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Flexibility of the metabolism of *Corynebacterium glutamicum* 2262, a glutamic acid-producing bacterium, in response to temperature upshocks

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In order to test the temperature sensitivity of glutamate production metabolism, several temperature shifts, from 33 to 37, 38, 39, 40 or 41°C, were applied to the temperature-sensitive strain, *Corynebacterium glutamicum* 2262, cultivated in a 24-h fed-batch process. Whereas glucose was entirely dedicated to biomass synthesis when cells were grown at 33°C, applying temperature upshocks, whatever their range, triggered a redistribution of the carbon utilisation between glutamate, biomass and lactate production. Although increasing the culture temperature from 33 to 37, 38, 39 or 40°C resulted in final glutamate titers superior to 80 g/l, temperatures resulting in the best chanelling of the carbon flow towards glutamic acid synthesis were 39 and 40°C. Moreover, this study showed that the higher the temperature, the slower the growth rate and the higher the lactate accumulation.
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Introduction

The production of glutamate by *Corynebacterium glutamicum* is an important part of the industrial exploitation of microbiology [12]. The current annual production of glutamate reaches approximately 900,000 tons. In view of this fermentation's economic impact, extensive studies were dedicated to selecting new *C. glutamicum* strains able to produce ever-increasing amounts of glutamate. Bacteria sensitive to various means of triggering glutamate excretion have been discovered. So far, the major processes developed industrially are biotin limitation [1,3,11,19], surfactant addition [14,17] and penicillin addition [16,17].

Glutamate production is strongly dependent on the treatment that induces glutamate excretion. Slight modifications often result in a decreased glutamate accumulation. In biotin-limited conditions, glutamate excretion triggering is closely related to biotin concentration in the culture medium. Whereas 20 μg/l of biotin are sufficient to allow *C. glutamicum* growth, glutamate excretion occurs only when the biotin external concentration falls to 3 μg/l [19]. In a process using surfactant addition, the amount of added surfactant, or more precisely the ratio of surfactant concentration to biomass concentration, is a key parameter. It has to be sufficient to increase membrane permeability; however, too great an elevated surfactant concentration should lead to cellular death and thus to the decrease of glutamate production [4].

To date, few data are available on the effect of temperature on glutamate production and on *C. glutamicum* metabolism. In 1978, Momose and Takagi [15] isolated several temperature-sensitive mutants of *Brevibacterium lactofermentum*. Performing either of two temperature shifts (from 30 to 37°C or from 30 to 40°C), they showed that, in their culture conditions, the less temperature sensitive the mutant was, the more severe condition had to be adopted for glutamic acid production. A temperature increase from 34 to 38°C was used as well during a process in which glutamate excretion was induced by Tween-40 addition [9]. This increase occurred 2 h after surfactant addition, in order to limit biomass accumulation and thus to favor the redirection of the carbon flow towards glutamic acid synthesis.

Recently, we presented a fed-batch process enabling the production of 85 g/l of glutamate using *C. glutamicum* in a biotin-rich medium by increasing the culture temperature from 33 to 39°C [6]. However, the effect of different temperature shifts was not reported. In the present paper, we investigated the sensitivity of the metabolism of *C. glutamicum* 2262 to various temperature shifts.

Materials and methods

Bacterial strain and medium composition

The strain used throughout this study was *C. glutamicum* 2262 [6]. The composition of the glutamate production medium used was based on MCGC medium [20] although citrate (used as chelating agent) was replaced by deferoxamine. Glucose was used as the sole carbon substrate. This medium consisted of: 60 g/l glucose, 3 g/l Na_2HPO_4, 6 g/l KH_2PO_4, 2 g/l NaCl, 8 g/l $(NH_4)_2SO_4$, 0.4 g/l $MgSO_4{\cdot}7H_2O$, 40 mg/l $FeSO_4{\cdot}7H_2O$, 3.9 mg/l $FeCl_3$, 0.9 mg/l $ZnSO_4{\cdot}7H_2O$, 0.3 mg/l $CuCl_2{\cdot}2H_2O$, 3.9 mg/l $MnSO_4{\cdot}H_2O$, 0.1 mg/l $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.3 mg/l $Na_2B_4O_7{\cdot}10H_2O$, 84 mg/l $CaCl_2$, 4 mg/l biotin, 20 mg/l thiamine, 3 mg/l deferoxamine, 2 g/l glycine betaine. In fed-batch cultures, this medium was supplemented with 1.3 g/l polypropylene glycol

