Journal of Industrial Microbiology (1996) 17, 104–109

D-Glucose does not catabolite repress a transketolase-deficient D-ribose-producing *Bacillus subtilis* mutant strain

P De Wulf[1], W Soetaert[1,2], D Schwengers[2] and EJ Vandamme[1]

[1]*Laboratory of Industrial Microbiology and Biocatalysis, Faculty of Agricultural and Applied Biological Sciences, University of Ghent, Coupure links 653, B-9000 Ghent, Belgium;* [2]*Pfeifer und Langen, Frankenstraße 25, 41539 Dormagen, Germany*

When *Bacillus subtilis* strain ATCC 21951, a transketolase-deficient D-ribose-producing mutant, was grown on D-glucose plus a second substrate which is metabolized via the oxidative pentose phosphate cycle (D-gluconic acid, D-xylose, L-arabinose or D-xylitol), D-glucose did not catabolite repress metabolism of the second carbon source. The D-ribose yield obtained with the simultaneously converted carbon substrates, significantly exceeded that when only D-glucose was used. In addition, the concentration of glycolytic by-products and the fermentation time significantly decreased. Based on these findings, a fermentation process was developed with *B. subtilis* strain ATCC 21951 in which D-glucose (100 g L^{-1}) and D-gluconic acid (50 g L^{-1}) were converted into 45 g L^{-1} of D-ribose and 7.5 g L^{-1} of acetoin. A second process, based on D-glucose and D-xylose (100 g L^{-1} each), yielded 60 g L^{-1} of D-ribose and 4 g L^{-1} of acetoin plus 2,3-butanediol. Both mixed carbon source fermentations provide excellent alternatives to the less efficient D-glucose-based processes used so far.

Keywords: D-ribose; *Bacillus subtilis*; transketolase; catabolite repression; pentose phosphate cycle

Introduction

D-Ribose is used commercially on a large scale to synthesize riboflavin (vitamin B_2) and flavor enhancers (5′-nucleotides) [10]. Since the 1970s, this pentose has been produced by fermentation with transketolase (EC 2.2.1.1)-defective *Bacillus subtilis* mutants. The mutant strains not only convert the substrate D-glucose into D-ribose (up to 92 g L^{-1} from 200 g L^{-1} D-glucose) [5,6], but also yield D-gluconic acid [5–7,10], which disturbs the recovery of D-ribose. When *B. subtilis* strain ATCC 21951, the best and most often used D-ribose-producing strain [5,6], was cultured as described in patent publications [5,6], only a low amount of D-ribose was obtained, even under subsequently optimized process conditions (maximally 40 g L^{-1} from 200 g L^{-1} of D-glucose) [2]. In addition, significant quantities (21 g L^{-1}) of acetoin and 2,3-butanediol were co-produced, no D-gluconic acid was formed, and the process took much longer than described (1 week *vs* 2 days) [2]. The low D-ribose titer achieved was due to the fact that the so-called transketolase-negative *B. subtilis* strain ATCC 21951 still contained 60% of its original transketolase activity as measured relative to the *B. subtilis* type strain ATCC 6051 [2]. To increase the D-ribose productivity of *B. subtilis* strain ATCC 21951, D-glucose was quantitatively replaced with D-gluconic acid, D-xylose, L-arabinose or D-xylitol. By using these carbon substrates, which are converted only via the D-ribose-yielding oxidative pentose phosphate cycle, we tried to increase the D-ribose yield and to limit the formation of glycolytic end-products. Also, we tried to overcome the problem of slow D-glucose utilization, attributed to transketolase-deficiency [8], to minimize the process time.

