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Influence of glucose, fructose and sucrose as carbon sources on kinetics and stoichiometry of lysine production by *Corynebacterium glutamicum*

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Batch cultivations of L-lysine-producing *Corynebacterium glutamicum* ATCC 21253 were carried out on the different carbon sources, glucose, sucrose and fructose. The time profiles of substrate and product concentrations were evaluated to compare kinetics and stoichiometry of lysine production. The lysine yield (mol C/mol C) on glucose was 8% higher than on sucrose and 30% higher than on fructose. The highest final biomass concentration of 5.0 g/l was obtained on glucose, whereas fructose and sucrose yielded 20% less biomass. Compared to glucose, fructose resulted in significantly higher respiration rates, a higher substrate uptake rate but a lower lysine production rate during the cultivation process. This was probably due to a higher tricarboxylic cycle activity combined with a lower activity of the pentose phosphate pathway. On sucrose, specific rates and yields differed significantly from those on fructose and glucose. Transport and metabolism of sucrose, therefore, are not a simple superposition of its building blocks, glucose and fructose.
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Introduction

Corynebacterium glutamicum is widely used for the production of amino acids such as glutamate or lysine [5]. In recent decades, optimization of fermentation strategies and producer strains have led to increased yields and rates of industrial amino acid production by corynebacteria. Notwithstanding the achieved progress, little attention has been paid to the carbon sources. For industrial lysine production, which is carried out to a high extent on molasses, sucrose and fructose are of major importance in addition to glucose. In research, glucose is used almost exclusively as the carbon source, whereas only a few reports consider alternative carbon sources [4,10]. Investigations with glutamate-producing *C. glutamicum* ATCC 17965 revealed significant differences in stoichiometry and carbon flux distribution in central metabolism between the two substrates fructose and glucose. Other investigations with substrate mixtures such as glucose and acetate/lactate [2,19] targeted the identification of metabolic network topology. Despite its importance as the main carbon source in industrial amino acid production, investigations of sucrose are only sparsely reported [3,10]. The present work deals with the influence of the carbon source on kinetics and stoichiometry of lysine production by *C. glutamicum* using comparative batch cultures of *C. glutamicum* ATCC 21253 on glucose, fructose and sucrose. To our knowledge, a direct comparison of these three carbon sources for a lysine-producing strain of *C. glutamicum* has not been examined.

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Materials and methods

Microorganism and medium

C. glutamicum ATCC 21253 was obtained from the American Type Culture Collection (Manassas, VA, USA). This homoserine auxotrophic strain excretes L-lysine under limitation of threonine and methionine due to the bypass of concerted aspartate kinase inhibition. The strain was cultivated on PMB medium [18] with a reduced citrate concentration of 0.57 g/l. Glucose, fructose and sucrose were used as carbon sources with initial concentration of 15 g/l. Precultures were inoculated from LB5G plates, grown overnight on LB5G medium in shake flasks [18] containing the carbon source for the main culture and washed twice with 0.9% NaCl solution.

Cultivation

Precultivations were performed in 500-ml baffled shake flasks with 50 ml of medium at 30°C and 150 rpm. The main cultivations were carried out in a 1.5-l stirred bioreactor (Fairmentec, Göttingen, Germany) under batch conditions at 30°C with a 1.2-l working volume and a shaking rate of 1000 rpm. The pH was measured by a pH probe (Sentek, Essex, England, UK) and kept between 6.93 and 7.0 by automatic addition of 2 N NH_3. Air flow was constant at 1.2 l/min by a mass flow controller. Aeration gas and exhaust gas were measured on-line by a quadruple mass spectrometer (Omnistar; Balzers, Vaduz, Liechtenstein) at 2-min intervals. Oxygen uptake rate (OUR), carbon dioxide production rate (CPR) and respiratory quotient (RQ) were calculated *via* inert gas balance. Dissolved oxygen in the medium was monitored by an oxygen probe (Sentek).

Chemicals

All chemicals were from Sigma (St. Louis, MO, USA), Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland), and were of analytical grade.