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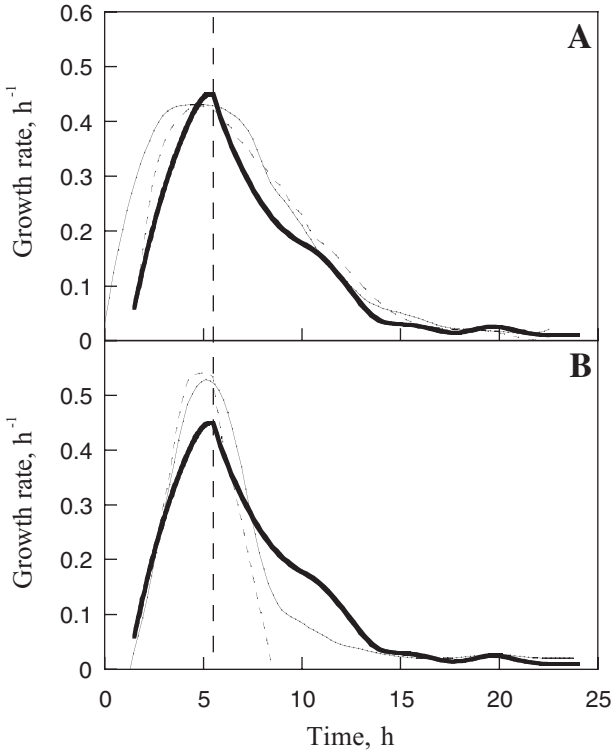

Figure 1 The specific growth rate in fed-batch cultures using *C. glutamicum* 2262 with a temperature upshock from 33 to 37 (A, dashed line), 38 (A, full thin line), 39 (A and B, full thick line), 40 (B, full thin line) or 41°C (B, dashed line). The vertical dashed line indicates the time when the broth temperature was increased.

used as antifoaming agent, and additional amounts of glucose were added, as required, during the production phase.

Fermentation conditions

The seed was grown in shake-flask cultures at 33°C in modified MCGC medium, supplemented with Na_2HPO_4, 3.8 g/l lactic acid and 1.9 g/l urea. The glucose concentration was decreased to 34 g/l and the initial pH was adjusted to 7.6 with 12 N NaOH.

For fed-batch cultures, 40 ml of an overnight culture of *C. glutamicum* was used to inoculate a 2-l fermentor (Biolafitte, Saint Germain en Laye, France) containing 0.75 l of modified MCGC. The culture was grown at 33°C with an aeration rate of 60 l/h. The pH set point was 7.6, regulated with 12 N NH_4^+. When the biomass concentration reached 5.6 g/l, the culture temperature was increased (from 33 to 37, 38, 39, 40 or 41°C) to provoke glutamate production. Three hours after the temperature shift, the culture was pulsed with a concentrated glucose solution (500 g/l) to avoid periods of glucose limitation.

Analytical methods

During fermentation, samples were collected to determine biomass, glucose, glutamate, lactate and trehalose concentrations. The cell concentration was measured by absorbance at 570 nm and by a direct gravimetric method to avoid artifacts due to changes in cell morphology during the fermentation. After centrifugation, trehalose and lactic acid analyses were performed on the supernatant by HPLC (Waters model 712, St Quentin, France) using a Brownlee Polypore H column, operated at 65°C and with H_2SO_4 (0.02 M) elution, UV and refractometer as detectors. The amount of glucose and glutamate in the supernatant were determined enzymatically (Sigma, Saint Quentin Fallavier, France, ref. 510-A and Diffchamb, Lyon, France, ref. 139 092, respectively).

