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Effect of biotin on transcription levels of key enzymes and glutamate efflux in glutamate fermentation by *Corynebacterium glutamicum*

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Abstract Biotin is an important factor affecting the performance of glutamate fermentation by biotin auxotrophic *Corynebacterium glutamicum* and glutamate is over-produced only when initial biotin content is controlled at suitable levels or initial biotin is excessive but with Tween 40 addition during fermentation. The transcription levels of key enzymes at pyruvate, isocitrate and α-ketoglutarate metabolic nodes, as well as transport protein (TP) of glutamate were investigated under the conditions of varied biotin contents and Tween 40 supplementation. When biotin was insufficient, the genes encoding key enzymes and TP were down-regulated in the early production phase, in particular, the transcription level of isocitrate dehydrogenase (ICDH) which was only 2 % of that of control. Although the cells' morphology transformation and TP level were not affected, low transcription level of ICDH led to lower final glutamate concentration (64 g/L). When biotin was excessive, the transcription levels of key enzymes were at comparable levels as those of control with ICDH as an exception, which was only 3–22 % of control level throughout production phase. In this case, little intracellular glutamate accumulation (1.5 mg/g DCW) and impermeable membrane resulted in non glutamate secretion into broth, even though the quantity of TP was more than 10-folds of control level. Addition of Tween 40 when biotin was excessive stimulated the expression of all key enzymes and TP, intracellular glutamate content was much higher (10–12 mg/g DCW), and final glutamate concentration reached control level (75–80 g/L). Hence, the membrane alteration and TP were indispensable in glutamate secretion. Biotin and Tween 40 influenced the expression level of ICDH and glutamate efflux, thereby influencing glutamate production.

Keywords Glutamate fermentation · Biotin · Transcription level · Glutamate secretion · Transport protein

Introduction

Corynebacterium glutamicum is a typical strain producing nucleotides and amino acids, especially L-glutamate. Glutamate is widely used in food, pharmaceutical, and other industry with a production scale of more than 2.2 million tons per year (Sano 2009). Biotin is the most important trace element significantly affecting cell growth and glutamate productivity during glutamate fermentation. Almost no glutamate was accumulated when biotin was in excess, while about 0.3 mol glutamate was produced from 1 mol glucose when biotin was insufficient. It was reported that 0.45–0.8 mol glutamate was produced from 1 mol glucose when 1.5 g/L Tween 40 or 2,200 U/L penicillin was added in the biotin-excessive medium (Hasegawa et al. 2008). Therefore, glutamate over-production could be realized under the conditions that biotin amount in medium is suitable, or certain fatty acid ester surfactant (such as Tween 40) or penicillin is added into the medium with excessive biotin.

Many researches regarding the effects of biotin content and addition of Tween 40 or penicillin on glutamate

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fermentation were reported, and most of them were focused on the activities of key enzymes involved and membrane integrity. Pyruvate carboxylase (PC) was activated and the activities of pyruvate dehydrogenase (PDH) and α-oxoglutarate dehydrogenase complex (ODHC) were repressed in the cases of biotin limitation and addition of Tween 40 or penicillin (Hasegawa et al. 2008; Kimura 2002). It was reported that biotin was the coenzyme of phospholipids synthesis, and addition of Tween 40 or penicillin resulted in down-regulation of *dtsR* encoding the subunit of acetyl-CoA carboxylase involved in fatty acid synthesis. 30 % of the membrane phospholipids was lost under the conditions of biotin starvation or addition of surfactant (Kimura 2002; Clément and Lanéelle 1986). Mycolic acids content of the outer cell layer decreased under the conditions that glutamate over-production was induced (Hashimoto et al. 2006). Membrane became permeable due to the change of membrane structure and thus glutamate secretion was induced. However, it was found that specific carrier was required for glutamate secretion in *C. glutamicum* (Hoischen and Krämer 1990). Some common amino acids, such as L-lysine, L-isoleucine and L-threonine, were exported by special carriers respectively (Vrljic et al. 1996; Simic et al. 2001; Kennerknecht et al. 2002). A putative transport carrier of glutamate encoded by gene NC*gl*1221 was found in *C. glutamicum* and the structure of this carrier protein altered with the efflux of glutamate in the overproduction strain (Yao et al. 2009; Nakamura et al. 2007). However, it remains unclear whether the carrier is affected by biotin or Tween 40.

