


# Hydrolysing the soluble protein secreted by *Escherichia coli* in *trans*-4-hydroxy-L-proline fermentation increased dissolve oxygen to promote high-level *trans*-4-hydroxy-L-proline production

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## ABSTRACT

*Trans*-4-hydroxy-L-proline (Hyp) production by *Escherichia coli* (*E. coli*) in fermentation is a high-oxygen-demand process. *E. coli* secretes large amounts of soluble protein, especially in the anaerobic phase of fermentation, which is an important factor leading to inadequate oxygen supply. And acetic acid that is the major by-product of Hyp production accumulates under low dissolved oxygen (DO). To increase DO and achieve high-level Hyp production, soluble protein was hydrolysed by adding protease in Hyp fermentation. The optimal protease, concentration, and addition time were trypsin, 0.2 g/L, and 18 h, respectively. With the addition of trypsin, the soluble protein in Hyp fermentation decreased by 43.5%. The DO could be maintained at 20–30% throughout fermentation. Hyp production and glucose conversion rate were 45.3 g/L and 18.1%, which were increases of 24.1% and 8.4%, respectively. The accumulation of acetic acid was decreased by 52.1%. The metabolic flux of Hyp was increased by 44.2% and the flux of acetate was decreased by 51.0%.

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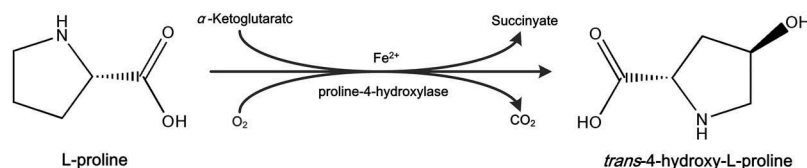
*Trans*-4-hydroxy-L-proline; fermentation; protease; dissolve oxygen; acetic acid; metabolic flux

## 1. Introduction

*Trans*-4-hydroxy-L-proline (Hyp) is a valuable amino acid that is widely used in pharmaceutical, chemical, food and cosmetic industries[1]. It is present in gelatin, collagen, and cell wall proteins and is also an important component of secondary metabolites of certain microorganisms[2]. Hyp has conventionally been produced by biological extraction, a complex process which has many disadvantages such as high cost, low recovery rate, and serious pollution[3]. With the increase of raw material price and people's attention to environmental issues, the traditional method will be gradually eliminated, while microbial fermentation becomes a promising method due to its economic advantages and environmentally-benign features[3].

In a recombinant *Escherichia coli* (*E. coli*) strain, proline-4-hydroxylase (P4H), which is a member of dioxygenase family [4–6], catalyses the hydroxylation of L-proline to produce Hyp in the presence of  $\alpha$ -ketoglutarate and Fe [2]<sup>+</sup> (Figure 1)[7]. In this hydroxylation reaction, an oxygen atom is introduced into L-proline to obtain Hyp, while another oxygen atom is introduced into  $\alpha$ -ketoglutarate to

obtain succinic acid and carbon dioxide [8,9]. Due to the catalytic properties of P4H, the production of Hyp is a high oxygen-demand process. However, the soluble protein secreted by *E. coli* during Hyp fermentation generates high-viscosity fermentation broths, hindering oxygen transfer, and reducing dissolved oxygen (DO) levels below that required to efficiently produce Hyp[10]. In addition, acetic acid, which is the primary by-product of Hyp fermentation in *E. coli*, has inhibitory effects on cell growth and product synthesis[11]. The accumulation of acetate is inversely correlated with DO levels, and acetate accumulation can be reduced by maintaining high DO levels at stationary growth phase [12,13]. Therefore, oxygen supply is generally considered to be a major limiting factor in Hyp production by recombinant strains[14]. At present, there are two strategies for solving the problem of insufficient oxygen supply during high-density fermentation. One is the introduction of *Vitreoscilla* haemoglobin gene (*vgb*) into cells through metabolic engineering methods to enhance the cells' respiration and energy metabolism under oxygen-limited conditions [10,15–17]. The other strategy is to increase the stirring speed, aeration, and pressure



**Figure 1.** The catalysis of proline-4-hydroxylase.

of bioreactor in fermentation. However, these methods require a long production cycle or high energy consumption, which are not conducive to controlling production costs [18].