Results

Growth of the temperature-sensitive C. glutamicum 2262 submitted to various temperature shifts

When *C. glutamicum* 2262 cells were cultivated in a fed-batch process at 33°C, cell growth rapidly reached a specific maximal rate of 0.5 h^{-1} without significant production of organic or amino acids in the culture medium. This rapid growth resulted in a high biomass concentration (52 g/l) exceeding the oxygen transfer capacity of the reactor, thus leading to an oxygen limitation and a stop of biomass synthesis (data not shown).

In order to assess the effect of the temperature sensitivity of *C. glutamicum* 2262, different temperature upshocks were imposed on fed-batch cultures. The culture was first grown at 33°C; then, when the biomass concentration reached 5.6 g/l, the temperature was rapidly increased to 37, 38, 39, 40 or 41°C, and was maintained during the rest of the 24-h fed-batch process.

The temperature shift between 33 and 39°C was described in a previous publication [6]. It resulted in a decrease in the specific growth rate to 0.03 h^{-1} (6% of the specific growth rate measured at 33°C) at which the culture remained for the rest of the culture period. Figure 1A and B clearly show that, whatever its range, the temperature upshock induced a deceleration of growth. However, the higher the temperature, the sooner cell division stopped (Figure 2A). This explained why the final biomass concentration decreased as the temperature upshock increased (Figure 2B). Indeed, after

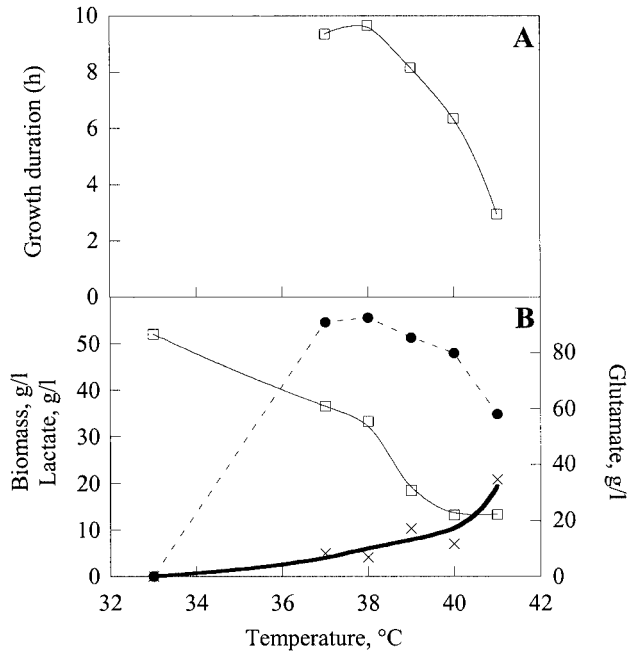

Figure 2 Growth duration (specific growth rate above 0.05 h^{-1}) after the temperature shift (A), final concentrations of biomass (B, □), lactate (B, ×) and glutamate (B, ●) during fed-batch cultures without (33°C throughout the culture) or with a temperature increase from 33 to 37, 38, 39, 40 or 41°C.

only 3 h at 41°C the specific growth rate was below 0.05 h^{-1} and the final biomass concentration was as low as 13 g/l, whereas at 37 and 38°C, growth stopped after only 9.5 h after the temperature shift, and the final biomass reached, respectively, 36.5 and 33.2 g/l. Thus, *C. glutamicum* 2262 growth is highly sensitive to medium temperature.

Glutamate and lactate production

Contrary to a culture maintained at 33°C throughout the process during which no glutamate accumulation occurred, glutamate excretion occurred whatever the temperature shift was (Figure 2B). Surprisingly, whereas the final biomass concentration was strongly affected by temperature elevation between 37 and 40°C, few modifications were measured in the final glutamate concentration. More than 80 g/l glutamate were obtained with all these temperature shifts. Compared to the glutamate accumulation observed during the reference process (33 to 39°C), a difference of 7 g/l glutamate at most (8%) was measured in the final amino acid titer among the processes ranging from 37 to 40°C. The highest final glutamate concentration was obtained at 38°C (92 g/l). Interestingly the elevation of the broth temperature to 41°C resulted in a low glutamate production of 58 g/l. Whatever the temperature shift range, the glutamate-specific production rates displayed similar profiles: after a rapid increase over a few hours, the production rate slowly declined till the end of the culture period (Figure 3A and B). Thus, the bacteria were unable to maintain a fast glutamate-specific production rate throughout the 24-h fed-batch process. From 37 to 39°C the glutamate specific production rate was enhanced throughout the glutamate accumulation phase. Similar specific production rates were obtained at 39 and 40°C, but a dramatic decrease in the glutamate-specific production rate was measured at 41°C. Thus, though the thermal process could be considered as quite robust in terms of final glutamate concentration, the temperature during the glutamate production phase had a dramatic effect on the bacterial physiology. Indeed, a deviation of 1°C induced important changes in the cells' capacity to excrete glutamate. At 37 and 38°C, the lowered capacity of cells to excrete glutamate was corrected by an increased biomass.