Transcription is an essential step in gene expression and transcriptional regulation determines the molecular machinery for homeostasis and adaptation (Balleza et al. 2009). The abundance of 139 proteins in cytoplasmid and cell wall biosynthesis proteins changed when *C. glutamicum* grew on different carbon sources (Haussmann and Poetsch 2012). Expression of about 10 % of the genes in *C. glutamicum* was shown to respond to heat shock (Ehira et al. 2009). The fermentative production of glutamate has been carried out for more than 45 years and the complete DNA sequence of *C. glutamicum* has been already reported, but knowledge on the molecular basis of the glutamate over-production remained limited (Ikeda and Nakagawa 2003; Kalinowski et al. 2003; Yukawa et al. 2007). Glutamate synthetic pathway was consisted of complicated enzymatic reactions and the change in the expression of any enzyme could lead to different fermentation performance. Therefore, the studies concerning transcriptional response to different biotin content in the medium and Tween 40 addition during glutamate fermentation are important, but relevant reports are seldom available. In this study, as the first step to understand the molecular mechanisms for glutamate over-production by *C. glutamicum*, the transcription levels of the enzymes at key metabolic nodes (PYR, ICIT and α-KG) and transport carrier during production phase were investigated, when biotin content in the medium was different and Tween 40 was supplemented during fermentation. The results would elucidate the transcriptional regulation nature of glutamate over-production by *C. glutamicum*. Relevant useful information and reference would be provided for optimizing glutamate fermentation by development of new strain and operation mode.

Materials and methods

Strain and culture conditions

Strain: *Corynebacterium glutamicum* ATCC13032, kept by the laboratory was used throughout this study.

The seed microorganism was grown in shake flasks at 30 °C, 220 r/min for 9 h in the liquid medium containing (in g/L): glucose 25, K_2HPO_4 1.5, $MgSO_4$ 0.6, $MnSO_4$ 0.005, $FeSO_4$ 0.005, corn slurry 40, and urea 3 (sterilized separately). Initial pH was adjusted to 7.0–7.2.

The medium for jar fermentation contained (in g/L): glucose 140, K_2HPO_4 1.5, $MgSO_4$ 0.6, $MnSO_4$ 0.002, $FeSO_4$ 0.002, thiamine 5.0×10^{-5} and urea 3 (sterilized separately). In the control, 15 g/L corn slurry was added in the medium which was equivalent to a biotin content of 12 μg/L (Diao 2008), and this amount was considered to be the optimal in this study. 10 μg/L pure biotin and 15 g/L corn slurry were added at the beginning of fermentation to create the condition of biotin in excess (about 22 μg/L biotin). Only 5 g/L corn slurry (about 4 μg/L biotin) was added in the medium to cause biotin insufficiency. 5 g/L Tween 40 was added at 10 h according to the experimental requirement.

The seed (8 %, v/v) was inoculated in a 5 L fermentor (BIOTECH-5BG, Baoxing Co., China) containing 3 L medium for glutamate fermentation at 32 °C. pH was controlled at 7.0 ± 0.1 by automatically adding 25 % (w/v) ammonia water which also supplied nitrogen source for glutamate synthesis. Dissolved oxygen (DO) concentration was controlled at 20 % by manually adjusting agitation speed. The air aeration rate and pressure inside fermentor were maintained at 1.33 vvm and 0.07 MPa respectively. Concentrated glucose was fed when glucose concentration decreased to a low level (<20 g/L) to maintain glucose concentration at suitable levels (10–20 g/L).

Analytical methods

The concentration of cells was assayed by spectrophotometer at 620 nm (OD_{620}). Glucose and glutamate concentration were measured by a biosensor (SBA-40C,

Shandong Science Academy, China). Oxidation–reduction potential (ORP) was assayed by an ORP electrode (Suzhou Han Star Electrochemical Co. Ltd., China). The partial pressure of CO_2 and O_2 in exhaust gas was measured on-line by a gas analyzer (LKM2000A, Lokas Co. Ltd, Korea), and then O_2 uptake rate (OUR), CO_2 evolution rate (CER) and respiratory quotient (RQ) were on-line calculated by a computer connected with gas analyzer based on the standard calculation formula. The data of ORP, CER, OUR and RQ was recorded on line by the computer every minute and they were smoothed by mean filtering for analysis. Cells were collected by centrifugation at 8000 r/min for 15 min and then suspended in deionized water. The suspension was sonicated by a sonifier for 10 min (pulse on 4 s, pulse off 2 s). Cell debris was removed by centrifugation at 8,000 r/min for 20 min and the supernatant was used to measure intracellular glutamate content.

