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Intra- and extracellular concentrations of glutamate, lactate and acetate during growth of *Corynebacterium glutamicum* on different media

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Corynebacterium glutamicum **ATCC 17965 was cultivated in a 4-L batch aerated fermentor with glucose, fructose and mixtures of these two sugars in various proportions as carbon sources and with different concentrations of minerals and vitamins. A multilayer centrifugation technique was devised to obtain cell extracts in order to assess intracellular production of glutamate and partitioning between intracellular and extracellular spaces for lactate and acetate, the main by-products produced during the growth phase. Glutamate production increased with the proportion of glucose in the carbon source. The average value for the intracellular concentration of glutamate obtained with basic glucose medium was increased three-fold when initial concentrations of vitamins and minerals were increased four-fold. In this case, overall production of glutamate (16.3 mM) reached the highest value obtained. Production of acetate was weak on all media types (< 1.6 mM). It was the same for lactate synthesis in media where glucose remained the major carbon source (< 2.3 mM). Production of lactate was significantly higher on media where fructose was the main carbon source (> 10 mM to 60 mM). The increase in lactate production and the decrease in glutamate production were correlated to a modification of carbon flux distribution between the metabolic pathways as the fructose proportion was increased. An increase in the concentration of minerals favoured production of glutamate during growth. This was correlated with an increase in the NADPH,H+ production rate.**

Keywords: *Corynebacterium glutamicum*; glutamate; intracellular concentrations; carbon fluxes

Introduction

Members of the group of 'glutamic acid bacteria', such as *Corynebacterium glutamicum*, have been used for the last 30 years to produce amino acids industrially. During the growth phase of batch cultures, *C. glutamicum* [3] overproduces glutamic acid, which accumulates in the cell. Its excretion is promoted by biotin limitation [6] or by adding penicillin [9] or surfactant to make the membrane permeable. The industrial production process has been optimized empirically and is directly linked to the characteristics of bacterial growth. Thus, further increases in glutamic acid production require a better understanding of the extracellular and intracellular parameters that characterize the growth of this bacterium. Extracellular data have been obtained in kinetic studies of the growth phase of *C. glutamicum* on different carbon substrates alone or in mixtures [2,16]. The amino acid efflux mechanism has also been investigated [11], but there is little information available on intracellular concentrations of metabolites during the growth phase. Shiio *et al* [18] measured intracellular concentrations of phosphate sugars (fructose 6-phosphate and fructose 1,6-biphosphate) during the growth phase of *Brevibacterium flavum* on glucose, fructose and on equimolar mixtures of these two sugars. Hoischen and Krämer [7] showed that the intracellular concentration of glutamate during the growth phase of *Corynebacterium glutamicum* is in the range 150–200 mM with glucose as carbon source and 100–400 mM with other sugars [11]. Since glutamate is not normally excreted during the growth phase, knowledge of how glutamate intracellular concentrations vary appears vital for understanding of the metabolism of this type of amino acid-producing organism.

The objective of this work was to determine the influence of medium composition on intracellular concentrations of the end-products of metabolism. Metabolic flux distributions were also calculated by a mass balance technique from the measurement of conversion yields [15,17]. For this strain, glutamic acid is the main metabolite accumulated in the cell. Lactic acid and acetic acid were also selected for study as they are the main catabolic by-products. Glucose, fructose and mixtures of these two sugars were tested as carbon sources as they are main components of beet and cane molasses, which are industrial raw materials the sugar composition of which is highly variable. The effects of different concentrations of minerals and vitamins were also studied.

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Materials and methods

Microorganism and media

Cornybacterium glutamicum ATCC 17965 was used in this study. The growth medium was adapted from the BMCG (Basal Medium for *Corynebacterium* Growth) of Liebl *et al* [13]. It contained six groups of components, the initial concentrations of which in the fermentor are:

- BMCG-Trace elements (medium T) in $mg\ L^{-1}$: $MnSO_4 \cdot H_2O$ (2), $Na_2B_4O_7 \cdot 10H_2O$ (0.2), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (80), $ZnSO_4 \cdot 7H_2O$ (20),

$CuSO_4 \cdot 5H_2O$ (0.5), $MnCl_2 \cdot 4H_2O$ (15), $FeCl_3 \cdot 6H_2O$ (0.2)