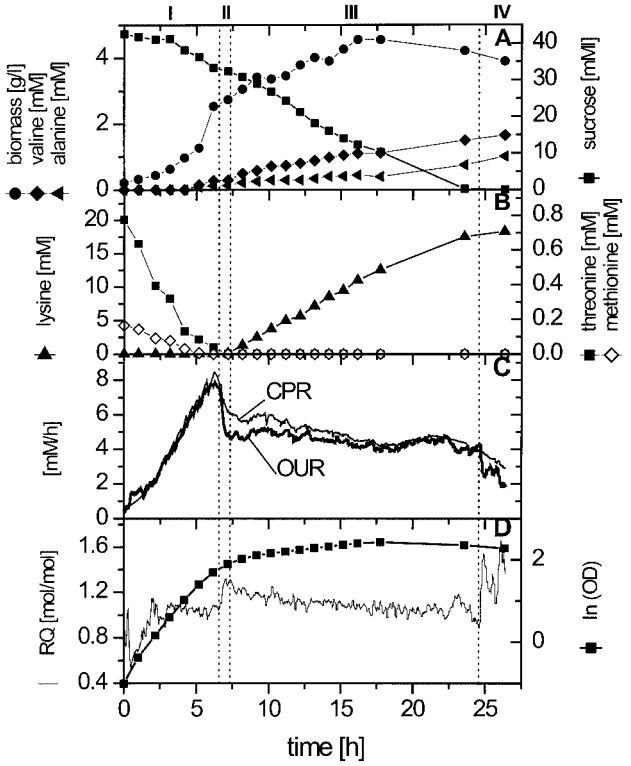

Figure 1 Cultivation of lysine-producing *C. glutamicum* ATCC 21253 on sucrose: concentration of sucrose, biomass, valine and alanine (A), concentration of lysine and the essential amino acids threonine and methionine (B), OUR and CPR (C), RQ and growth (ln OD) (D). The different phases of the cultivation are marked.

Substrate and product analysis

Biomass concentration was determined by measurement of optical density at 660 nm (Marsha Pharmacia Biotech, Freiburg, Germany) or by measurement of dry cell weight. The latter was determined as follows: 10 ml of broth was centrifuged at 4°C for 10 min at 3900 g, washed and centrifuged again. Washed cells were dried at 80°C to constant weight. The correlation factor (g biomass/$OD_{660\ nm}$) between dry cell dry mass and $OD_{660\ nm}$ was 0.294.

Further analysis was carried out with the supernatant phase of the samples, obtained *via* 5-min centrifugation at 14,200 g (Heraeus Biofuge Pico, Hanau, Germany). Fructose, glucose, sucrose and trehalose were quantified by GC using an HP 6890 gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) with an HP-5 column (5% diphenyl-dimethylpolysiloxane) and FID detection. The sample preparation included lyophilization of the culture supernatant mixed with β-D-ribose as internal standard, dissolution in pyridine and a subsequent two-step derivatization of the sugars with hydroxylamine [11] and (trimethylsilyl)trifluoracetamide (BSTFA) forming the oxime trimethylsilyl derivatives [13]. The temperature program for GC analysis was: 170°C (0–2 min), 10°C/min (2–5 min), 200°C (5–7 min), 8°C/min (7–22 min), 310°C (22–26 min). Helium was used as carrier gas with a split flow of 1.7 l/min and a total flow of 5.5 ml/min. The inlet temperature was 310°C and the detector temperature was 350°C. Acetate, lactate, pyruvate, succinate, 2-oxoglutarate and glycerol were determined by HPLC using an Aminex-HPX-87H Biorad Column (300×7.8 mm; Biorad, Hercules, CA, USA) with 4 mM sulfuric acid as mobile phase at a flow rate of 0.6 ml/min, and UV detection at 210 nm. Amino acids were analyzed by HPLC [AQC fluor reagent, AccQ column (150×3.9 mm); Millipore, Milford, MA, USA] as described in the instruction manual at a flow rate of 1 ml/min, and UV detection at 250 nm.

Data processing

For calculation of rates and yields from the experiment data, the following data processing was performed: Data on high data density obtained from on-line gas analysis were smoothed with a Savitzky–Golay filter. Other data were fitted using cubic splines from the MATLAB signal processing toolbox. Data consistency was verified *via* the carbon balance for each cultivation. The elemental composition of *C. glutamicum* ATCC 21253 was taken from the literature [18].