RNA purification, cDNA synthesis, and real-time fluorescence quantitative PCR analysis

The transcription level of pyruvate dehydrogenase (PDH), pyruvate carboxylase (PC), isocitrate dehydrogenase (ICDH), isocitrate lyase (ICL), α-oxoglutarate dehydrogenase complex (ODHC), glutamate dehydrogenase (GDH) and transport protein (TP) was analyzed. The genes corresponding to the enzymes and TP were *pdh*, *pc*, *icdh*, *icl*, *odhc*, *gdh* and *tp*. Total bacterial RNA was extracted using Trizol Plus RNA Purification Kit (Invitrogen^{TM}), and the purification method described in the manual of the kit. Total RNA was used as template to synthesize cDNA, and cDNA products were amplified by the method of real-time fluorescence quantitative PCR with primers of *pdh*-F (5′-GCCAAACCAGATCCGTGAA-3′), *pdh*-R (5′-TGGGCGGGTATCAGAGAAAC-3′), *pc*-F (5′-CCGAAGCCAACCGAAGAGT-3′), *pc*-R (5′-CGCATCCAGGCGAACAAG-3′), *idhc*-F (5′-ACGCAGCACTGGGTTCACA-3′), *idhc*-R (5′-CAGAAGGTAGGCAACGCACTC-3′), *icl*-F (5′-ACACCCCAACTGTTGTTATCG-3′), *icl*-R (5′-CGTATGGTGCGTAGGACTTTG-3′), *odhc*-F (5′-GTTGTAGCGGGGGTAGTATTTG-3′), *odhc*-R (5′-AGACGAGATGTTCCAGCAGTTC-3′), *gdh*-F (5′-CTTCACAATGCCCAGGTTTACA-3′), *gdh*-R (5′-GCAGTGGCAGAGGTTTTGGA-3′), *tp*-F (5′-TGATGGGCTTCGGATTTTTC-3′), and *tp*-R (5′-ACGAGGTCGGTGCGGTAA-3′). 16SrRNA was adopted as reference gene. The following PCR conditions were adopted: an initial denaturation step at 95 °C for 10 min, followed by an amplification and quantification program repeating for 40 cycles (95 °C for 10 s, 60 °C for 60 s with a single fluorescence measurement), and then a melting curve program (a continuous fluorescence measurement raising temperature from 60 to 95 °C with a slow heating unit).

Results

Fermentation performance under different operation conditions

The fermentation performance under the conditions of different initial biotin contents and addition of Tween 40 during fermentation was shown in Fig. 1. Glutamate fermentation is non-growth associated fermentation process. Generally, cells grow exponentially during cells growth phase (0–10 h) and glutamate production does not occur. In production phase (10–34 h), cells enlarge gradually and cells' morphological shape transform into long-narrow shape to begin to produce glutamate (Yu 2009). Final glutamate production was about 75–80 g/L in the control and the data of control was used as comparison basis.

When biotin was insufficient, cells grew slowly and the maximum OD_{620} was only half of that of control. Although the transformation of cells' morphology occurred normally, the glutamate concentration ended at 64 g/L. CER and OUR were lower than those of control during the entire production phase and RQ was always at low level (0.75 → 0.45). Under this condition, TCA cycle was weakened (lower RQ) and less NADH was produced (Xiao et al. 2006). NAD^+/NADH ratio in vivo was higher than that of control, which was reflected by the higher oxidation–reduction potential (ORP) values (Li et al. 2010). When biotin was in excess, cell growth was vigorous and the maximum OD_{620} was 50 % higher than that of control, but the morphological shape of cells did not change. CER and OUR stayed at a much higher levels and RQ kept around 1.0 steadily throughout the production phase. It was indicated that, in this case, glucose was dominantly directed into TCA cycle (Xiao et al. 2006), and a large amount of NADH was produced leading to lower ORP (−230 to −150 mV) in the production phase. The intracellular metabolic flux was redistributed and channeled out of glutamate synthesis route due to improper ORP when biotin was insufficient or excessive (Sridhar and Eiteman 1999).

