Lee and J. C. Bae (1985) The fermentation process for L-phenylalanine production on using an auxotrophic regulatory mutant of *Escherichia coli*. *Appl. Microbiol. Biotechnol.*, 22:108-113. 15.Ikeda, M., K. Nakanishi, K. Kino and R. Kasumata (1994) Fermentative production of tryptophan by a stable recombinant strain of *Corynebacterium glutamicum* with a modified serine-biosynthetic pathway *Biosci. Biotech. Biochem.*, 58 (4) :674-678. 16.Ikeda, M., and R. Katsumata, (1995) Tryptophan Production by Transport Mutants of *Corynebacterium glutamicum* *Biosci. Biotech. Biochem.*, 59 (8) :1600-1602. 17.Ishiwata, K., N. Fukuhara, M. Shimada, N. Makiguchi and K. Soda (1990) Enzymatic production of L-tryptophan from DL-serine and indole by a coupled reaction of tryptophan synthase and amino acid racemase. *Biotechnol. Appl. Biochem.*, 12 (2) :141-149. 18.Kupfer, D., and D. E. Atkinson (1964) Quantitative method for determination of indole, tryptophan and anthranilic acid in the same aliquot. *Anal. Biochem.*, 8:82-94. 19.Methews, C. K., and K. E. van Holde (1990) *Biochemistry*, p.716-726. The Benjamm/Cummings Publishing. 20.Sano, K., K. Yokkozeki, C. Eguchi, T. Kagaw, I. Noda and K. Mitsugi (1977) Enzymatic production of L-tryptophan from L- and DL-5-indolymethylhydation by newly isolated bacterium. *Agri. Bio. Chem.*, 41 (5) :819-825. 21.Shiio, I., H. Sato and M. Nakagawa (1972) L-tryptophan production by 5-methyltryptophan-resistant mutant of glutamate-producing bacteria. *Agri. Bio. Chem.*, 36 (13) :2315-2322. 22.Shiio, I., S. I. Sugimoto and M. Nakagawa (1975) Production of L-tryptophan by mutants *Brevibacterium flavum* resistant to both tryptophan and phenylalanine analogues. *Agri. Bio. Chem.*, 39 (3) :627-635. 23.Shiio, I., S. I. Sugimoto and M. Nakagawa (1982) Production of L-tryptophan by azaserine-resostant mutants of *Brevibacterium flavum*. *Agri. Bio. Chem.*, 46 (7) :1849-1854. 24.Shiio, I., S. I. Sugimoto and K. Kavamura (1988) Breeding of phenylalanine-producing *Brevibacterium flavum* strains by removing feedback regulation of both the two key enzymes in its biosynthesis. *Agri. Bio. Chem.*, 52 (9) :2247-2253. 25.Sugimoto, S. I., and I. Shiio (1977) Enzymes of the tryptophan synthetic pathway in *Brevibacterium flavum*. *J. Biochem.*, 81:823-833. 26.Su, Y. C., and K. Yamada (1960) Studies on L-glutamic acid-producing strain and its taxonomical studies. *Bull. Agri. Chem. Soc. Jpn.*, 24 (1):69-74. 27.Terasawa, M., M. Inui, Y. Uchida, M. Kobayashi, Y. Kurusu and H. Yukawa (1991) Application of the tryptophanase promoter to high expression of the tryptophan synthase gene in *Escherichia coli*. *Appl. Microbiol. Biotechnol.*, 34:623-627. 28.Udenfriend, S., and R. E. Peterson (1957) *Methods of Enzymology* (Colowick, S. P., and N. O. Kaplan), 4:613-614. Academic Press, New York. 29.Windholz, M. (1983) *The Merck Index an Encyclopedia of chemicals and drugs*.10th Ed., p. 9601. Merck, NJ.