Results

Batch cultivations of *C. glutamicum* ATCC 21253 using glucose, fructose and sucrose as carbon sources revealed distinct differences between the different substrates as can be seen from the data on growth, lysine production, by-product formation and respiration activity (Figures 1–3). When cultivated on fructose, the strain produced 14.9 mM lysine, which was significantly lower compared to sucrose and glucose (18.2 and 18.8 mM lysine, respectively). Marked differences were also observed for biomass formation. The

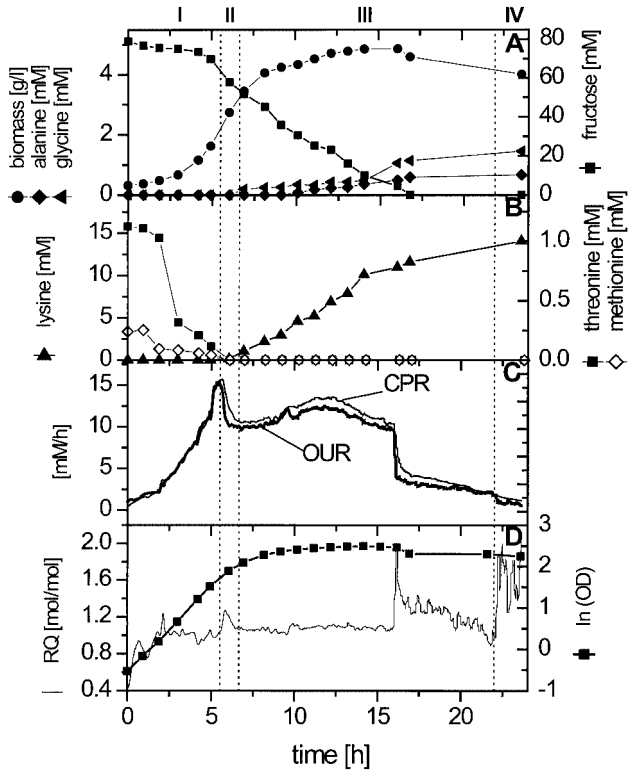

Figure 2 Cultivation of lysine-producing *C. glutamicum* ATCC 21253 on fructose: concentration of fructose, biomass, glycine and alanine (A), concentration of lysine and the essential amino acids threonine and methionine (B), OUR and CPR (C), RQ and growth (ln OD) (D). The different phases of the cultivation are marked.

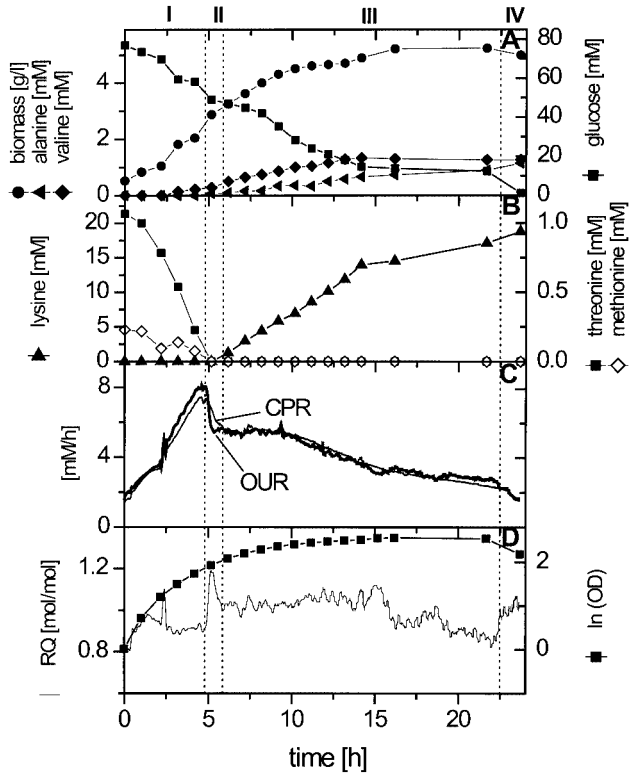

Figure 3 Cultivation of lysine-producing *C. glutamicum* ATCC 21253 on glucose: concentration of glucose, biomass, valine and alanine (A), concentration of lysine and the essential amino acids threonine and methionine (B), OUR and CPR (C), RQ and growth (ln OD) (D). The different phases of the cultivation are marked.