Correspondence: EJ Vandamme, Laboratory of Industrial Microbiology and Biocatalysis, Faculty of Agricultural and Applied Biological Sciences, University of Ghent, Coupure links 653, B-9000 Ghent, Belgium
Received 6 November 1995; accepted 23 July 1996

Materials and methods

Microorganisms and media

The D-ribose-producing *B. subtilis* strain ATCC 21951 and the *B. subtilis* type strain ATCC 6051 were stored at −74°C in a glycerol-based (25%, v/v) inoculation medium which contained (g L^{-1}): D-glucose 30, yeast extract (MC 1, Lab M) 10, K_2HPO_4 5, KH_2PO_4 5 and $MgSO_4 \cdot 7H_2O$ 1. The initial pH was set at 7.0. Medium 1, used in experiments involving flask cultures, consisted of (g L^{-1}): D-glucose 15, D-fructose 15 or D-mannitol 15; plus (g L^{-1}): D-gluconic acid (Na-salt) 15, D-xylose 15, L-arabinose 15 or D-xylitol 15; yeast extract 10, K_2HPO_4 5, KH_2PO_4 5 and $MgSO_4 \cdot 7H_2O$ 1. The initial pH was set at 7.0. Medium 2, used in fermentors, consisted of (g L^{-1}): D-glucose 100, D-gluconic acid (Ca-salt) 15 or D-xylose 100, corn-steep liquor (CSL, Roquette, Solulys L 48B) 20 and $(NH_4)_2SO_4$ 5. The pH was controlled at 6.5 (D-glucose plus D-gluconic acid) or 6.3 (D-glucose plus D-xylose). All medium ingredients were graded highly pure and were sterilized by autoclaving them (21 min, 121°C, +13.2 Pa). When CSL was used, the lactic acid bacteria and particulate matter present in CSL, were removed by centrifugation (15 min; 2000 × *g*). The supernatant fluid was autoclaved without pH-adjustment to avoid the formation of complexes between CSL components.

Process conditions

The inoculum was prepared by transferring 20 μl of a frozen stock culture to a baffled 2-L Erlenmeyer flask, containing 100 ml of inoculation medium. The flask was cultured on a rotary shaker (200 rpm, New Brunswick G10,

Edison, USA) at 37°C, until the cells reached the post-exponential growth phase. This suspension, or part of it (inoculum volume of 10%, v/v), was then transferred to the Erlenmeyer or fermentor in which the fermentation took place. For experiments involving shake flasks, a baffled 2-L Erlenmeyer was filled with 90 ml of medium 1, inoculated as described above, and incubated (200 rpm) at 37°C. The process was monitored on-line for cell density and pH. The substrate and product concentrations were determined enzymatically and/or via high performance liquid chromatography (HPLC).

Fermentations run in a fermentor (1 L of medium 2; Biostat B2, B Braun Co, Melsungen, Germany), were controlled regarding temperature (37°C), stirrer speed (1000 rpm) and pH (6.5 or 6.3). The aeration level was 3 (D-glucose plus D-gluconic acid) or 2 (D-glucose plus D-xylose) volumes of air per volume of medium per minute. The pH of the broth was controlled with 1 M H_2SO_4 and 1 M NaOH. The fermentations were monitored on-line for cell density and pH. The substrate and product concentrations were determined enzymatically and/or via HPLC.

Assay methods

Bacterial biomass concentration (by optical density, OD) was determined spectrophotometrically. The absorbance of a 15-fold diluted fermentation sample was measured at 600 nm relative to distilled water. When $CaSO_4$ precipitated during the fermentation, the aliquot was diluted with 3 M HCl. The carbohydrate substrate(s) and the products D-ribose, acetic acid, acetoin and 2,3-butanediol were quantified by HPLC. Before analysis, the cells were removed from the aliquot by centrifugation (15 min; $2000 \times g$), the supernatant fluid was filtered (0.45-μm pore size) and injected (20 μl) into the HPLC, which was equipped with an Aminex HPX87H column (Bio-rad, 0.78 cm × 30 cm) and a differential refractometer (Bio-rad, model 1755). The assay conditions were: column temperature 65°C; mobile phase 5 mM H_2SO_4; pressure 924 Pa; flow rate 0.6 ml min^{-1}. D-Gluconic acid was quantified enzymatically using a Boehringer-Mannheim kit (No. 428191).