- BMCG-Vitamins (medium V) in mg L^{-1}: deferoxamine (3), biotin (30), thiamine (2), nicotinic acid (3), pantothenic acid (3), choline chloride (3), myoinositol (3)
- BMCG-Salts (medium S) in mg L^{-1}: $MgSO_4 \cdot 7H_2O$ (400), $FeSO_4 \cdot 7H_2O$ (20)
- BMCG-Base (medium B) in g L^{-1}: $(NH_4)_2SO_4$ (7), Na_2HPO_4 (6), KH_2PO_4 (3), NaCl (0.5), NH_4Cl (1)
- $CaCl_2$ (medium C): (5.5 mg L^{-1})
- Carbon source (glucose and/or fructose): (10 g L^{-1})

The carbon sources used were glucose or fructose in various proportions, but the initial concentration of the carbon source was always 10 g L^{-1}. The different media tested were referred to as media G: glucose (10 g L^{-1}); 3GF: glucose (7.5 g L^{-1}) and fructose (2.5 g L^{-1}); GF: glucose (5 g L^{-1}) and fructose (5 g L^{-1}); G3F: glucose (2.5 g L^{-1}) and fructose (7.5 g L^{-1}); F: fructose (10 g L^{-1}). For all these media the concentrations of salts, minerals and vitamins are those of media T, V, S, B and C. Additional media involved multiplying the concentrations of the groups of trace elements, vitamins, salts and $MgSO_4$ by four with glucose or fructose alone as the carbon source. These culture media were referred to as G4T when medium T was multiplied by four with glucose as carbon source and so on for media G4V, G4S, G4T4V4S, F4S and F4Mg.

Finally, one experiment (medium G+Fe) was performed with glucose as carbon source with additions of 2 ml of a 30-mM solution of $FeSO_4$ to the culture broth at 4, 5 and 6 h of running time in order to compensate the formation of a precipitate which occurred between Fe and dissolved CO_2.

Concentrated solutions of sugars were added during the course of the cultures to prevent carbon limitation and to keep the sugar concentration within the range 1–10 g L^{-1}. Those two or three additions were taken into account in the calculation of a dilution factor estimated on line and corrected for each variation of the liquid volume in the fermentor. The dilution factor lay within the range 1–1.2 for all cultures.

Taurine was added to the medium at a concentration of 1 g L^{-1} as an internal standard to evaluate the extracellular medium content in the cell extracts.

Culture conditions

C. glutamicum was grown in batch cultures at 33.5°C in a 6-L Biostat ED fermentor (B Braun, Melsungen, Germany) with a 4-L working volume. The pH was kept at 7.3 by adding 4 M NaOH. The aeration rate was maintained at 0.37 vvm. To prevent oxygen limitation, the dissolved oxygen concentration was maintained above 20% of the saturation value by adjusting the stirrer speed.

Analyses

Biomass concentration was determined by both dry cell weight analysis and measurement of optical density at 570 nm. Lactate, acetate and taurine concentrations were determined by high performance liquid chromatography (Waters, Milford, MA, USA), using two identical cation exchange columns in series (PHENOMENEX ROA-OOH-0138-KO). Glutamic acid concentration was measured using an enzymatic assay method (Boehringer Mannheim, Mannheim, Germany: kit No. 139092).

Extraction of intracellular metabolites

Cell extracts were prepared from samples taken during the growth phase for biomass concentrations in the range 2–11 gdw L^{-1}. Samples were taken when sugar concentrations were greater than 2 g L^{-1} in order to avoid the effect of carbon limitation on the intracellular concentrations.