To solve the problem of an insufficient oxygen supply in Hyp fermentation, a new method that adding protease to hydrolyse the soluble protein secreted by *E. coli* was proposed in this study. The added protease could degrade the proteins into small molecules, such as peptides and amino acids, which are used as nitrogen sources by microorganisms [19–21], resulting in the decrease of protein content and the increase of oxygen transmission efficiency in fermentation broth. In the present study, different proteases, concentrations, addition times, and the effect of adding protease on Hyp fermentation were examined. The results confirmed the feasibility of improving DO by hydrolysing the soluble protein to promote high-level Hyp production by this method.

## 2. Materials and methods

### 2.1. Microorganism

The Hyp-producing *E. coli* THLP strain used in this study was obtained from the Center of Culture Collection of Department of Life Science of Shanxi Datong University.

### 2.2. Culture conditions

The off-line culture of fermentation broth was carried out in 500 mL shake flasks containing 30 mL of Hyp fermentation broth with various concentrations of alkaline protease, neutral protease, trypsin and complex protease, and cultured on a rotary shaker at 200 r/min, 36°C, and pH 7.0 for 6 h. The Hyp fermentation broth was prepared in a 5 L stirred fermenter (Shanghai Baoxing Bioengineering Equipment Co., Ltd) for 18 h.

The Hyp fermentation was conducted in a 5 L fermenter. The strains cultured on agar slant (10 g/L

tryptone, 5 g/L yeast extract, 1 g/L  $KH_2PO_4$ , 0.5 g/L  $MgSO_4 \cdot 7H_2O$ , 3 g/L NaCl and 20 g/L agar) at 37°C for about 12 h. Seed cultures were prepared by transferring an appropriate amount of agar slant cultured cells into 5 L seed fermenter containing 2 L seed medium (20 g/L glucose, 10 g/L yeast extract, 5 g/L  $(NH_4)_2SO_4$ , 3 g/L  $KH_2PO_4$ , 1 g/L sodium citrate, and 1 g/L  $MgSO_4 \cdot 7H_2O$ ) at 36°C and pH 7.0 for about 10 h. The culture grown in the seed fermenter was transferred with 10% (V/V) inoculum into 5 L fermenter containing 3 L fermentation medium (20 g/L glucose, 5 g/L yeast extract, 3 g/L  $(NH_4)_2SO_4$ , 4 g/L  $KH_2PO_4$ , 2 g/L sodium citrate, 1 g/L  $MgSO_4 \cdot 7H_2O$ , and 0.1 g/L  $FeSO_4 \cdot 7H_2O$ ) in total. The fermentation temperature was maintained at 36°C, and the pH was adjusted to 7.0 by automated addition of 25% (V/V) ammonium hydroxide. The DO was maintained at 20–30% by adjusting the stirring speed and aeration. When the initial glucose was exhausted, 80% (m/V) glucose solution was fed into the fermenter at an appropriate rate to maintain the glucose concentration at 0–5 g/L.

### 2.3. Analysis of substrate and product

The biomass concentration was measured as the optical density at 600 nm ( $OD_{600}$ ) using a spectrophotometer [22]. The glucose concentration was determined by a biological sensor (Shandong Academy of Sciences Institute of Biology). The concentrations of acetic acid and lactate were detected by high performance liquid chromatography (HPLC; Agilent 1200) using an Aminex HPX-87H column with  $H_2SO_4$  (0.004 mol/L) as the mobile phase at a flow rate of 0.60 mL/min [23]. The detection wavelength, temperature and injection volume were set as 210 nm, 35°C, and 20  $\mu$ L, respectively. The Hyp concentration was measured according to the chloramine-T method [24]. The protein concentration was determined with a kit (Nanjing Jiancheng Bioengineering Institute).

## 2.4. Analysis of metabolic flux

The distribution of metabolic flux with the control fermentation (CF) and the added-protease fermentation (APF) was calculated by MATLAB based on the analysis of metabolic flux balance and stoichiometry according to the method of Su et al. [6,25]

## 2.5. Statistical analysis

All experiments were conducted in triplicate and the dates represent mean values and standard deviation.