Results

D-Gluconic acid, D-xylose, L-arabinose and D-xylitol are converted to D-ribose by B. subtilis strain ATCC 21951, but cell lysis occurs

When a carbohydrate, converted only via the oxidative pentose phosphate cycle, was used as the carbon source (30 g L^{-1} of D-gluconic acid, D-xylose, L-arabinose or D-xylitol in medium 1), the molar D-ribose productivities (calculated on the amount of carbon substrate utilized) exceeded that obtained with 30 g L^{-1} D-glucose (Table 1). Only a minimal amount of glycolytic by-products was formed from the carbon source (Table 1). In the presence of these pentose phosphate cycle-converted substrates, the growth of *B. subtilis* strain ATCC 21951 was not maintained, leading to rapid cell lysis (see time of fermentation during which cell metabolism occurred, Table 1) and incomplete conversion of the carbon substrate (Table 1). Extending the fermentation time did not lead to extra consumption of carbon source or product synthesis (data not shown). This may have been due to the 'low' activity of the enzyme transketolase, such that energy and biomass synthesis were not sustained via the non-oxidative pentose phosphate cycle and the subsequent gluconeogenesis. To maintain cell growth better, the pentose phosphate cycle-converted substrate was half-replaced with D-glucose (15 g L^{-1} of each in medium 1).

D-Glucose does not catabolite repress B. subtilis strain ATCC 21951

Grown on a mixture of D-glucose and a pentose phosphate cycle-metabolized substrate, *B. subtilis* strain ATCC 21951 converted both completely (Figure 1, Table 2). The molar D-ribose productivities obtained significantly exceeded that achieved with D-glucose alone (30 g L^{-1}), while the concentration of glycolytic by-products significantly decreased (compare Tables 1 and 2). This was presumably since cell metabolism was shifted more pronouncedly towards the oxidative pentose phosphate cycle, relative to the glycolysis. Since *B. subtilis* strain ATCC 21951 simultaneously converted both carbon sources (Figure 1), D-glucose did not exert catabolite repression on metabolism of the second substrate. When the *B. subtilis* type strain ATCC 6051 was cultured on D-glucose plus each of the mentioned co-substrates, D-glucose catabolite repressed the use of the second substrate, resulting in a diauxic growth profile with extensive cell lysis (Figure 2). Interestingly, when *B. subtilis* strain ATCC 21951 was grown on D-gluconic acid plus D-fructose or D-mannitol, D-gluconate catabolism was partially repressed, the effect being most pronounced with D-fructose (Figure 3). From the time when D-fructose or D-mannitol was depleted, D-gluconate was not further utilized, probably since it could not maintain cell activity, resulting in cell lysis (Figure 3). *B. subtilis* strain ATCC 21951 actively converted D-gluconic acid and D-fructose or D-mannitol via the glycolysis, leading to decreased D-ribose productivity and a significantly increased concentration of glycolytic by-products (Table 2).

Since the D-glucose-based double substrate fermentations remained most efficient to produce D-ribose, these were further studied to maximize D-ribose synthesis with *B. subtilis* strain ATCC 21951.

D-Glucose-based double substrate fermentations lead to high D-ribose titers with B. subtilis strain ATCC 21951

The optimal pH to produce D-ribose at fermentor level from D-glucose and D-gluconic acid was 6.5 [2]. The combined use of $Ca(gluconate)_2$ and H_2SO_4 to control the pH (the pH increased due to D-gluconate consumption) was important, since the $CaSO_4$ formed did not disturb cell growth and D-ribose synthesis (contrary to the salts formed from other D-gluconate salts and pH-controlling agents) [2]. Starting from 100 g L^{-1} each of D-glucose and D-gluconic acid, *B. subtilis* strain ATCC 21951 produced 75 g L^{-1} of D-ribose and 7.5 g L^{-1} of acetoin plus 2,3-butanediol within 3 days of fermentation [2]. Despite the very high molar D-ribose productivity (0.7), the process remained suboptimal, since part of the D-glucose (46 g L^{-1}) and the D-gluconic acid (23 g L^{-1}) remained unused, even when the fermentation

Table 1 Fermentation data obtained with *B. subtilis* strain ATCC 21951 grown on diverse carbon substrates in shaken flasks