Cells were separated from the culture broth by a multilayer centrifugation technique [5,8], based on the technique of centrifugal filtration developed by Palmieri and Klingenberg [14]. The following solutions were layered (from bottom to top) in 10-ml Nalgene tubes: 2 ml of $HClO_4$ (18% w/w, d = 1.116), 2.5 ml of silicone oil (Fluka [Buchs, Switzerland] 85419, d = 1.049) and 1–5 ml of culture broth. Tubes were centrifuged for 10 min at $10000 \times g$ in a Sigma 3K30 centrifuge (Sigma, St Louis, MO, USA) equipped with a swinging bucket rotor and the supernatant was stored at −20°C for subsequent analysis. The silicone layer was then removed from the tube. After homogenization, the $HClO_4$ layer which contained the cells, was centrifuged for 10 min at $10000 \times g$ to remove cell walls and membranes. The $HClO_4$ was neutralized with 6 M K_2CO_3 and centrifuged again for 5 min at $5000 \times g$ to eliminate the $KClO_4$ precipitate. Silicone oil traces were removed by extraction with diethyl ether. The remaining supernatant was kept frozen (−20°C) for subsequent analysis. The metabolism quenching time (the time needed to transfer the cells from the bulk culture broth to the $HClO_4$ solution) was estimated to be lower than 1 min. The ratio of the extracellular volume over the intracellular volume in the cell extract was in the range of 2 to 3.

Determination of intracellular metabolite concentrations

Intracellular concentrations were calculated by difference between the total quantities in the extracts and the extracellular concentrations in the supernatants [12]. In order to validate the multilayer centrifugation technique, the glutamate concentration, which is an exclusively intracellular product, was also measured by a direct technique for which the broth sample was put directly in the $HClO_4$ 18% (w/w) solution. The results obtained by both methods were in excellent agreement. The multilayer technique was chosen because the proportion of extracellular medium in the extract was 20–30 times lower than with the other technique.

Results

The growth phase of *C. glutamicum* was studied during batch culture on 12 different culture media with glucose, fructose or mixtures of these two sugars as carbon source and with different concentrations of trace elements, salts and vitamins. Experiments were duplicated up to six times on medium G. Extracellular data were analyzed to check that conversion yields remained constant during the growth phase for all media. The redundant experimental information was used to test the consistency of experimental

data by checking the elemental balances (ie the carbon, nitrogen, available electron and proton balances). The balances closed within ±6% [15,16].

Intracellular concentrations are reported in Table 1 and extracellular concentrations and/or the specific productivities are reported in Table 2; the total final volumetric quantities produced in the reactor at the end of the growth phase are reported in Table 3.

For all media, the intracellular glutamate per unit volume of culture medium appeared to be proportional to the biomass concentration (Figure 1). Intracellular concentrations of glutamate did not vary significantly during the growth phase for biomass concentrations ranging from 2 to 11 gdw L^{-1}. It was thus possible to calculate an average value of glutamate intracellular concentration for each medium from samples taken during the growth phase (Table 1).

Extracellular concentrations of glutamate remained almost constant during the growth phase (Figure 1). Average values of extracellular concentrations of glutamate for each medium are presented in Table 2. Most of the glutamate synthesized during the growth phase remained within the cells (between 82 and 98% of the total glutamate) with only a weak excretion to the medium.

The variability of the intracellular concentration of lactate was greater than for that observed with glutamate. The results can however be interpreted in terms of a normally distributed variable, which gives an average value of lactate concentration for each medium (Table 1). As the calculated means were in the range 0.04 to 0.08 mmol gdw^{-1} except on medium F4S (0.14 mmol gdw^{-1}) with relative deviations up to 50%, it was justified to consider an overall intracellular lactate concentration for all media equal to 0.06 ± 0.01 mmol gdw^{-1}.

The range of variation of acetate concentration was of the same order of magnitude as that of lactate concentration, giving an overall average acetate concentration of 0.04 ± 0.01 mmol gdw^{-1} for all media types.

Influence of the proportion of fructose

When the proportion of fructose was increased, the intracellular concentration of glutamate decreased from 0.37 mmol gdw^{-1} (medium G) to 0.24 mmol gdw^{-1} (medium F) (Table 1). Assuming an intracellular water content of 1.86 ml gdw^{-1} [12], these concentrations were in the range 130–200 mM and agreed with the values found by Krämer [11] during the growth phase of *C. glutamicum* on various sugars (100–400 mM). The overall production of glutamate decreased with the proportion of glucose in the medium from 4.9 mM on medium G to 2.2 mM on medium F (Table 3).

