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A novel metabolic model incorporating directed signal flow diagram with enzymatic activities data for evaluating glutamate yield in glutamate fermentation

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ABSTRACT

Glutamate conversion yield is one of the most important performance indexes in glutamate fermentation. The experimental results showed that anaplerotic reaction could be enhanced by jointly manipulating pH regulation and $NaHCO_3$ supplement during fermentations by *Corynebacterium glutamicum*, leading to a 36% increase in the yield and comparably high glutamate productivity. A novel metabolic model incorporating directed signal flow diagram and enzymatic activities data was proposed to interpret the yield enhancement. The simulation and experimental results revealed that singly regulating each individual enzyme could not increase the yield, and glutamate yield could be enhanced only when six key enzymes of pyruvate carboxylase (PC), pyruvate dehydrogenase (PDH), isocitrate dehydrogenase (ICDH), isocitrate lyase (ICL), glutamate dehydrogenase (GDH) and α-oxoglutarate dehydrogenase (ODHC) works in a coordinated way. Namely, relative activities ratios of enzymatic pairs of PC/PDH should be controlled at moderate level of 6:4, while those of ICDH/ICL and GDH/ODHC at higher level of 8:2 simultaneously. The model could cluster data pairs of glutamate yields and enzymatic activities obtained under different operation conditions into different categories, indicating its abilities in guiding optimal enzyme regulation ways for fermentations characterized with multiple enzymatic reactions and closed reaction loops.

1. Introduction

L-Glutamate is widely used in food, pharmaceutical and other industries, and its fermentative production amount exceeds 2.2 million tons per year [1]. In industrial glutamate production, final glutamate concentration varies at about 100–120 g/L, glutamate yield (from glucose) and productivity range at 55–60% (w/w) and 3.0–3.8 g/(L h), respectively. In glutamate fermentation, if oxaloacetate in tricarboxylic acid (TCA) cycle could be supplemented by anaplerotic reaction, maximal glutamate yield could reach a level of 81.7% theoretically. Conversely, the yield would stay at lower level of 54.4% if anaplerotic reaction completely fails to work [2]. Glutamate is a food ingredient with huge market requirement in oriental countries (China, Japan, Korea, Taiwan, etc.). In China, the selling price of raw glutamate crystals (90% glutamate, w/w) is relatively low ($1080/ton), and profit margin by fermenting glucose ($400/ton, industrial class) is very limited. Assuming a 60% glutamate yield, glucose cost would occupy more than 60% of the gross revenue. Therefore, it would be of great commercial importance to enhance glutamate yield while maintaining a comparably high productivity. Our previous studies showed that glutamate yield could be significantly enhanced by raising pH from 7.0 to 8.0 and adequately adding $NaHCO_3$ simultaneously, or raising pH first followed by $NaHCO_3$ supplement during the fermentations [3]. The enzymatic activities of 6 key enzymes involved in glutamate fermentations under different operation modes were also investigated. However, the very informative enzymatic data could not directly used in quantifying or evaluating the yields, because the enzymatic system was complex and each enzyme interacted with the others in complicated ways. For example, singly regulating pH or supplementing $NaHCO_3$ during fermentations could increase pyruvate carboxylase (PC) activity leading to a higher CO_2 anaplerotic rate, but glutamate yield could not be enhanced as expected. Glutamate fermentation is a complex metabolic network characterized with existence of multiple products, enzymatic

Abbreviations: Ac-CoA, acetyl coenzyme A; GLC, glucose; GLU, glutamate; GLY, glyoxylate; ICIT, isocitrate; MAL, malate; OAA, oxaloacetate; PYR, pyruvate; SUC, succinate; α-KG, α-oxoglutarate; GDH, glutamate dehydrogenase; ICDH, isocitrate dehydrogenase; ICL, isocitrate lyase; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; ODHC, α-oxoglutarate dehydrogenase complex.

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reactions and closed reaction loops, it is very important to reveal or clarify the natures/reasons of the yield enhancement both theoretically and practically. Directed signal flow diagram was firstly proposed by Mason [4], who represented complicated electronic circuits with current directions and closed-loops by signal flow graph, called Mason theory. After that, a couple of its applications have been reported in the fields of simplifying metabolic networks, analysis/estimation of carbon/energy metabolisms and recognition of physiological states in yeast and *E. coli* cultivations [5–7]. Mason theory treats metabolic network as a directed signal flow diagram, with substrates and products as inputs and outputs. The overall transfer function between output and input could be formulated by integrating all sub-transfer functions between the involved intermediate nodes. In general, transfer function from intermediate node (A) to another (B) could be represented by stoichiometric coefficient of reaction A → B. However, if metabolic flow branches into 2 or more than 2 paths at certain node, then the transfer functions from this node to the downstream nodes are difficult to calculate. Shimizu et al. investigated flux redistribution at a key branch node of α-ketoglutarate when varying activities of three key enzymes [8]. Their results indicated the possibility of incorporating enzymatic data into a metabolic model for representing carbon flux distributions at branched nodes. In this study, a novel metabolic model incorporating directed signal flow diagram with enzymatic activities data was proposed to reveal the natures of glutamate yield enhancement achieved under optimal $NaHCO_3$/pH manipulating conditions. We also attempted to use this generalized model to estimate product yield and to guide optimal enzymatic regulation way for other fermentations characterized with multiple products, enzymatic reactions and closed loops.