Carbon source (30 g L^{-1})	Products (g L^{-1})					Time of fermentation (h)
	D-Ribose	Acetoin + 2,3-butanediol	Acetic acid	Carbon source used (g L^{-1})	D-Ribose productivity (mol/mol)	
D-Glucose	2.7	2.9	5.2	30	0.1	50
D-Gluconic acid	4.2	0	1.8	9.5	0.6	36
D-Xylose	2	0	0	4	0.5	9
L-Arabinose	3.5	0.5	0.5	7	0.5	12
D-Xylitol	4	0.5	0	7.5	0.5	12

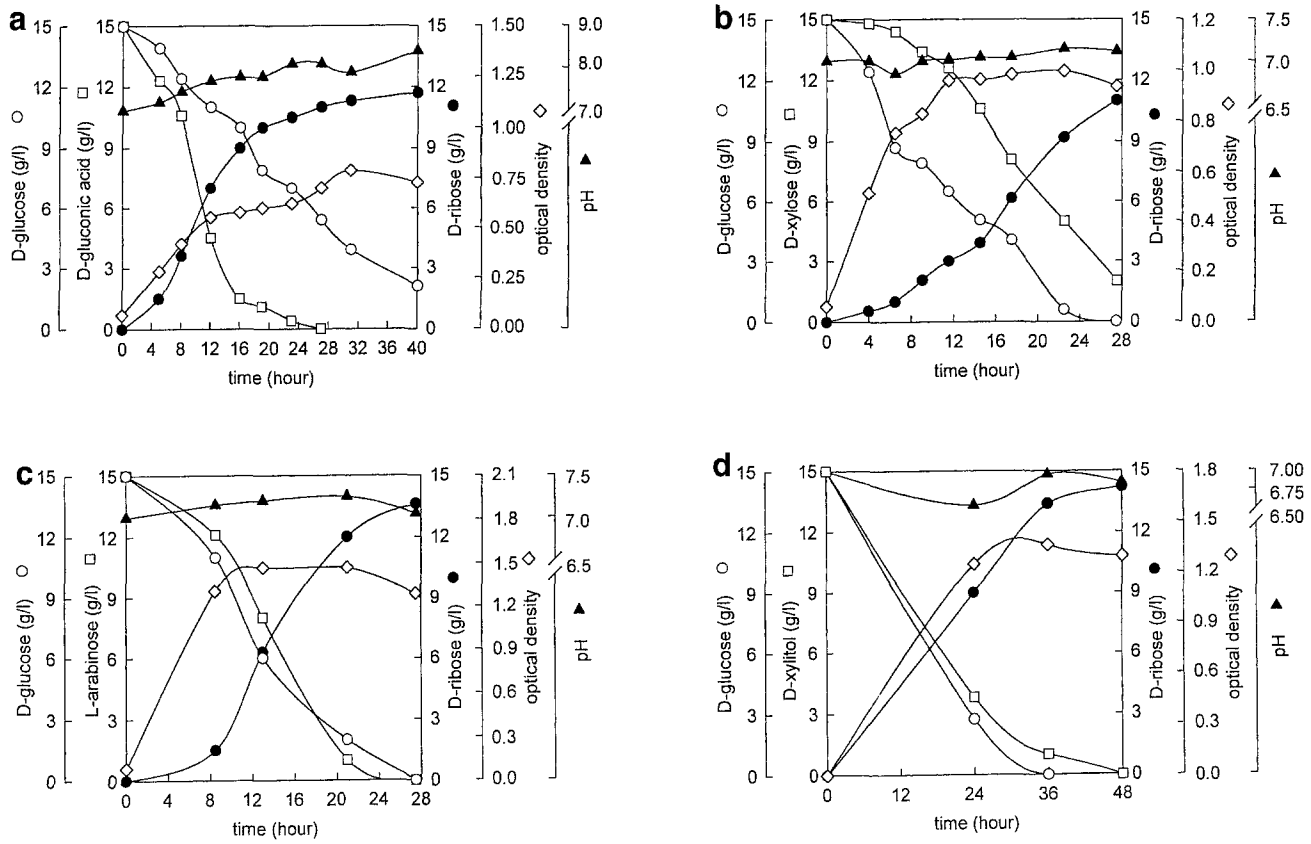


Figure 1 Fermentation profiles of *B. subtilis* strain ATCC 21951 grown on D-glucose plus D-gluconic acid (a), D-glucose plus D-xylose (b), D-glucose plus L-arabinose (c) and D-glucose plus D-xylitol (d) (each 15 g L^{-1}, in shaken flasks).