On media in which the carbon source was at least 50% glucose (G, 3GF, GF, G4V, G4S, G4T, G4T4V4S and G+Fe), the extracellular concentration of lactate fluctuated during the growth phase. Average values were calculated to allow comparison between the different media types (Table 2). It must be noted that the extracellular lactate concentration remained low on these media (between 0.3 and 1.5 mM). This can be explained by the fact that lactate can be exchanged continuously between intracellular and extracellular compartments.

On media in which fructose was the main component of the carbon source (G3F, F, F4S and F4Mg), the extracellular concentration of lactate increased during the growth phase. The lactate extracellular concentration was almost proportional to the biomass concentration (Figure 2), allowing calculation of the average ratio of lactate excretion to biomass synthesized (Table 2). The intracellular/ extracellular ratio remained constant showing that the synthesis and excretion rates of lactate were proportional. The percentage of intracellular lactate was less than 10% on these media.

On media where fructose was a minor carbon source (G, 3GF, GF, G4V, G4S, G4T and G4T4V4S), the overall production of lactate (Table 3) was low (between 0.9 and 2.3 mM). Lactate production increased with the proportion of fructose in the medium up to 28.3 mM on medium F. The extracellular concentrations of acetate were of the same order of magnitude as for lactate (Table 2), except that for fructose media no significant changes were observed. As acetate can be exchanged between intracellular and extracellular compartments, the same comments are valid as for lactate concerning the partition of acetate.

Influence of mineral and vitamin concentrations

On media G4V, G4S and G+Fe, the average values of intracellular concentrations of glutamate (0.33–0.44 mmol gdw^{-1}) were in the range of those found with medium G (Table 1). On media G4T and G4T4V4S, the intracellular glutamate concentrations were 0.56 and 1.12 mmol gdw^{-1} respectively, showing a significant influence of trace elements on the intracellular pools and the synergy effect between the three groups of components tested (medium G4T4V4S).

However, the overall production of glutamate (Table 3) was significantly higher on medium G4S (6.3 mM) than on media G and G4V (4.9 and 3.9 mM respectively). This resulted from the significantly higher biomass concentration obtained at the end of the culture G4S (21 gdw L^{-1}). On medium G4T the overall production of glutamate (7.2 mM) was about the same as on medium G+Fe (7.1 mM) for an identical maximal biomass concentration (16 gdw L^{-1}). The highest overall production of glutamate was obtained on medium G4T4V4S (16.3 mM).

Lactate production was increased when the concentration of $MgSO_4$ (medium F4Mg) or salts (medium F4S) were multiplied by four. The overall production of acetate (Table 3) was about the same on all media types, ranging between 0.6 and 1.6 mM.

Discussion

Two different effects on the general regulation of metabolism during growth were noted in this study: the influence of the proportion of fructose and the influence of the mineral and vitamin composition of the culture medium. In order to improve our understanding of the observed variations of the intracellular and extacellular concentrations of glutamate, lactate and acetate, the corresponding metabolic flux distribution during the growth phase on each medium was calculated by a mass balance method [15,17,19]. The main inputs of this computation method were the

Table 1 Effects of growth medium on the final biomass and average intracellular concentrations (mmol gdw^{-1}) of glutamate, lactate and acetate in batch cultures of *C. glutamicum*. Confidence intervals are for a significance level of 95%

	Growth medium											
	G	G4T	G4V	G4S	G4T4V4S	G+Fe	3GF	GF	G3F	F	F4S	F4Mg
Number of extracts	27	9	6	8	8	5	9	8	9	16	5	5
Final biomass (gdw L^{-1})	13	15.5	13	21	16	16	12	11	9	8	13	14
Glutamate	0.37±0.02	0.56±0.09	0.33±0.09	0.33±0.04	1.12±0.19	0.44±0.07	0.38±0.04	0.34±0.06	0.30±0.06	0.24±0.04	0.33±0.13	0.30 ± 0.07
Lactate	0.06±0.01	0.06±0.04	0.05±0.01	0.04±0.01	0.07±0.03	0.04±0.02	0.07±0.03	0.05±0.03	0.08±0.04	0.07±0.01	0.14±0.12	0.04±0.03
Acetate	0.04±0.02	0.03±0.06	0.02±0.02	0.04±0.04	0.02±0.01	0.03±0.02	0.07±0.06	0.02±0.02	0.06±0.04	0.03±0.02	0.04±0.03	0.02±0.01

