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Fed-batch production of D-ribose from sugar mixtures by transketolase-deficient *Bacillus subtilis* SPK1

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Abstract D-Ribose, a five-carbon sugar, is used as a key intermediate for the production of various biomaterials, such as riboflavin and inosine monophosphate. A high D-ribose-producing *Bacillus subtilis* SPK1 strain was constructed by the chemical mutation of the transketolase-deficient strain, *B. subtilis* JY1. Batch fermentation of *B. subtilis* SPK1 with 20 g l⁻¹ xylose and 20 g l⁻¹ glucose resulted in 4.78 g l⁻¹ dry cell mass, 23.0 g l⁻¹ D-ribose concentration, and 0.72 g l⁻¹ h⁻¹ productivity, corresponding to a 1.5- to 1.7-fold increase when compared with values for the parental strain. A late-exponential phase was chosen as the best point for switching to a fed-batch process. Optimized fed-batch fermentation of *B. subtilis* SPK1, feeding a mixture of 200 g l⁻¹ xylose and 50 g l⁻¹ glucose after the late-exponential phase reduced the residual xylose and glucose concentrations to less than 7.0 g l⁻¹ and gave the best results of 46.6 g l⁻¹ D-ribose concentration and 0.88 g l⁻¹ h⁻¹ productivity which were 2.0- and 1.2-fold higher than the corresponding values in a simple batch fermentation.

Introduction

D-Ribose is a five-carbon carbohydrate present naturally in ribonucleic acid, NAD, NADP, and FAD as a ribosyl residue. In the context of commercial applications, D-ribose has long been used as a starting material for the chemical synthesis of riboflavin, which can be used not only for pharmaceuticals but also for animal feed additives, cosmetics, and foods. Chemical methods for D-ribose synthesis were established using arabinose, glucose, and gluconic acid. D-Arabinose can be epimerized in the presence of molybdc ion and boric acid to obtain D-ribose with a yield of 60–94% (Hiroshi et al. 1986). Esterification of glucose at the C₃ hydroxyl group was devised to synthesize D-ribose (Smith 1955). Chemical processes for D-ribose synthesis suffered from disadvantages such as low yield, a complex scheme, and inefficient recovery and purification. Microbial production of D-ribose was developed in 1966. *Pseudomonas reptilivora* and *Candida pelliculose* strains isolated from soil had an ability to produce D-ribose from glucose (De Wulf and Vandamme 1997). Several *Bacillus subtilis* and *B. pumilus* strains accumulating D-ribose were selected and characterized as transketolase mutants. Transketolase is a metabolic enzyme involved in the non-oxidative pentose phosphate (PP) pathway and creates a bridge between glycolysis and the PP pathway, as shown in Fig. 1. If transketolase is deactivated, all glucose metabolites catalyzed by glucose-6-phosphate dehydrogenase are not directed into glycolysis. In addition, five carbon sugars including xylose and arabinose may accumulate as the PP-pathway intermediate, D-ribose-5-phosphate. Biological production of D-ribose is carried out by the dephosphorylation of D-ribose-5-phosphate. Many attempts have been made to establish the mass production of D-ribose using the transketolase mutants of *Bacillus* species. Glucose can be converted to D-ribose with 48% yield by *B. subtilis* ATCC 21915 (De Wulf and Vandamme 1997). Various carbon sources (glucose, sorbitol, mannitol, maltose) are useful for the production of D-ribose and corn steep liquor is effective for large-scale production (Sasajima and Yoneda 1971).

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Dibasic organic acids are conducive to increase yields of D-ribose; and maleic acid is particularly effective (Sasajima and Yoneda 1975). Aromatic amino acids are necessary for the growth of a D-ribose producing *Bacillus* sp. strain; and supplementation with L-tryptophan, L-tyrosine, and L-phenylalanine increases the yield of D-ribose synthesis and suppresses the formation of gluconic acid (Kishimoto et al. 1990). When a D-ribose producing mutant was grown on glucose plus a second substrate (D-gluconate, D-xylose, L-arabinose, xylitol), no catabolite repression on utilization of the second substrate was found (De Wulf et al. 1996) and hence L-gluconate was selected as a good second-carbon source (De Wulf et al. 1997).