Table 2 Fermentation data obtained with *B. subtilis* strain ATCC 21951 grown on diverse double substrates in shaken flasks

Carbon sources (each 15 g L^{-1})	Glycolytic by-products (g L^{-1})		D-Ribose productivity (mol/mol)
	Acetoin + 2,3-butanediol	Acetic acid	
D-Glucose + D-gluconic acid	1	0	0.5
D-Glucose + D-xylose	0.4	0	0.4
D-Glucose + L-arabinose	0.9	2.5	0.5
D-Glucose + D-xylitol	0.5	1	0.5
D-Fructose + D-gluconic acid	4.5	0	0.3
D-Mannitol + D-gluconic acid	4.5	2.2	0.3

In all glucose-based media, the carbon substrates were completely used. In the D-fructose- and D-mannitol-based fermentations, the data mentioned are those determined when no further conversion of the carbon sources or extra product formation was observed (see Figure 3). Molar D-ribose productivity is calculated as per amount of each carbon source utilized. The times of fermentation are shown in Figures 1 (D-glucose-based fermentations) and 3 (D-fructose- and D-mannitol-based fermentations).

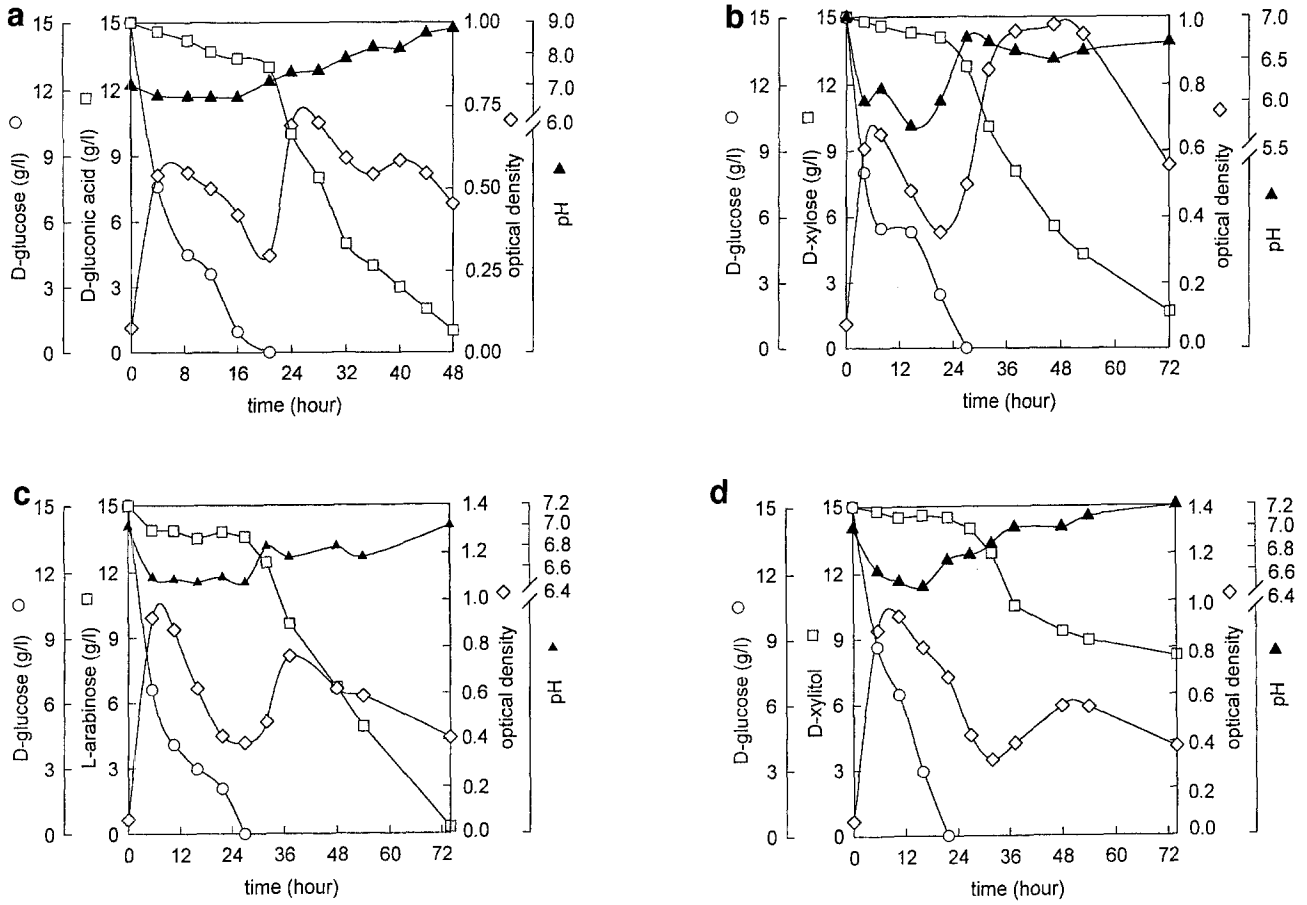


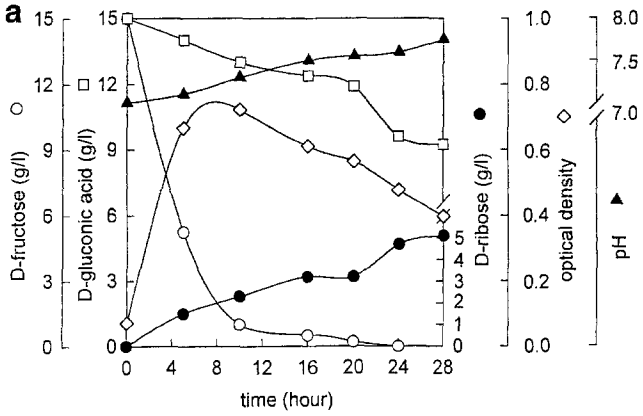
Figure 2 Fermentation profiles of *B. subtilis* type strain ATCC 6051 (transketolase-positive and does not produce D-ribose) grown on D-glucose plus D-gluconic acid (a), D-glucose plus D-xylose (b), D-glucose plus L-arabinose (c) and D-glucose plus D-xylitol (d) (each 15 g L^{-1}, in shaken flasks).

time was prolonged [2]. The high D-gluconic acid concentration was toxic leading to cell lysis [2]. Only by lowering the D-gluconate concentration, was this lethal effect overcome, and both D-glucose (100 g L^{-1}) and D-gluconic acid (50 g L^{-1}) were completely converted into 45 g L^{-1} of D-ribose and 7.5 g L^{-1} of acetoin (no 2,3-butanediol was formed) (Figure 4). Since the toxic effect of the high D-gluconate concentration limited a maximal exploitation of the double substrate process, D-gluconate was replaced with D-xylose.

A preliminary experiment showed that D-glucose and D-xylose were optimally converted in a pH range between pH 7.0 and pH 6.3 [2]. In the next fermentor experiment, based on D-glucose and D-xylose (100 g L^{-1} each), the initial pH 7.0 was allowed to drop to and maintained at 6.3 (Figure 5). Both substrates were quickly, completely, and simultaneously converted into 60 g L^{-1} of D-ribose, 4 g L^{-1} of acetoin plus 2,3-butanediol and 1 g L^{-1} of acetic acid (not shown) within 4.5 days of fermentation (Figure 5).