## 3. Results and discussion

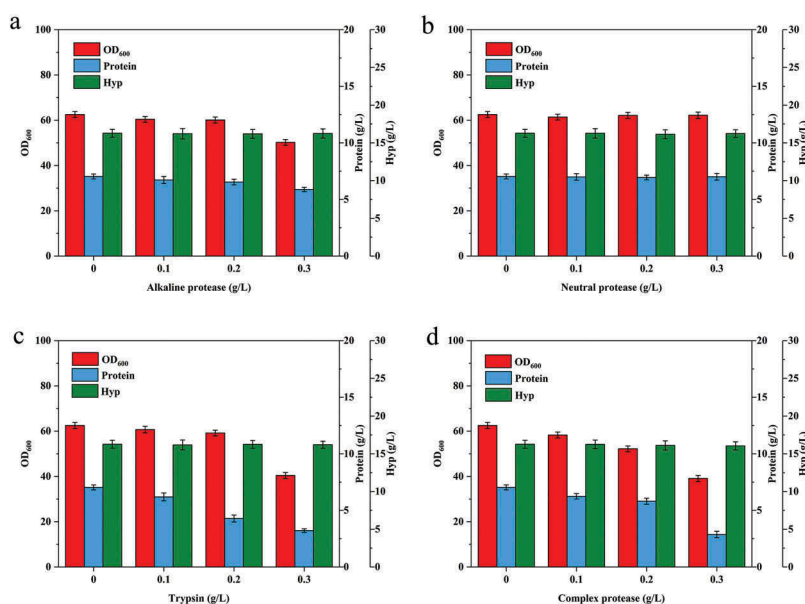
### 3.1 Effect of different protease types and concentrations on hyp fermentation broth

The soluble protein produced by *E. coli* during Hyp fermentation was hydrolysed by adding protease. To evaluate the effect of different protease types and concentrations on degrading soluble protein in Hyp fermentation broth without destroying Hyp and OD<sub>600</sub> under the conditions of Hyp fermentation, the off-line culture was carried out. As shown in Figure 2, there was no significant decrease in Hyp concentration following the addition of proteases, regardless of types or concentrations, indicating that Hyp was not destroyed by these proteases. When alkaline protease was added to Hyp fermentation broth at 0.1, 0.2, or 0.3 g/L

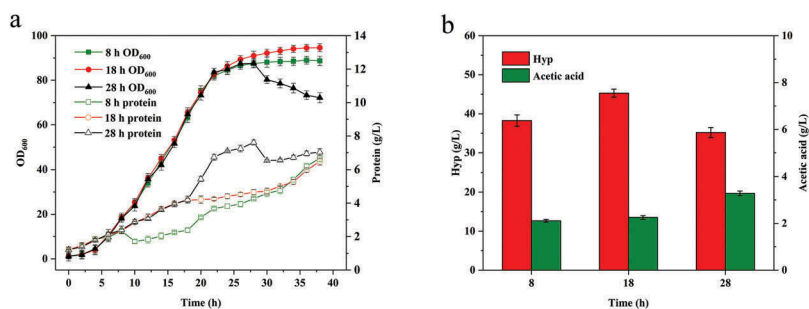
(Figure 2a), the protein content was decreased by 4.41%, 6.97%, and 16.36%, respectively. Figure 2b shows that the neutral protease could not play a role on degrading protein under the conditions of Hyp fermentation. When 0.3 g/L trypsin was added to the fermentation broth (Figure 2c), the protein content was decreased by 54.34% from 7.03 g/L to 3.21 g/L, but OD<sub>600</sub> decreased by 35.36%. In contrast, when trypsin was added at 0.2 g/L, the protein content was decreased by 39.12% to 4.28 g/L with a slight decrease in the OD<sub>600</sub> of 5.28%. When the complex proteases were added (Figure 2d), OD<sub>600</sub> decreased significantly as the protein content decreased. Therefore, trypsin was chosen as the optimal protease type, and the optimum amount was 0.2 g/L.

### 3.2 Effect of protease addition time on hyp fermentation

Based on the results of above experiments, Hyp fermentation experiments were carried out in 5 L fermenters. 0.2 g/L trypsin was added at 8, 18, and 28 h of Hyp fermentation respectively to determine the optimal time for addition. The OD<sub>600</sub>, protein, Hyp, and acetic acid contents are showed in Figure 3. The addition of trypsin at 8 or 18 h had no adverse effect on bacterial growth. However, when adding trypsin at 28 h, OD<sub>600</sub> decreased from 87.6 to 72.2 in the late stage of fermentation. From the perspective of protein



**Figure 2.** Effect of different protease types and concentrations on the OD<sub>600</sub>, protein content, and *trans*-4-hydroxy-L-proline concentration in the fermentation broth. (a) Alkaline protease; (b) neutral protease; (c) trypsin; (d) complex protease.