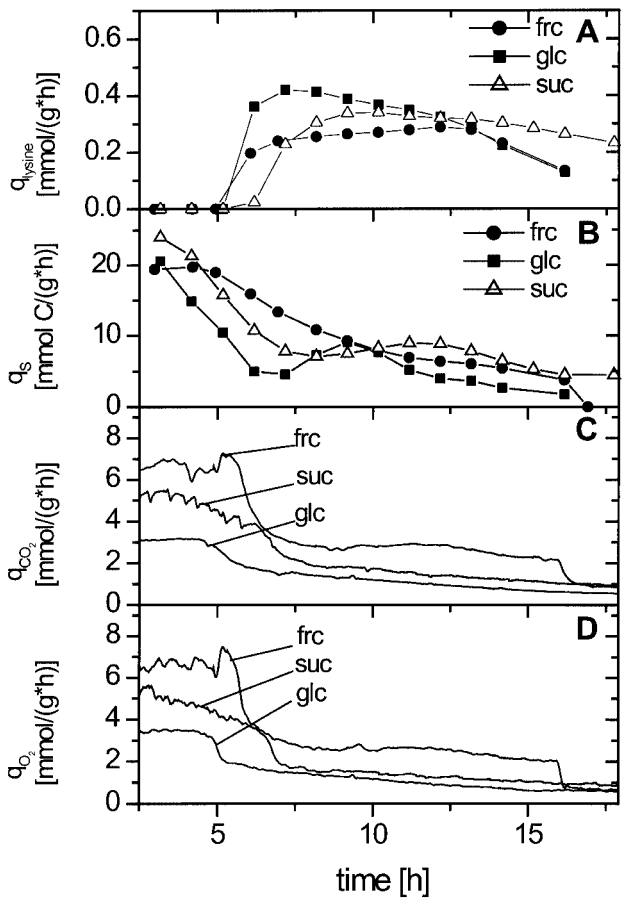

Figure 4 Specific rates during cultivation of lysine-producing *C. glutamicum* ATCC 21253 on sucrose, fructose and glucose, respectively: Specific lysine production rate (q_{lysine}) (A), specific substrate uptake rate (q_s) (B), specific CPR (q_{CO_2}) (C), specific OUR (q_{O_2}) (D).

highest final biomass concentration of 5.0 g/l was obtained on glucose, whereas fructose (4.0 g/l) and sucrose (4.0 g/l) yielded 20% less biomass. The carbon recovery was 93% on sucrose, 92% on glucose and 95% on fructose.

Culture profiles

With respect to the dynamics of growth and lysine production, the cultivations can be divided into four different phases: (I) growth without lysine formation, (II) transient toward lysine production, (III) lysine production and (IV) terminal phase (Figures 1–3). Subdivision of the cultivations into four different phases was carried out following characteristic changes of OUR, CPR and RQ. During Phase I, lysine formation was completely suppressed with all carbon sources due to the presence of threonine and methionine. After a short lag phase, the cells grew exponentially with stable RQ values of about 1.0 for fructose and sucrose, and 0.9 for glucose. The specific growth rates during this phase were 0.46 h^{-1} (glucose), 0.28 h^{-1} (fructose) and 0.42 h^{-1} (sucrose). Whereas with sucrose no organic acids accumulated during Phase I, 0.5 mM lactate and 0.5 mM acetate were formed with glucose. With fructose, elevated levels of 1.2 mM lactate and 0.5 mM acetate were found at the end of Phase I. Alanine accumulated with all three substrates. Valine was produced with sucrose or glucose as carbon source, but not with fructose, where glycine accumulation was observed. Concentrations of the by-products further increased during cultivation. With complete consumption of the essential amino acids after 5–6 h, the specific growth rate decreased, significantly linked to drastic changes in respiration activity. Lysine secretion did not start immediately with the depletion of threonine and methionine in the medium. The strain underwent a transient phase (Phase II) of about 30–60 min, characterized by a drastic decrease of CPR and OUR and an increase of the RQ value of about 0.3 U. During further cultivation, lysine concentration steadily increased with all substrates (Phase III). The terminal phase of cultivation was indicated by a high degree of noise in RQ data as a consequence of reduced metabolism (Phase IV). The carbon source used showed a strong influence on the formation pattern of by-products such as organic acids or amino acids. With regard to amino acids, alanine, valine and glycine were the major by-products. The final concentrations of alanine were 1.14 mM (on glucose), 1.00 mM (on sucrose) and 0.66 mM (on fructose).