Discussion

When the transketolase-defective D-ribose-producing *B. subtilis* strain ATCC 21951 was grown on D-glucose and a second carbon source, D-glucose did not catabolite repress the second carbon compound. Normally, glucose-derived D-fructose-1,6-biphosphate triggers a reaction which results in formation of a phospho-serine[46]-HPr-CcpA protein complex [1,4]. The latter then binds to a DNA-located catabolite responsive element (CRE), localized inside the operon which codes for utilization of the second carbon source. Consequently, the co-substrate is not utilized. Only when D-glucose is depleted, the protein complex leaves the CRE, the catabolic operon becomes expressed and the second carbon source is metabolized [3]. By growing the *B. subtilis* type strain ATCC 6051 on D-glucose plus a pentose phosphate cycle-converted co-substrate, glucose-based catabolite repression occurred and diauxic growth was observed. The reason why *B. subtilis* strain ATCC 21951 avoided glucose-based catabolite repression, might be explained by the fact that this mutant strain less actively converted D-glucose via glycolysis, relative to D-fructose and D-mannitol. Since D-glucose was more intensely catabolized via the oxidative pentose phosphate cycle and since it left the cell more quantitatively as D-ribose (higher D-ribose productivity with D-glucose relative to D-fructose and D-mannitol), the amount of D-fructose-1,6-biphosphate formed from D-glucose via the glycolysis and the non-oxidative pentose phosphate cycle, may have been too low to trigger catabolite repression in *B. subtilis* strain ATCC 21951. Since D-fructose and D-mannitol were more actively converted via glycolysis, both may have generated a higher intracellular concentration of D-fructose-1,6-biphosphate, leading to repression of D-gluconate utilization by *B. sub-*



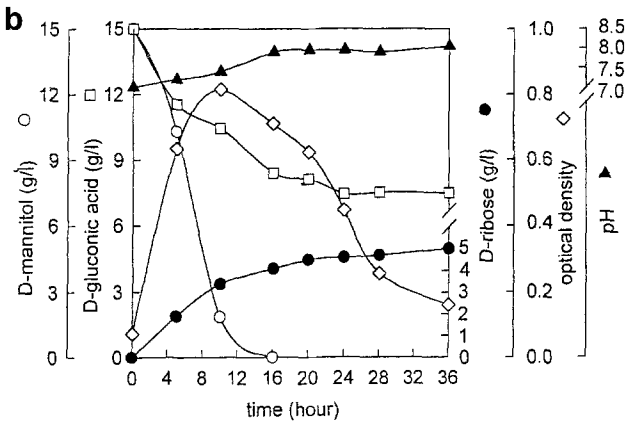


Figure 3 Fermentation profiles of *B. subtilis* strain ATCC 21951 grown on D-fructose plus D-gluconic acid (a) and D-mannitol plus D-gluconic acid (b) (each 15 g L^{-1}, in shaken flasks).

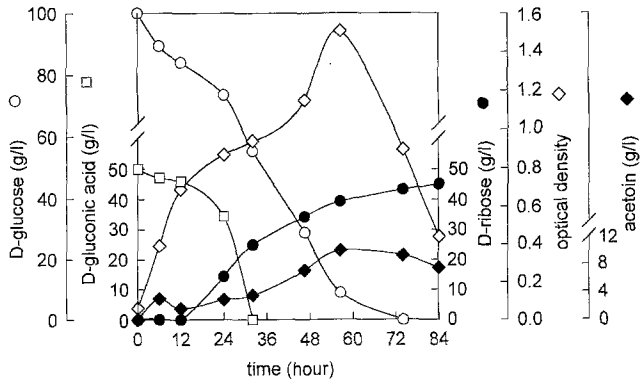


Figure 4 Fermentation profile of *B. subtilis* strain ATCC 21951 grown on D-glucose (100 g L^{-1}) and D-gluconic acid (50 g L^{-1}, Ca-salt) in a fermentor.

tilis strain ATCC 21951. But, since *B. subtilis* strain ATCC 21951 was developed via random (UV and nitrosoguanidine-based) mutagenesis [9], one may not exclude that enzymes which (indirectly) play a part in carbon catabolite control, were also mutationally affected.