Table 2 Effects of growth medium on average extracellular concentrations (mmol L^{-1}) of glutamate, lactate and acetate in batch cultures of *C. glutamicum*

	Growth medium											
	G	G4T	G4V	G4S	G4T4V4S	G+Fe	3GF	GF	G3F	F	F4S	F4Mg
Glutamate	0.12	0.22	0.24	0.06	0.39	0.14	0.14	0.26	0.36	0.09	0.13	0.13
Lactate	0.59	0.79	0.32	0.51	0.51	0.92	0.91	1.55	0.95[a]	3.40[a]	4.54[a]	2.87[a]
Acetate	0.38	0.31	0.27	0.80	0.31	0.16	0.27	0.74	1.17	1.01	0.73	1.01

[a]The extracellular concentrations did not remain constant during the cultures and were proportional to the biomass concentration; these values are expressed as yields (mmol extracellular lactate gdw^{-1}).

Table 3 Effects of growth medium on final total concentrations (mmol L^{-1}) of glutamate, lactate and acetate in batch cultures of *C. glutamicum*

	Growth medium											
	G	G4T	G4V	G4S	G4T4V4S	G+Fe	3GF	GF	G3F	F	F4S	F4Mg
Glutamate	4.9	7.2	3.9	6.3	16.3	7.1	4.6	3.9	3.0	2.2	4.3	3.9
Lactate	1.4	1.4	0.9	1.2	1.5	1.5	1.5	2.3	9.2	28.3	59.0	40.2
Acetate	0.9	0.7	0.6	1.5	0.6	0.5	1.1	1.0	1.6	1.3	1.1	1.2

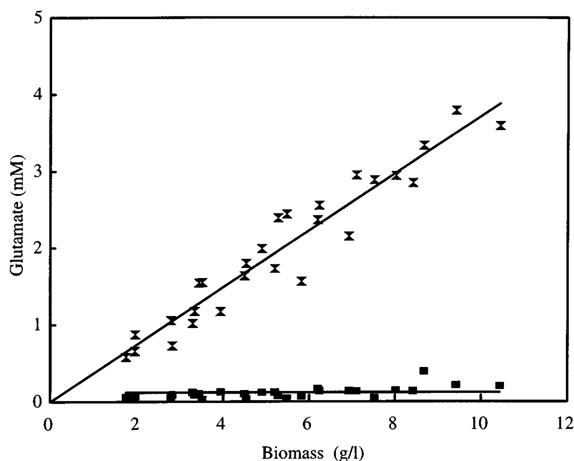

Figure 1 Relationship between biomass and extracellular (■) and intracellular (⧗) glutamate concentrations during the growth phase of *C. glutamicum* on medium G.

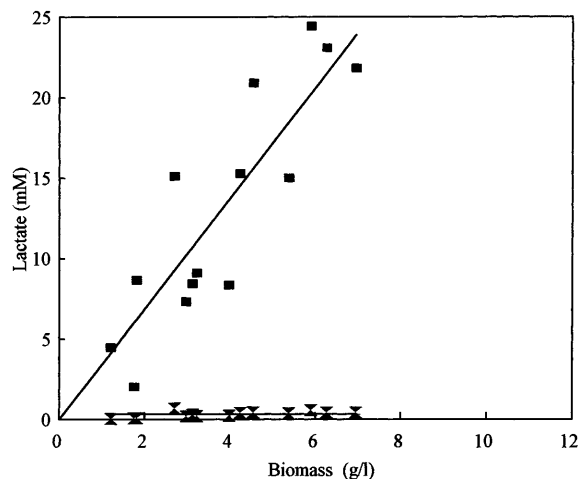

Figure 2 Relationship between biomass and extracellular (■) and intracellular (⧗) lactate concentrations during the growth phase of *C. glutamicum* on medium F.

oxygen/biomass yields which were experimentally measured by a gas balance technique [17]. The metabolic network considered 86 reactions from the assimilation of the carbon source to the synthesis of biomass. It was assumed that no transhydrogenase was operative [10] and that all the anaplerotic reactions could be represented by the PEP carboxylase [19]. In order to compute the metabolic fluxes, the slight production of glycerol, glyoxylate and pyroglutamate during growth was taken into account. The most significant results are reported in Table 4, either in terms of normalised fluxes for a 100 moles total uptake of sugars, or in terms of specific rates obtained by multiplying the normalised fluxes by the specific growth rate and the corresponding yields.