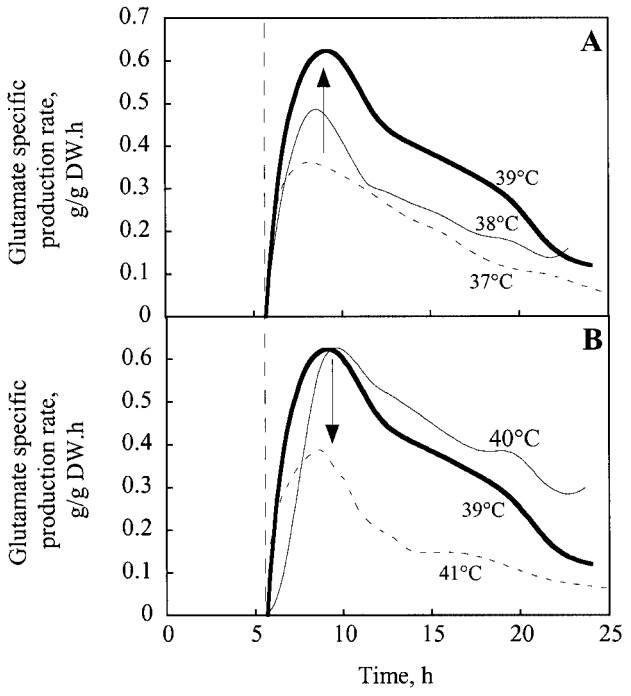

Figure 3 The glutamate specific production rate during fed-batch cultures using *C. glutamicum* 2262 with a temperature upshock from 33 to 37 (A, dashed line), 38 (A, full thin line), 39 (A and B, full thick line), 40 (B, full thin line) or 41°C (B, dashed line). The vertical dashed line indicates the time when the broth temperature was increased.

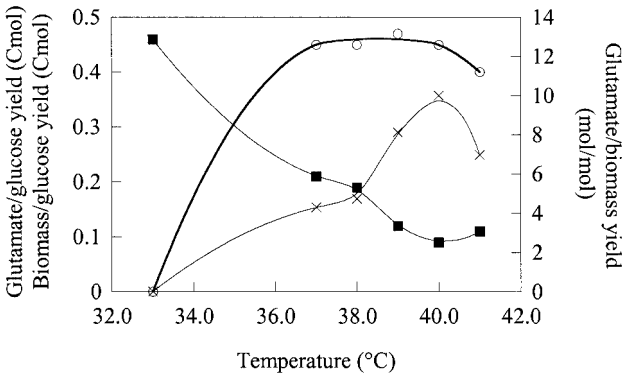

Figure 4 Effects of glutamate on glucose (○), biomass on glucose (■) and glutamate on biomass (×) ratios during fed-batch cultures without (33°C throughout the culture) or with a temperature increase from 33 to 37, 38, 39, 40 or 41°C.

As shown in Figure 4, the glucose to glutamate conversion yield remained relatively constant around 0.45 for temperatures between 37 and 40°. Conversely, the glutamate/biomass yield was extremely sensitive to temperature: up to 40°C, the higher the temperature, the higher the glutamate produced per gram of biomass. This result confirmed that 39 and 40°C were the best temperatures to redirect the carbon flux towards glutamate production at the expense of biomass synthesis. At 41°C, a decrease in both glutamate/glucose and glutamate/biomass ratios were observed.