Under the condition of adding 5 g/L Tween 40 at 10 h when initial biotin was excessive, CER and OUR decreased gradually from high levels to the comparable levels of control with a 4–5 h time delay after adding Tween 40. Then cells' morphological shape transformation occurred and cell growth ceased and glutamate began to accumulate. In addition, RQ declined and ORP rebounded immediately after adding Tween 40, and then they were maintained at the desirable levels (RQ at about 0.75, ORP at about −100 mV). Glutamate concentration eventually reached the level of the control (76 g/L). In order to unravel the intrinsic difference among different operation conditions, the expression level of the key enzymes catalyzing the main reactions in glutamate synthetic pathway was analyzed.

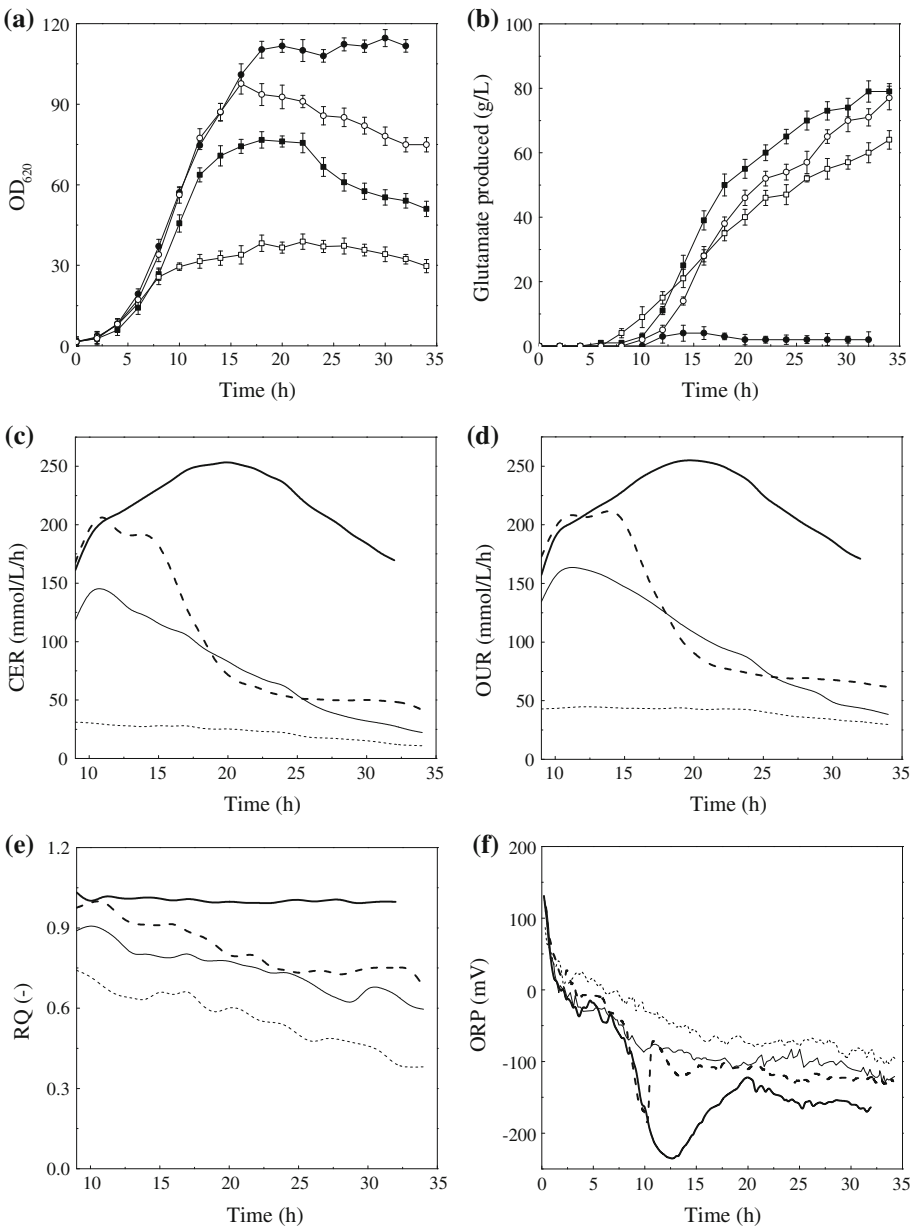

Fig. 1 Changing pattern of glutamate fermentation performance under different operation conditions. **a** The changing pattern of cells concentration; **b** the changing pattern of glutamate concentration; **c** the changing pattern of CO_2 evolution rate (CER); **d** the changing pattern of O_2 uptake rate (OUR); **e** the changing pattern of respiratory quotient (RQ); **f** the changing pattern of oxidation–reduction potential (ORP). *Filled square* control; *filled circle* initial biotin in excess; *open square* initial biotin in shortage; *open circle* initial biotin in excess with addition of 5 g/L Tween 40 at 10 h; *thin solid line* control; *thick solid line* initial biotin in excess; *thin dash line* initial biotin in shortage; *thick dash line* initial biotin in excess with addition of 5 g/L Tween 40 at 10 h

The effect of biotin and Tween 40 on the transcription level of key enzymes

Glutamate synthetic pathway includes glycolytic pathway, pentose phosphate pathway, CO_2 fixing reaction, TCA cycle and glyoxylate shuttle. The carbon flux proportion of pentose phosphate pathway was almost not affected by biotin content. However, the ratio of carbon flux distributions at the nodes of pyruvate (PYR), isocitrate (ICIT) and α-ketoglutarate (α-KG) were affected by biotin content (Yu 2009). Figure 2 showed the simplified glutamate synthetic pathway accordingly. The key enzymes catalyzing the branched reactions at PYR, ICIT and α-KG nodes largely affected glutamate production. The differences in glutamate production mainly occurred in the early (10–20 h) and late (26–34 h) production phase (Fig. 1b), and the typical characteristics of glutamate synthesis could be fully displayed by the data obtained at 14 and 32 h. Therefore, the transcriptional level of the genes encoding the key enzymes at 14 and 32 h under different conditions were shown in Fig. 3. Here, the relative transcriptional level (RTL) was used for comparison or interpretation as described by Eq. (1).