Table 1 Maximum specific lysine production rates $q_{lysine,\ max}$ (mmol/g/h) and duration of high lysine during cultivation of *C. glutamicum* ATCC 21253 on glucose, fructose and sucrose, respectively

Parameter	Glucose	Fructose	Sucrose
$q_{lysine,\ max}$ (mmol/g/h)	0.42	0.28	0.34
$t_{lysine,\ 50}$ (h)	8.7	10.3	14.1

The time span of high lysine production activity ($t_{lysine,\ 50}$) was defined as time interval of $q_{lysine} > 0.50 * q_{lysine,\ max}$.

Table 2 Yields of biomass, lysine and carbon dioxide (mol C/mol C) during lysine production by *C. glutamicum* ATCC 21253 on glucose, fructose and glucose

C mol/C mol	Glucose	Fructose	Sucrose
$Y_{X/S}$	0.373	0.311	0.300
$Y_{lysine/S}$	0.224	0.157	0.205
$Y_{CO_2/S}$	0.210	0.371	0.236

The concentrations of valine were 1.26 mM (on glucose) and 1.65 mM (on sucrose), and no valine was formed on fructose. On sucrose, additional small amounts of phenylalanine (0.08 mM) and glutamine (0.13 mM) were detected. In order to exclude higher quantities of organic acids that stemmed from lysis of cells (Phase IV), organic acid concentrations after 16 h of cultivation, linked to growth cessation, were considered. Accumulation of organic acids originating from the pyruvate node was highest on fructose, with concentrations of 2.6 mM lactate, 5.5 mM acetate and 0.7 mM pyruvate. With glucose, lower levels of 0.6 mM lactate, 1.8 mM acetate and 0.2 mM pyruvate were detected, whereas a small accumulation of 0.3 mM lactate, 0.1 mM acetate, but no pyruvate was observed for sucrose. Similar characteristics were found for organic acids stemming from the TCA cycle. Fructose as carbon source resulted in secretion of 6.8 mM succinate and 0.3 mM 2-oxoglutarate, whereas much lower levels were detected for glucose and sucrose. Additional products were glycerol, with a final concentration of 2.45 mM (on fructose), 8.11 mM (on glucose) and 1.19 mM (on sucrose), and trehalose, with a final concentration of 0.4 mM (on glucose) and 1.2 mM (on sucrose). With fructose, no trehalose was formed. In summary, sucrose as carbon source led to minor formation of organic acids, whereas cultivation on fructose yielded markedly higher amounts of organic acids.

Substrate uptake was highest for fructose, with complete depletion of the substrate after about 16 h. However, this significantly higher substrate input resulted mainly in higher respiration activity, as is shown from the total amount of CO_2. With identical initial amounts of C moles supplied by the carbon source, 166 mmol CO_2 were produced on fructose. In comparison, the production of CO_2 was only 101 mmol on glucose and 121 mmol on sucrose. In contrast, lysine production on fructose was by far the lowest among the carbon sources investigated.

Effects of substrates on kinetics

In order to compare the effects of the different substrates on process kinetics, specific rates were calculated for lysine production (q_{lysine}), substrate uptake (q_s), carbon dioxide production (q_{CO_2}) and oxygen uptake (q_{O_2}).

Lysine production

At the beginning of lysine production, q_{lysine} increased markedly for all substrates. Maximum values of q_{lysine} were reached after about 8 h with glucose, 10 h with sucrose and 12 h with fructose. During further cultivation, q_{lysine} decreased (Figure 4A). Referring to the time interval of q_{lysine} higher than 90% of the maximum, there was a short phase of 3 h on glucose, whereas on fructose and sucrose, the specific lysine production rate stayed above 90% of the maximum level for an extended period of about 6 and 5 h, respectively. Table 1 displays the achieved maximal values ($q_{lysine,\ max}$) and the time interval with q_{lysine} higher than 50% of the maximum ($t_{lysine,\ 50}$) for the different substrates. On glucose, a $q_{lysine,\ max}$ of 0.42 mmol/g/h was observed, about 30% higher than for fructose and about 19% higher than for sucrose. On sucrose, $t_{lysine,\ 50}$ lasted for about 14 h, whereas it lasted for 10.3 h with fructose and only 8.7 h with glucose.