2. Materials and methods

2.1. Microorganism

Biotin auxotrophic *Corynebacterium glutamicum* S_{9114} was used. It is an industrial production strain of last generation, supplied by Shandong Linghua Co., Ltd., China. The major technical specifications of fermentation by this strain under standard operation condition (biotin limitation) are: glutamate yield of 45–50%, final glutamate concentration of 80 g/L (8%, w/w), and fermentation period of 34 h [9].

2.2. Fermentation medium and condition

The compositions of medium and fermentation conditions were the same as those previously reported [3,9–11], where biotin-limited condition was strictly controlled. In glutamate fermentation, ammonia water is a sole nitrogen source for glutamate synthesis and it also automatically regulates pH. During production phase, glucose consumption rate is closely correlated with ammonia consumption rate [11]. Two electronic balances connecting with an industrial computer via a multi-channels A/D–D/A converter (PCL-812 PG, Advantech Co., Taiwan) were used to on-line measure the weight losses of ammonia water (25%, w/w) and glucose (50%, w/w) feeding reservoirs, so that both ammonia water and glucose feeding amount could be monitored. By on-line measuring ammonia water consumption amount during certain control interval, 50% glucose was automatically fed into a 5 L fermentor (initial medium volume of 3.0 L) to maintain glucose concentration in the broth around 10–15 g/L by the control strategy previously reported [11]. The CO_2 and O_2 partial pressure in exhaust gas were on-line measured by a gas analyzer (LKM2000A, Lokas Ltd., Korea), oxygen uptake rate (OUR) and CO_2 evolution rate (CER) were then on-line calculated by the standard calculation formula. The accumulated CO_2 evolution amount $A_{CO_2}(t)$ and respiratory quotient RQ(t) at instant t ($t > t_0$) were then determined by the following equations:

$$A_{CO_2}(t) = \int_{t_0}^{t} \mathrm{CER}(t)\,dt \tag{1}$$

$$\mathrm{RQ}(t) = \frac{\mathrm{CER}(t)}{\mathrm{OUR}(t)} \tag{2}$$

Here, t_0 represented the time when production phase was considered to start ($t_0 \approx 10$ h).

Upon requirements, pH was manually shifted from 7.0 to 8.0 by adding ammonia water, and at this moment, the glucose automatic feeding system was temporarily suspended to avoid over-feeding. $NaHCO_3$ solution was added intermittently if its supplement is required. When $NaHCO_3$ addition was initiated, it was consecutively supplemented for 4 times at 2 h interval and $NaHCO_3$ amounted of 25 mmol/L-broth was pumped into the fermentor each time. The following five operations were manipulated: the control (pH controlled at 7.0 without any pH shift and $NaHCO_3$ addition); consecutively supplementing $NaHCO_3$ solution beginning at 10 h but controlling pH at 7.0 without shift; raising pH from 7.0 to 8.0 at 10 h but without $NaHCO_3$ supplement; consecutively adding $NaHCO_3$ and raising pH from 7.0 to 8.0 at 10 h simultaneously; raising pH to 8.0 at 10 h but consecutively adding $NaHCO_3$ solution beginning at 16 h. The data of control batch were used as the comparison base.

2.3. Analytical methods

The cell concentration was determined by measuring the optical density at 620 nm (OD_{620}). Glucose and glutamate concentrations were measured by a biosensor (SBA-40 C, Shandong Science Academy, China). The activities of 6 key enzymes involved with glutamate synthesis, namely those of pyruvate dehydrogenase (PDH), pyruvate carboxylase (PC), isocitrate dehydrogenase (ICDH), isocitrate lyase (ICL), α-oxoglutarate dehydrogenase complex (ODHC) and glutamate dehydrogenase (GDH) were assayed using the reported methods [11–13]. The enzymatic activities were expressed as U/ml-broth, where 1 U was defined as the volume of broth that converted 1 μmol NAD(P)H (or substrate) per minute. OD, glutamate concentration and all the enzymatic activities were measured in triplicate, the average values as well as the upper and lower deviation limits were determined accordingly.

2.4. Metabolic network and directed signal flow diagram of glutamate synthesis

Metabolic network of glutamate fermentation during production phase is shown in Fig. 1a according to the map depicted by Shimizu et al. [8]. The metabolic network is then reorganized and simplified as the directed signal flow diagram (Fig. 1b). Here, according to the metabolic reactions and their stoichiometric coefficients, transfer functions between intermediate nodes are marked with symbols of "a", "b_1", "b_2", ..., "h", and their values were at the range of [0,1] (except for a). It should be addressed that carbon numbers are not completely balanced at the nodes of "SUC" and "MAL" (refer to abbreviations) in the directed signal flow diagram, because glyoxylate shuttle was simplified by deleting the GLY node. However, this simplification does not affect the relevant transfer functions between the nodes. The two enzymes converting acetyl-CoA (Ac-CoA) into isocitrate (ICIT) and malate (MAL) via glyoxylate shuttle, namely citrate synthase and malate synthase, are difficult to measure. Therefore, it is assumed that the amount of