$$RTL_i^j(t) = \frac{TL_i^j(t)}{TL_i^c(t)} \quad (1)$$

where TL refers to the measured gene transcriptional values, i represents the value for i-th gene encoding the corresponding key enzymes ($i = 1, 2, \ldots, 6$; *pdh*, *pc*, *icdh*, *icl*, *odhc* and *gdh*), j accounts for the different operation conditions ($j = 1, 2, 3, 4$; control, insufficient biotin, excessive biotin, excessive biotin but with Tween 40 addition during operation), t is the instance of measurement of data (14 and 32 h), c represents the case of control. According to Eq. (1), the RTL of all the 6 key enzymes in the control case was 1.

When biotin was in shortage, RTL of most key enzymes was 30–50 % of control level at 14 h, while RTL_{icdh} was only 2 % of that of control. ICDH was the key enzyme for formation of the glutamate precursor (α-KG), and 10–20 h was the main glutamate production phase during which glutamate accumulation dominantly occurred. In this case, less glutamate was produced, although RTL of all the key enzymes could recover to comparable level with control in the late production phase (32 h). These results indicated that the negative effect due to insufficient biotin on glutamate synthesis during the main production phase was irreparable.

When biotin was in excess, there were no big differences in RTLs of the key enzymes as compared with those of control at 14 and 32 h with RTL_{icdh} as exception. RTL_{icdh} was only 3 and 22 % of that of control at 14 h and 32 h respectively. Similar to the case of biotin in shortage, low expression of *icdh* also limited the supplement of α-KG. However, RTL_{odhc} in the case of excessive biotin was threefolds of that in the case of biotin in shortage. The higher RTL_{odhc} in the former could not promote glutamate over-production even though the RTL_{gdh} was also higher. The carbon flux distribution at α-KG node was determined by ODHC instead of GDH, and it was reported that glutamate production decreased by 45 % when the strain with *gdh* amplification was used (Shimizu et al. 2003). Therefore, in the case of biotin in excess, most of the carbon flux at α-KG node was directed into TCA cycle rather than glutamate synthesis route.