Substrate uptake

Figure 4B displays q_s for glucose, fructose and sucrose (mmol C/g/h). The highest specific substrate uptake rates were found during the growth phase. During the phase of lysine production, q_s was significantly lower than q_s during growth. The specific substrate uptake rate of fructose was significantly higher compared to glucose throughout lysine production. The highest differences in q_s between fructose and glucose were found during the first part of the process, where the substrate uptake rate for fructose was up to 50% higher compared to glucose. Sucrose uptake exhibited intermediate values. After 12 h, the specific substrate uptake rate for sucrose was even higher than the q_s for fructose. Nevertheless, the molar-specific substrate uptake rate was highest for fructose throughout cultivation (data not shown).

O_2 uptake and CO_2 production

Fructose, glucose and sucrose as carbon sources resulted in significantly different values for q_{O_2} and q_{CO_2}. Cultivation on fructose resulted in higher values for q_{O_2} and q_{CO_2} during the whole cultivation until the fructose was completely consumed. During the growth period, q_{O_2} and q_{CO_2} reached the highest values for all substrates with values of 6.5 mmol/g/h with fructose, 5.0 mmol/g/h with sucrose and 3.0 mmol/g/h with glucose. Specific respiration rates during this phase were about two times higher compared to the period of lysine production (Figure 4C and D).

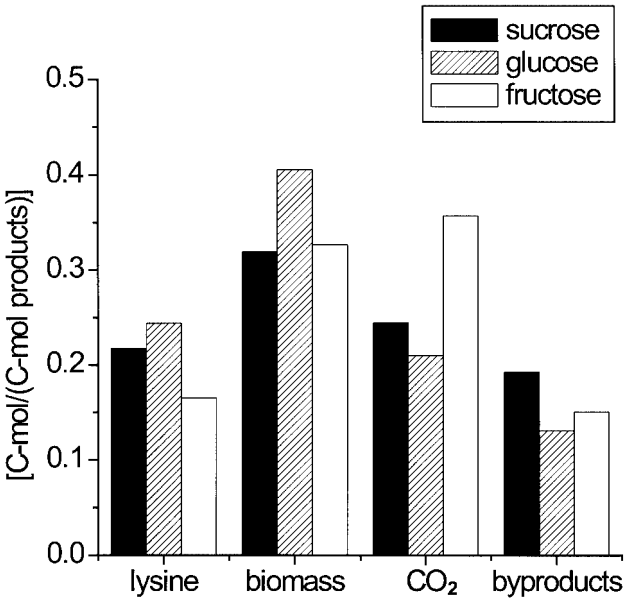

Figure 5 Cultivation of lysine-producing *C. glutamicum* ATCC 21253 on sucrose, fructose and glucose, respectively. Comparison of the individual product fractions with respect to the overall products formed (mol C/mol C). All byproducts were lumped together.

The transition from growth to lysine production was indicated by a sharp drop in specific respiration rates, especially for fructose.

Effects of substrates on yields

C. glutamicum ATCC 21253 produced significantly different quantities of biomass, lysine and CO_2 depending on the substrate. Table 2 shows the corresponding yields at the end of the culture process. Glucose resulted in the highest yields for lysine and biomass. By comparison, lysine yield was about 8% less for sucrose and about 30% less for fructose. The biomass yield was 16.5% lower on fructose and 19.5% lower on sucrose than on glucose. On the other hand, fructose resulted in the highest CO_2 yield, about 36% higher than with sucrose and 43% higher than with glucose. The amount of lysine produced relative to the biomass was 3.50 mol/g for fructose, 4.37 mol/g for glucose and 4.47 mol/g for sucrose. The organism produced similar quantities of lysine relative to biomass with glucose and sucrose as carbon sources, whereas with fructose about 20% less lysine was formed per unit biomass. Figure 5 displays the carbon distribution of the particular products' respective product groups referred to the carbon of the summarized products (mol C/mol C) at the end of cultivation. The results are comparable to yields per unit biomass. Twenty-four percent of total product carbon was found in the lysine fraction with glucose, 22% with sucrose and 16.5% with fructose. Biomass constituted 41% on glucose, 32% on sucrose and 33% on fructose. For fructose, 35% of the product carbon was found in CO_2, which was higher than the biomass fraction on fructose and higher than the CO_2 fractions of 24% and 21% on sucrose and glucose, respectively. The fraction of other products, lumped together as by-products, was 19% on sucrose, 15% on fructose and 13% on glucose.