We selected a D-ribose-producing *B. subtilis* JY1 strain which had lost transketolase activity and designed a batch process for synthesizing D-ribose from xylose (Park and Seo 2004). In this study, a chemical mutant of *B. subtilis* was constructed to possess an improved ability of D-ribose production. Fed-batch fermentations were optimized to maximize D-ribose biosynthesis from a mixture of xylose and glucose, the two major components of lignocellulosic materials.

Materials and methods

Bacterial strains and mutation

A D-ribose-producing *B. subtilis* strain was isolated from highly concentrated sugar solution and donated by Bolak

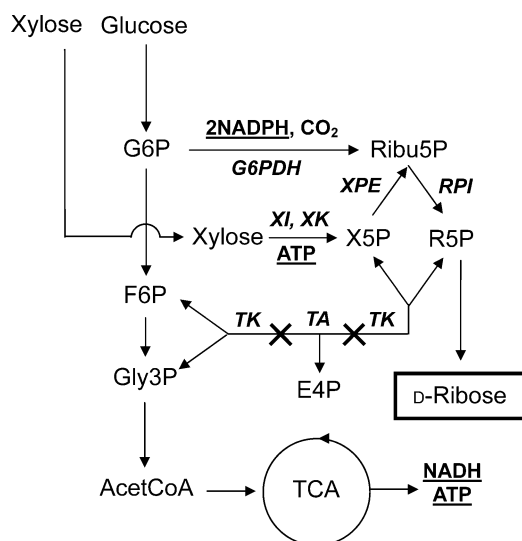


Fig. 1 Metabolic pathway for D-ribose biosynthesis from xylose and glucose. **Bold and underlined** names show cofactors produced or consumed in catabolic steps. **Bold and italic** letters indicate enzymes involved in D-ribose biosynthesis. **Crosses** indicate disruption of the transketolase gene. *G6PDH* Glucose-6-phosphate dehydrogenase, *XPE* xylulose-5-phosphate epimerase, *RPI* ribulose-5-phosphate isomerase, *TK* transketolase, *TA* transaldolase, *XI* xylulose isomerase, *XK* xylulokinase. *G6P* glucose-6-phosphate, *F6P* fructose-6-phosphate, *Gly3P* glyceraldehyde-3-phosphate, *AcetCoA* acetyl-CoA, *Ribu5P* ribulose-5-phosphate, *X5P* xylulose-5-phosphate, *R5P* ribose-5-phosphate, *E4P* erythrose-4-phosphate

Co. (Kyonggi, Korea). It was characterized as a transketolase-deficient mutant like other D-ribose-producing strains and was named *B. subtilis* JY1 (KCCM10407), as described by Park and Seo (2004). Chemical mutation of *B. subtilis* JY1 with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG; Sigma-Aldrich, St. Louis, Mo., USA) was used to construct a mutant strain with increased D-ribose production ability. A cell suspension in 9 ml of 0.1 M citrate buffer (pH 5.5) was mixed with 1 ml of 1 mg ml⁻¹ NTG, followed by incubation at 37°C for 30 min. After removing the NTG solution by centrifugation and washing cell pellets twice with 0.05 M KH₂PO₄/NaOH buffer (pH 7.0), a cell suspension in KH₂PO₄/NaOH buffer was applied onto complex medium containing 20 g l⁻¹ agar and 40 g l⁻¹ glucose. Among several clones incubated at 37°C for 24 h, a mutant strain able to consume carbon sources more rapidly than the parental strain (*B. subtilis* JY1) was selected. It was named *B. subtilis* SPK1 (KCCM10408) and deposited at the Korean Culture Collection for Microorganisms (Hongjaedong, Seodaemonku, Korea).