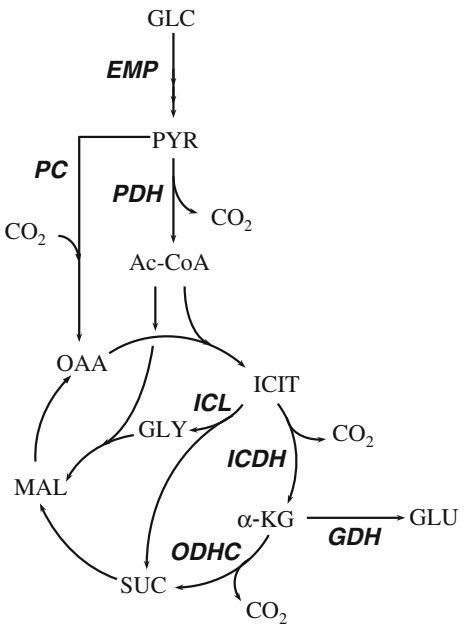

Fig. 2 Simplified pathway of glutamate synthesis. *GLC* glucose, *PYR* pyruvate, *Ac-CoA* acetyl-CoA, *ICIT* isocitrate, *α-KG* α-oxoglutarate, *GLU* glutamate, *MAL* malate, *OAA* oxaloacetate, *PDH* pyruvate dehydrogenase, *PC* pyruvate dehydrogenase, *ICDH* isocitrate dehydrogenase, *ICL* isocitrate lyase, *GDH* glutamate dehydreogenase, *ODHC* α-oxoglutarate dehydrogenase complex

When initial biotin was in excess but 5 g/L Tween 40 was added at 10 h, the RTL of most key enzymes was slightly higher than that of control at 14 h and increased significantly (≥threefolds) at 32 h. Under this condition, all the metabolic pathways were accelerated, particularly that of TCA cycle in downstream after α-KG node (RTL_{odhc} was about 17-folds of control level). Consequently, the carbon flux at the node of α-KG towards glutamate synthesis did not increase significantly, even though RTL_{gdh} also increased. Furthermore, the respiratory chain was active and most of NAD(P)H was used to produce ATP, thus the higher GDH was not fully utilized for glutamate synthesis because it was limited by the less coenzyme [NAD(P)H] regeneration rate. Therefore, the final glutamate production was not accordingly improved, but merely reached the control level.

From the above results, it could be concluded that the expression level of *icdh* was very low when biotin was in excess and in shortage, while glutamate was accumulated in the broth only when biotin was insufficient. It was commonly believed that intracellular glutamate was not secreted into broth when biotin was excessive due to the impermeable membrane (Yu 2009; Hashimoto et al. 2006). However, it also might be true that no glutamate was synthesized, while the expression of exporter might be regulated by the concentration of cell-internal glutamate (Eggeling and Sahm 2001). Hence, intracellular glutamate content and the transcription level of transport protein (TP) under different operation conditions were analyzed.

Transcription level of TP and intracellular glutamate under different operation conditions

The transcription level of TP and intracellular glutamate content under different operation conditions were shown in Fig. 4. When biotin was in shortage, RTL_{tp} was 50 and 70 % of control level respectively at 14 and 32 h, and the changing pattern of intracellular glutamate content in the production phase was same as that of control. Under this condition, glutamate content in vivo increased to the maximum at 14 h, then it rapidly declined and then