Discussion

The carbon source has an important influence on kinetics and stoichiometry of growth and lysine production of *C. glutamicum* ATCC 21253. Cultivation on fructose exhibited a 30% lower lysine yield compared to glucose and sucrose, whereas rates of substrate uptake and respiration were substantially higher. Similar findings were described for glutamate production by *Corynebacterium* [12]. For *C. melassecola* ATCC 17695, the glutamate yield on fructose was about 30% less compared to glucose. Cultivation on fructose was linked to significantly higher values for q_{CO_2}. The similarities observed by Pons *et al* [12] and in this study might point to similar consequences of the carbon source used for amino acid production by different strains of *C. glutamicum*. As shown in the present work, the specific OUR of *C. glutamicum* ATCC 21253, which is correlated to oxidative phosphorylation activity, was highest for fructose and lowest on glucose. According to this, the CPR on fructose was markedly increased compared to glucose, which is attributed to a higher activity of the TCA cycle. A possible way of regeneration of the high amounts of NADH and FADH formed is indicated by the pattern of by-products on fructose, which included increased levels of lactate, that is produced from pyruvate linked to an oxidation of NADH. The large secretion of various by-products on fructose linked to the observed high rate of substrate uptake might indicate overflow phenomena in central metabolism. In contrast, glucose and especially sucrose resulted in much lower levels of by-products. The suppression of by-product formation on sucrose can be of interest for industrial production processes.

The observed differences between the different substrates are probably the consequence of different substrate uptake and entrance into metabolism. Previous work showed different specific PTS systems for fructose, glucose and sucrose in glutamate-producing *C. glutamicum* ATCC 17695 [3]. Fructose uptake predominantly leading to fructose-1-phosphate involves an entry of fructose into central metabolism, which is different from glucose. Fructose-1-phosphate enters glycolysis *via* fructose-1,6-bisphosphate, which inhibits glucose-6-phosphate dehydrogenase [9]. This is underlined by the observation that no trehalose was formed on fructose, which indicates a low availability of glucose-6-phosphate, the starting substance for trehalose phosphate formation [6,16]. The entry of fructose into central metabolism can consequently lead to a reduced pentose phosphate pathway (PPP) activity [17]. PPP represents the main source of NADPH for biosynthesis and lysine formation. As previously shown, lysine production by *C. glutamicum* is linked to the flux through the PPP [14]. The decreased lysine yield on fructose might therefore be a consequence of a decreased carbon flux through the PPP. The NADPH demand for product formation and growth was estimated as four NADPH per lysine produced [8] and 14,849 μmol NADPH/g cell dry weight [7]. With the yields observed in the present work, the overall NADPH demand of *C. glutamicum* is 1.73 mol NADPH/mol glucose, 1.33 mol NADPH/mol fructose and 0.75 mol NADPH/mol sucrose. Assuming that the difference in the NADPH demand is covered by the PPP, the flux through the PPP is about 20% lower on fructose compared to glucose. The higher CO_2 production on fructose and the presumed higher TCA cycle activity imply that the flux difference through the PPP between growth on glucose and on fructose might be even higher, since *C. glutamicum* is also able to generate NADPH *via* isocitrate dehydrogenase [1]. For lysine production on sucrose with an NADPH demand of 0.75 mol NADPH/mol sucrose, equal to 1.50 mol NADPH/mol C_6 U sucrose, a PPP flux difference of about 10% to glucose can be calculated, despite the fact that the lysine yield is relatively similar between the two substrates. The main reason is the lower biomass yield and the resulting lower NAPDH demand for anabolism on sucrose.

Sucrose as carbon source for *Corynebacterium* has received little attention. In *Brevibacterium flavum*, a species closely related to *Corynebacterium*, glucose and sucrose yielded similar phenylalanine production, whereas lower yields of about 70–75% were achieved from fructose [15]. Due to its precursor erythrose-4-phosphate, the formation of phenylalanine is correlated to PPP activity. The decreased phenylalanine yield on fructose might therefore be associated with a decreased carbon flux through the PPP.

The cultivations on sucrose and glucose with *C. glutamicum* ATCC 21253 differed significantly from each other. As shown, transport and metabolism of sucrose are not a simple superposition of transport and metabolism of its building blocks glucose and fructose. The reasons for the observed differences cannot be entirely understood from our results, but further investigations such as elucidation of metabolic fluxes in cultivations on sucrose and fructose will shed light on the metabolic consequences of the different carbon sources in lysine-producing *C. glutamicum*.

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