Metabolic flux distribution and intracellular concentrations provide two independent pictures of the functioning of the cell's metabolism, the former being calculated from the conversion yields determined by linear regression over the entire growth phase [16], the latter being obtained by direct measurements.

Growth rates and intracellular concentrations of glutamate appear to be dependent on the same control parameters. As previously shown, biomass production yields decrease when the glucose proportion in the carbon source is decreased [16]. When the fructose proportion is increased, flux through the pentose pathway decreases (Table 4) in accordance with the results of Shiio *et al* [18]. Since the transhydrogenase system has not been detected in *C. glutamicum* [10], the only sources of NADPH,H^+ are the pentose-P pathway (2 NADPH,H^+ produced at the glucose 6-phosphate dehydrogenase and the phosphogluconate dehydrogenase levels) and the Krebs cycle (1 NADPH,H^+

Table 4 Experimental values of biomass/oxygen yields and specific growth rates, and calculated metabolic fluxes obtained for the growth of *C. glutamicum* on different media

	Growth medium											
	G	G4T	G4V	G4S	G4T4V4S	G+Fe	3GF	GF	G3F	F	F4S	F4Mg
Y_{XO_2} (gdw g^{-1} O_2)[a]	1.75	1.87	1.74	1.97	1.90	1.68	1.71	1.57	1.56	1.36	0.83	1.05
μ_{max} (h^{-1})[a]	0.59	0.60	0.58	0.61	0.61	0.64	0.56	0.59	0.43	0.43	0.39	0.40
Anaplerotic reactions[b] (mol per 100 mol sugar)	37	38	36	38	41	37	36	35	34	27	22	26
Entrance of Pentose pathway[c] (mol per 100 mol sugar)	51	54	51	57	50	48	50	46	44	35	9	23
Glutamate synthesis[d] (mol per 100 mol sugar)	77	79	77	79	79	76	76	74	71	58	48	55
Lactate synthesis[e] (mmol gdw^{-1} h^{-1})	1.0	0.9	0.6	0.5	0.9	0.9	1.2	1.9	8.8	27	28	21
Total reduced cofactors production[f] (mmol gdw^{-1} h^{-1})	34	30	29	30	26	35	36	35	26	32	34	29
qo_2 (mmol gdw^{-1} h^{-1})	10.5	10	10.4	9.7	10	11.9	10.2	11.7	8.6	9.9	14.7	11.9

[a]Experimentally determined.
[b]All the anaplerotic reactions are expressed as the PEP carboxylase reaction: P-enolpyruvate + CO_2 + H_2O → Oxaloacetate + Pi.
[c]Glucose 6-P + 2 $NADP^+$ + H_2O → Ribose 5-P + 2 NADPH,H^+ + CO_2.
[d]2-Oxoglutarate + NH_3 + NADPH,H^+ → Glutamate + $NADP^+$ + H_2O.
[e]Pyruvate + NADH,H^+ → Lactate + NAD^+.
[f]The total reduced cofactor production rate (NADPH,H^+, NADH,H^+, $FADH_2$) is equal to the total cofactor consumption rate as specified by the quasi steady-state assumption.

produced at the isocitrate dehydrogenase level). A decrease in carbon flux through the pentose pathway leads to a decrease of the specific synthesis rate of NADPH,H^+, which is the main reduced cofactor used for anabolic reactions and also the obligate cofactor in the synthesis of glutamate by glutamate dehydrogenase. Therefore, there is less NADPH,H^+ to be directed to glutamate synthesis and to biomass synthesis in fructose media than in glucose media. This results in lower intracellular glutamate concentrations (Table 1), although the glutamate accumulation inside the cells represents only a small part (10%) of the overall glutamate production, the main part being directed to protein synthesis. This is confirmed by calculated values of normalised glutamate synthesis rates which decrease from 77 moles per 100 moles sugar on media containing glucose to 58 moles per 100 moles sugar for the medium containing only fructose. It can be concluded that there is a positive correlation between intracellular glutamate concentration during growth, the glutamate production rate, the percentage of carbon directed to the pentose pathway, and the production of NADPH,H^+ which appears to be a key variable for understanding the regulation of metabolism.