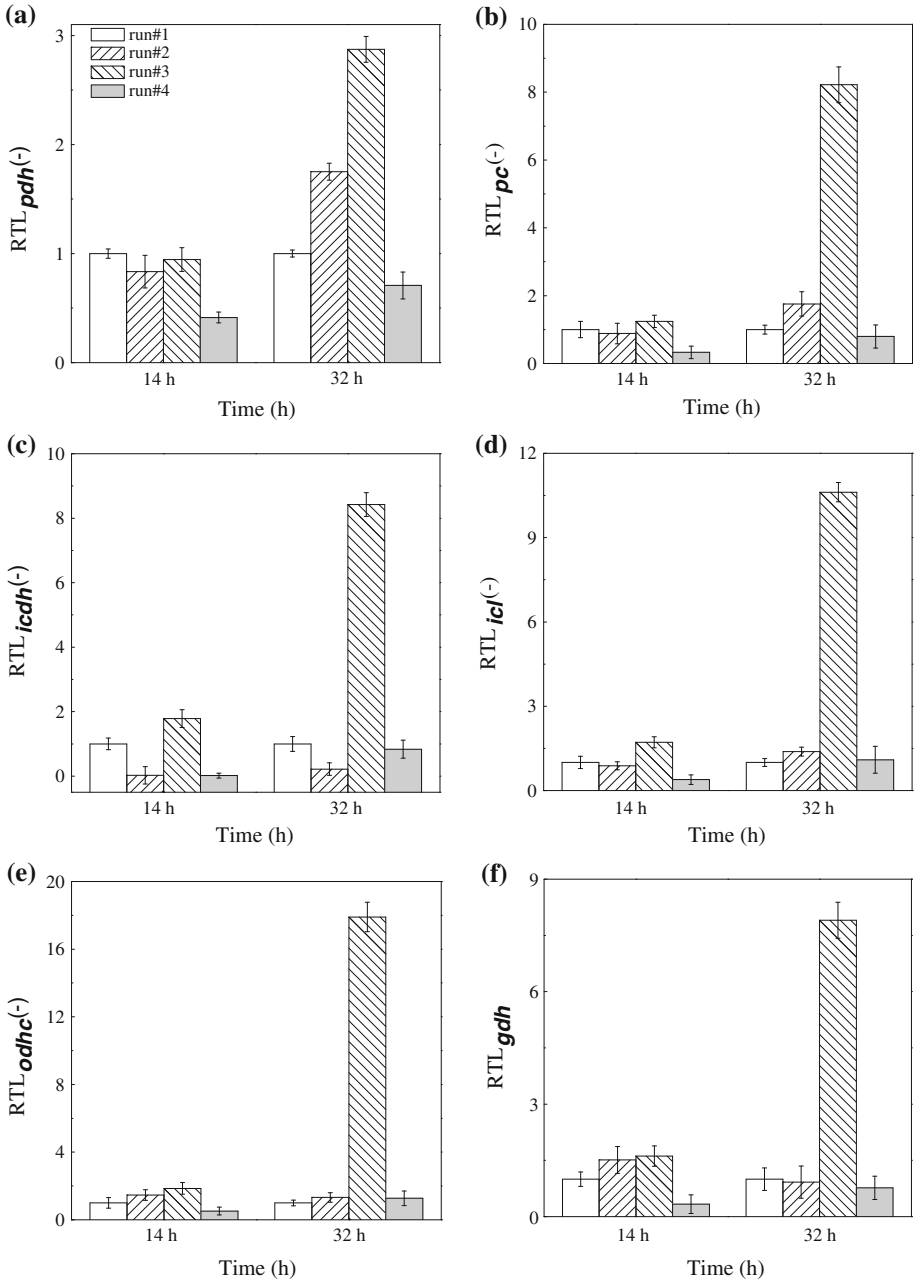

Fig. 3 The transcription level of key enzymes in the early (14 h) and late (32 h) production phase when initial biotin amount is different. *Run #1* control; *run #2* initial biotin in excess; *run #3* initial biotin in excess with addition of 5 g/L Tween 40 at 10 h; *run #4* initial biotin in shortage

maintained at the comparable level of control (2.5 mg/g DCW) after 20 h. However, intracellular glutamate content before 20 h was 30–70 % lower than that of control. When biotin was excessive, RTL_{tp} was more than 10-folds of that of control and the maximum intracellular glutamate content was achieved at 10 h (6.8 mg/g DCW), which was about twofolds of the control level at the same time. After that, intracellular glutamate concentration decreased and then maintained at a lower level constantly (1.5 mg/g DCW). Almost no glutamate was detected in the broth throughout the production phase when biotin was in excess (Fig. 1b), even though intracellular glutamate was higher or equivalent than/with that in the case of biotin in shortage at 10–12 h. It was demonstrated that increasing quantity of carriers alone could not lead to glutamate secretion and TP must be activated by alteration of membrane tension before transporting glutamate (Nakamura et al. 2007). Therefore, although glutamate formation was earlier in the cell when biotin was excessive, glutamate could not be transported to the outside of cell. As a result, further glutamate synthesis in vivo was hindered and RTL_{gdh} decreased in the late production phase (Fig. 3f).