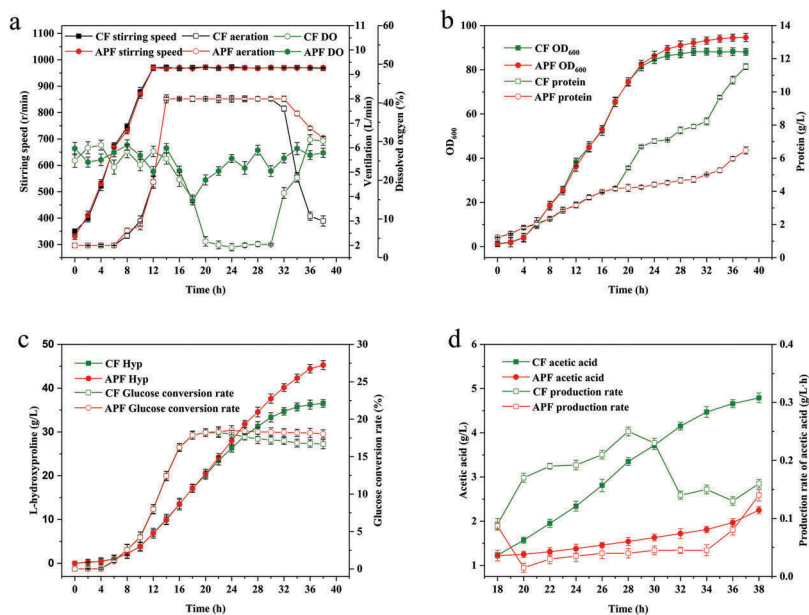
**Figure 3.** Effect of trypsin addition time on the fermentation of *trans*-4-hydroxy-L-proline. (a) OD<sub>600</sub> and protein content; (b) *trans*-4-hydroxy-L-proline concentration and acetic acid accumulation.

content in fermentation broth, although the final protein content was roughly the same after adding trypsin at the three time points, the addition at 8 or 18 h could keep soluble protein at relatively low levels throughout the whole fermentation, and this effect was not observed when trypsin was added at 28 h. Besides, compared with adding trypsin at 8 or 28 h, Hyp production was the highest when trypsin was added at 18 h, and the acetate accumulation was also at a low level. Taken together, 18 h of fermentation was the optimal time for the addition of trypsin.

### 3.3 Effect of the added-protease fermentation on hyp production

Trypsin (0.2 g/L) was added to Hyp fermentation at 18 h, and the process was monitored. The stirring

speed, aeration, DO, OD<sub>600</sub>, protein content, Hyp production, the rate of glucose conversion to Hyp, acetic acid content and production rate were evaluated (Figure 4). In the prophase of fermentation, oxygen consumption gradually increased with the growth of bacteria, and the stirring speed and aeration volume were continuously adjusted to maintain DO at 20–30%. The stirring speed and aeration were adjusted to the maximum of 970 r/min and 8.0 L/min at 12 and 14 h, respectively (Figure 4a). With the effect of biomass increase and product formation on oxygen consumption, DO dropped to 10–20% at 18 h. After 18 h of fermentation, the DO in CF decreased significantly to <5%, while that in APF did not drop below 10%, and slowly recovered from 10–20% to 20–30% at 20 h. In the CF, DO was increased after 30 h, and aeration gradually



**Figure 4.** Effect of trypsin addition on *trans*-4-hydroxy-L-proline production. CF is control fermentation (no protease added); APF is added-protease fermentation (trypsin was added at 18 h). (a) Stirring speed, aeration and dissolved oxygen; (b) OD<sub>600</sub> and protein content; (c) Hyp concentration and glucose conversion rate; (d) acetic acid concentration and production rate.





secreted by *E. coli* with the addition of protease was developed. The optimal protease, concentration, and addition time were trypsin, 0.2 g/L, and 18 h, respectively. The soluble protein in Hyp fermentation broth was decreased by 43.5%. DO in the APF was maintained at 20–30% throughout fermentation. Compared with the corresponding levels in the CF, Hyp titer and glucose conversion rate in the APF were 45.3 g/L and 18.1%, which were increases of 24.1% and 8.4%, respectively. The accumulation of acetic acid was decreased by 52.1%. Moreover, the metabolic flux of Hyp increased by 44.2% and the flux of acetate decreased by 51.0%. Thus the addition of trypsin appropriately during Hyp fermentation is an effectively approach to improving DO, reducing by-production acetate, and promoting Hyp production.

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