Redirection of carbon flow towards fermentative pathways as a consequence of oxygen limitation has already been described [7]. However, whatever the temperature between 37 and 41°C, lactate was produced, whereas no oxygen limitation occurred (data not shown). Therefore, during temperature-triggered glutamic acid fermentation, lactate could be considered as a co-metabolite as previously mentioned [6]. Lactate production slightly increased as the temperature was increased between 37 and 40°C (Figure 5A and B). At 37 and 38°C, a little production of lactate (below 0.03 g/g/h) was measured all during the culture. At 39 and 40°C, lactate excretion occurred mainly after 15 h of culture. At 41°C, a striking lactate titer of 21.5 g/l was measured: lactate production started ca. 3 h after the temperature was increased, when growth stopped, and rapidly reached the fast rate of 0.13 g/g/h (Figures 5B and 1B).

Discussion

The present work on temperature sensitivity of *C. glutamicum* metabolism demonstrated that temperature has an immediate effect on glutamate excretion. Whatever the range of the temperature shift up to 40°C, glutamate was excreted; however, the higher the temperature the higher the maximal glutamate excretion rate. The glutamate production rate can be considered as the result from both glutamate synthesis rate and glutamate excretion rate. Tuning of

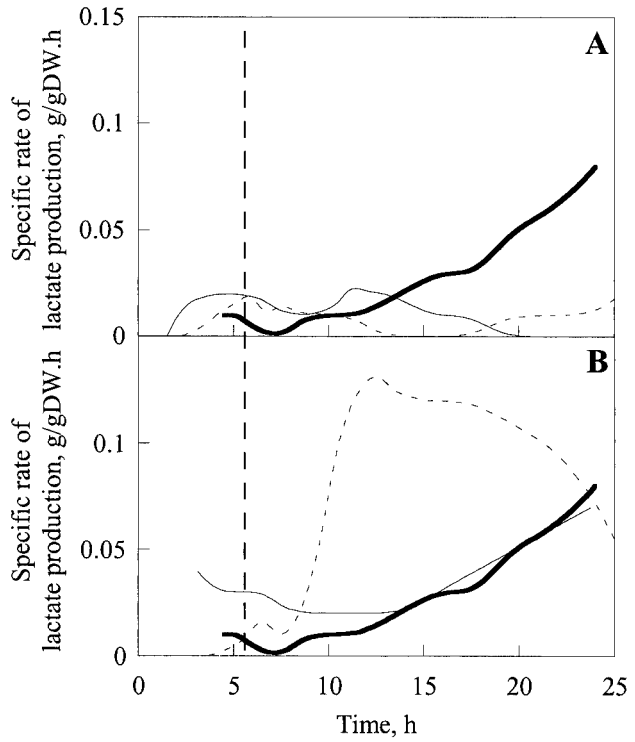

Figure 5 The lactate-specific production rate during fed-batch cultures using *C. glutamicum* 2262 with a temperature increase from 33 to 37 (A, dashed line), 38 (A, full thin line), 39 (A and B, full thick line), 40 (B, full thin line) or 41°C (B, dashed line). The vertical dashed line indicates the time when the broth temperature was increased.

these two rates could explain the increase in the glutamic acid specific production rate measured between 37 and 40°C. Indeed, glutamate synthesis could be enhanced by the activation of enzymes involved in its pathway, while glutamate excretion could be thermally activated. The impact of temperature was previously measured on the activity of two enzymes involved in the glutamate synthesis pathway in corynebacteria strains: the phosphoenolpyruvate carboxylase (PEPc) and the glutamate dehydrogenase (GDH). The *in vitro* PEPc activity in *C. glutamicum* 2262 was strongly reduced above 40°C. The *in vitro* maximal enzymatic activity was measured between 30 and 33°C whereas at 40°C the residual activity was 40% of this maximal activity [5]. The same behavior was observed with the GDH activity from *C. melassecola* ATCC 17965 (A Guyonvarch, personal communication). These results are in contrast to an increase in glutamate synthesis rate by a temperature increase. The influence of temperature variations on glutamate excretion was already studied during short-time fermentations using *C. glutamicum* 2262, with broth temperature varying from 33 to 40°C [13]. It was then suggested that the temperature could activate glutamate export and, as reported previously [2], this step could be a major kinetic bottleneck for glutamic acid production. Such a hypothesis could explain the increase in the glutamate production specific rate measured when the temperature was increased from 37 to 40°C during the production phase. The possible decrease in the glutamate synthesis rate could be corrected by an increase in the excretion rate. At 41°C, the decrease in glutamate production could be explained by a more dramatic reduction of glutamate synthesis or by a decrease in the excretion rate.