A D-glucose (100 g L^{-1})/D-gluconate (50 g L^{-1})-based fermentation, which exploited the higher observed findings, overcame the negative aspects encountered when D-glucose

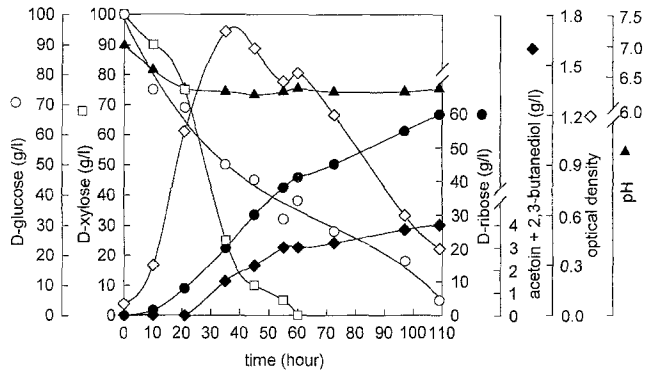


Figure 5 Fermentation profile of *B. subtilis* strain ATCC 21951 grown on D-glucose (100 g L^{-1}) and D-xylose (100 g L^{-1}) in a fermentor.

(200 g L^{-1}) was used as the sole carbon source [2]. Since cellular metabolism was shifted more pronouncedly into the oxidative pentose phosphate cycle, this by replacing part of D-glucose by pentose phosphate cycle-converted D-gluconic acid, the molar D-ribose productivity (0.4) increased by a factor of 1.5, and the amount of acetoin plus 2,3-butanediol decreased from 21 g L^{-1} to 7.5 g L^{-1}. In addition, the slow carbon source utilization observed when only D-glucose was used, was partly overcome by using D-gluconic acid, such that the fermentation time dropped from 1 week to 3.5 days.

A second fermentation process, based on D-glucose (100 g L^{-1}) and D-xylose (100 g L^{-1}), similarly increased D-ribose productivity 1.4-fold. In this case, the process time decreased to 4.5 days, and the concentration of glycolytic by-products dropped to 5 g L^{-1}. By decreasing the concentration of D-glucose to, for instance 50 g L^{-1}, and by increasing the D-xylose concentration to 150 g L^{-1}, the fermentation time may decrease further (D-glucose is utilized more slowly than D-xylose, Figure 5). Moreover, the co-secreted amount of glycolytic metabolites may decrease further as well. Since acetoin and 2,3-butanediol disturb the recovery of D-ribose, pentose isolation may occur more easily and with less expense. By cultivating a completely transketolase-deficient *B. subtilis* mutant strain under the process conditions described here, the D-ribose yield may even exceed the levels obtained with *B. subtilis* strain ATCC 21951.

Acknowledgements

We thank Pfeifer and Langen, Dormagen, Germany, for financial support of this research.

References

1 Deutscher J, E Küster, U Bergstedt, V Charrier and W Hillen. 1995 Protein kinase-dependent HPr/CcpA interaction links glycolytic activity to carbon catabolite repression in Gram-positive bacteria. Mol Microbiol 15: 1049–1053.
2 De Wulf P. 1995. Microbial synthesis of D-ribose. PhD thesis, University of Ghent, Belgium.
3 Fujita Y and Y Miwa. 1994. Catabolite repression of the *Bacillus subtilis gnt* operon mediated by the CcpA protein. J Bacteriol 176: 511–513.
4 Fujita Y, Y Miwa, A Galinier and J Deutscher. 1995. Specific recognition of the *cis*-acting catabolite-responsive element by a protein com-

plex formed between CcpA and seryl-phosphorylated Hpr. Mol Microbiol 17: 953–960.
5 Kintaka K, YH Sekitani and JP Yamguchi. 1986. Verfahrung zur Erzeugung von D-ribose. German Patent DE 35 24549 A1.
6 Kishimoto K, K Kintaka and N Ochiyama. 1990. Production of D-ribose. US Patent 4 904 587.
7 Miyagawa K, J Miyazaki and N Kanzaki. 1992. Method of producing D-ribose. European Patent 0 501 765 A1.
8 Sasajima KI and T Kumada. 1979. Deficiency of D-glucose transport in a transketolase mutant of *Bacillus subtilis*. Inst Ferment Res Commun 10: 3–9.
9 Sasajima KI and M Yoneda. 1974. Verfahrung zur Herstellung von D-Ribose. German Patent DE 2330426 C2.
10 Sasajima KI and M Yoneda. 1989. Production of D-ribose by microorganisms. In: Biotechnology of Vitamins, Pigments and Growth Factors (Vandamme EJ, ed), pp 167–197, Elsevier Science Publishing, New York.