When biotin was in excess and Tween 40 was added at 10 h, RTL_{tp} at 14 h was slightly higher than that of control and it increased to 22-folds of control level at 32 h. Under this condition, intracellular glutamate content was

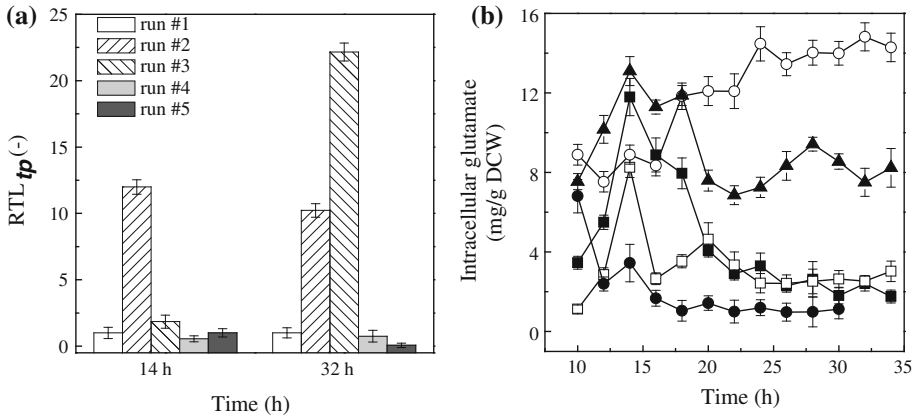

Fig. 4 The transcription level of TP (**a**) and changing pattern of intracellular glutamate content (**b**) under different operation conditions. *Filled square* control; *filled circle* initial biotin in excess; *open square* initial biotin in shortage; *open circle* initial biotin in excess with addition of 5 g/L Tween 40 at 10 h; *filled triangle* abnormal batch. *Run #1* control; *run #2* initial biotin in excess; *run #3* initial biotin in excess with addition of 5 g/L Tween at 10 h; *run #4* initial biotin in shortage; *run #5* abnormal batch

maintained at 8 mg/g DCW at 10–16 h and then increased to a further higher level (12–14 mg/g DCW). However, glutamate accumulation rate in the broth did not increase significantly, especially in the late production phase. It might be due to the fact that, glutamate efflux was an energy consuming process (Gutmann et al. 1992), and the transport rate was dependent on the energy when the carrier was saturated. The cell viability decreased (lower OUR in Fig. 1d) in the late production phase and the energy was not sufficient enough. Consequently, even though more carriers existed and intracellular glutamate was much higher, final glutamate concentration was not improved as expected.

On the other hand, glutamate accumulation did not occur if altering the membrane structure only (Hoischen and Krämer 1990). Glutamate production ceased in the late production phase in an abnormal batch (the data was not listed). In this abnormal fermentation, the cell morphology transformation occurred normally and intracellular glutamate content was at higher level, but there was nearly no transport carrier in the late production phase (RTL_{tp} was 1 % of control level at 32 h, as shown in Fig. 4). Therefore, the alteration of membrane structure and specific carrier were indispensable for glutamate secretion.

Conclusions

Initial biotin content had great impact on the transcription level of ICDH and ODHC and glutamate secretion during glutamate fermentation, accordingly influencing glutamate production. Insufficient biotin led to low expression of ICDH in the main production phase which directly caused the decrease of glutamate synthesis, but glutamate secretion was not affected. In the case of biotin in excess, low expression of ICDH and higher expression of ODHC resulted in low intracellular glutamate synthesis. In this case, glutamate efflux was hampered because the membrane structure did not alter, even though transport carriers were sufficient enough. Supplement of Tween 40 when biotin was excessive activated all the metabolic pathways and induced alteration of membrane structure. With the higher intracellular glutamate content and abundant glutamate transport carrier, final glutamate concentration reached control level. Therefore, up-regulation of *icdh*, down-regulation of *odhc* and normal glutamate secretion were simultaneously required for glutamate over-production. The alteration of membrane structure and the glutamate transport protein were indispensable for glutamate secretion.

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