Another striking effect of temperature is the slowing and early end of cell growth. Furthermore, the higher the temperature, the faster growth stopped. This effect could be explained by a thermal inactivation of enzymes involved in biomass synthesis. Concomitantly to this phenomenon, production of lactate, which could be related to growth decline, was observed (Figure 6). The accumulation of this organic acid could indicate a bottleneck at the level of its precursor: pyruvate. Indeed, the reduced utilisation of precursors coming from glycolysis or of NADPH from the pentose–phosphate pathway for growth may lead to an accumulation of glycolytic intermediates, resulting in an overflow of carbon at the pyruvate branchpoint. The easiest way for the bacteria to detoxify pyruvate is lactate synthesis and excretion. However, production of lactate could also reflect a metabolic imbalance provoking NADH accumulation. This last phenomenon was previously observed during the thermal process (temperature increase from 33 to 39.5°C) using *C. glutamicum* 2262 [10]. At the end of this process, an intracellular accumulation of NADH was determined. Such an increase in NADH content could be responsible for activation of lactate dehydrogenase concomitant to an inhibition of pyruvate dehydrogenase as previously noticed in other bacterial strains [8,18] and then could lead to the production of lactate.

In terms of industrial processes, and using 39°C as the reference temperature, a deviation of temperature during the glutamate production phase between 37 and 40°C should not lead to a dramatic modification in glutamate productivity. However, with 37 or 38°C, together with extracellular glutamate accumulation, the production of a high biomass occurred. Such a biomass could introduce some difficulties during the purification of glutamic acid from the fermentation. Further improvement of glutamate production may still be possible but requires a better understanding of the temperature effect on cellular metabolism. One question still unanswered is why are the cells not able to maintain a high glutamate production rate during a long period? One way to ameliorate glutamate production could be to maintain metabolic activity by modulating the temperature profile during the glutamic acid fermentation. For instance, a 1 or 2°C decrease in the broth temperature a few hours after the temperature shift could sustain residual metabolic activity and prolong glutamate production. However, it has to be kept in mind that though the temperature shift

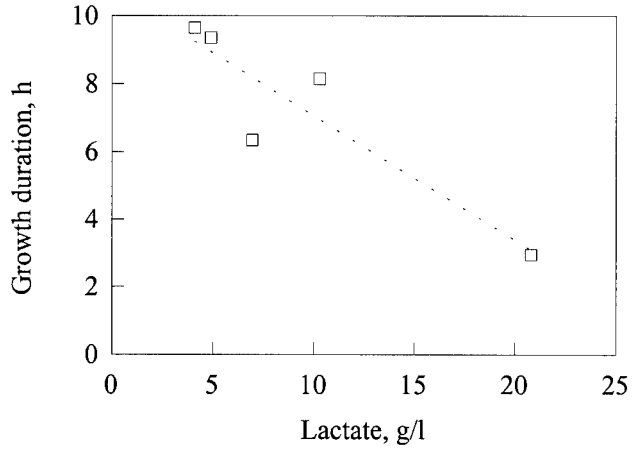

Figure 6 Relationship between growth duration after the temperature shift and lactate final concentration.

provoked a decrease in metabolic activity, this parameter could also allow enhancement of the excretion rate [13]. A compromise would have to be found in order to favor the excretion step without stopping metabolic activity. Thus, it appears that despite the quantity of work dedicated to glutamate production using *C. glutamicum*, more detailed studies are required to identify the physiological parameter(s) responsible for the decrease in the glutamate synthesis and/or excretion, and thereafter to enhance glutamate production.

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