A significant production of lactate occurred on media where fructose was the major carbon source (Table 3) even though metabolism was not under oxygen limitation [16]. This is in agreement with the calculated specific production rates of lactate at the lactate dehydrogenase level which were 25 times greater on fructose media than on glucose media (Table 4). Also, it should be noted that the specific respiration rates were slightly greater on fructose media (12 mmoles gdw^{-1} h^{-1}) than on media containing glucose (10 mmoles gdw^{-1} h^{-1}). As the specific growth rate is lower on fructose than on glucose media, the metabolism uses NADH,H^+ to produce cellular components at a lower rate. Therefore, the respiratory chain appears to be saturated during growth on fructose, the excess of NADH,H^+ being oxidised at the lactate dehydrogenase level. In the same way, additional experimental results showed that on fructose the high flux through the glycolysis pathway leads to an accumulation of fructose 1,6-biphosphate [1] which is known to activate the lactate dehydrogenase in *C. glutamicum* [4].

When glucose was the sole carbon source (G4S, G4T, G4T4V4S and G+Fe), changes in the concentrations of minerals and vitamins acted only on glutamate production, with the production of lactate and acetate remaining at a constant value (Tables 1 and 3). When fructose was the carbon source (F, F4S, and F4Mg), an increase in the concentration of minerals also restored intracellular glutamate concentration and the overall production of glutamate to the values obtained on glucose alone. An increase in the concentration of vitamins (medium G4V) did not affect glutamate production and metabolic flux distribution when compared to the reference medium G.

Comparisons between calculated metabolic fluxes and glutamate concentrations for media G4T, G4S and G4T4V4S indicate that the increase of glutamate production may be correlated with an increase in the metabolic flux through glutamate dehydrogenase though the specific respiration rates remain approximately the same (10 mmoles gdw^{-1} h^{-1}). This suggests that under these conditions metabolism is more directed to the synthesis of reduced NADPH,H^+ cofactors which are used for glutamate synthesis, than to the synthesis of NADH,H^+ cofactors which are oxidised via the respiratory chain. Thus, the increase of glutamate production appears to be linked either to an increase of the pentose pathway (media G4T and G4S), leading to an increase of NADPH,H^+ production at

glucose 6-phosphate dehydrogenase and phosphogluconate dehydrogenase levels, or to an increase of anaplerotic reactions, which may be activated by the increase of Mn^{2+} and Mg^{2+} concentrations. This could explain the increase of NADPH,H^+ production at the isocitrate dehydrogenase level. In any case, the increase of glutamate production is correlated to the increase of the specific rate of NADPH,H^+ synthesis which indicates that there is a satisfactory agreement between metabolic flux estimations and the measurement of intracellular concentrations.

When additions of $FeSO_4$ are made during the growth phase (medium G+Fe), the increase in overall glutamate production and biomass synthesis were correlated to an increase in the specific oxygen consumption rate (11.9 mmoles gdw^{-1} h^{-1}), suggesting that the respiratory chain could function more efficiently and oxidise NADH,H^+ at a higher rate. Interestingly, no such effect was observed when the salt concentrations were multiplied by four (medium G4S) even though Fe^{2+} ions were present in the salt solution. This may be explained by the fact that the actual Fe^{2+} concentration is determined by the low solubility product of $FeCO_3$ which precipitates in small quantities as CO_2 is produced during incubation. Therefore, it appears that the increase of the capacity of the respiratory chain enables the metabolism to recycle NADH,H^+ more efficiently. This leads to higher rates for the reactions of Krebs cycle and especially a high production of NADPH,H^+ at the isocitrate dehydrogenase level which in turn favours glutamate synthesis.

In conclusion, this study shows that there is an important flexibility of the metabolism of this strain and the results suggest intrinsic possibilities for increasing glutamate production during growth by adjusting medium composition. Although quantitative and predictive calculations of metabolic fluxes including intracellular concentrations of metabolites which play an important role in metabolic control remain difficult, the different observations obtained during the exponential growth phase were consistent.

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