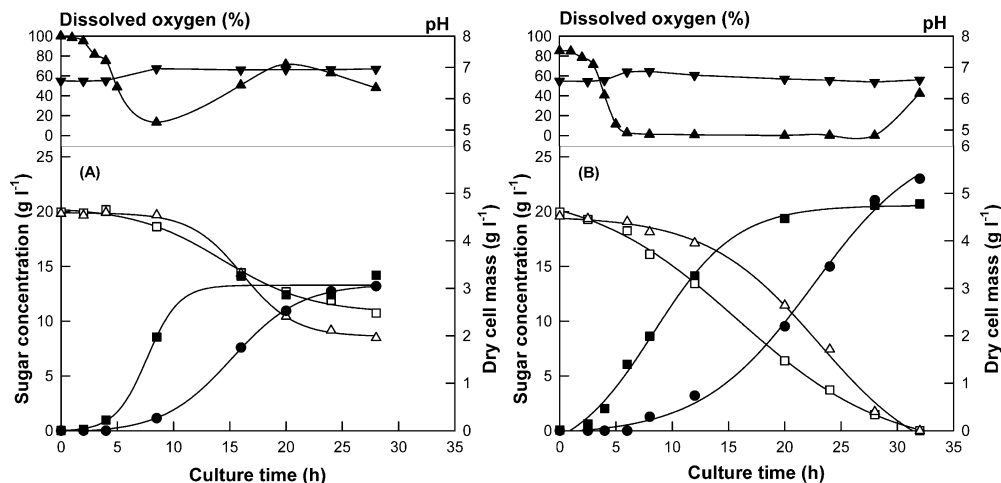
Medium and culture conditions

Pre-culture in a 500-ml baffled flask (Nalgene 4110-0500; Nalge Nunc Int., USA) was carried out with 200 ml of a culture medium consisting of 10 g l⁻¹ Bacto yeast extract (BD; Sparks, Md., USA), 5 g l⁻¹ KH₂PO₄, 5 g l⁻¹ K₂HPO₄, 1 g l⁻¹ MgSO₄·7H₂O (De Wulf et al. 1996), and 10 g l⁻¹ glucose at 37°C and 250 rpm in a shaking incubator (HK-S125C; Hankook Mechanics Co., Korea). Batch culture (1 l working vol. in a 3.7-l jar fermentor; ALF Bioengineering, Wald, Switzerland) was performed with 20 g l⁻¹ xylose and 20 g l⁻¹ glucose at 37°C, 600 rpm, and 1 vvm airflow. A pre-culture (10 ml) was inoculated into the culture medium. Dissolved oxygen (DO) content in culture broth was detected with a DO probe (Mettler-Toledo, Greifensee, Switzerland). One hundred percent DO content indicated oxygen saturation by the air supply at 37°C, 600 rpm, and 1 vvm. After batch cultivation with initial carbon sources of 20 g l⁻¹ xylose and 20 g l⁻¹ glucose, a mixture of 200 g l⁻¹ xylose and 200 g l⁻¹ glucose was fed continuously at a feed rate of 7.77 ml h⁻¹ either at the late-exponential phase, or at the early or mid-stationary phase to initiate the fed-batch mode of operation. Another mixture of 200 g l⁻¹ xylose and 50 g l⁻¹ glucose was used as a feeding solution in the optimized fed-batch culture. The feed rate was changed between 7.40 ml h⁻¹ and 9.25 ml h⁻¹ to keep the glucose level below 10 g l⁻¹. Other conditions of fed-batch fermentations were the same as in the batch case.

Dry cell mass and carbohydrate concentration

The optical density at 600 nm was converted to dry cell mass by multiplication with a conversion factor of 0.33. Concentrations of glucose, xylose, and D-ribose in culture

Fig. 2 Batch culture of **a** *B. subtilis* JY1 and **b** SPK1 with 20 g l⁻¹ xylose and 20 g l⁻¹ glucose at 37°C, 600 rpm, pH 7.0, and 1 vvm. Filled squares Dry cell mass, open squares glucose, open upright triangles xylose, filled circles D-ribose, filled up triangles DO, filled inverted triangles pH



broth were determined with a high performance liquid chromatography system (M930, Younglin Co., Korea; Lee et al. 2003). Twenty microliters of diluted sample were injected into a Carbohydrate analysis column (Waters, USA) at room temperature, with an 80% acetonitrile flow at 1.5 ml min⁻¹. To confirm their concentrations and determine the acetoin and acetic acid contents in culture broth, an Aminex HPX-87H ion exclusion column (300×7.8 mm; Bio-Rad Co., Hercules, Calif., USA) heated at 60°C was used to analyze the culture broth. H₂SO₄ (5 mM) was used as a solvent at a flow rate of 0.6 ml min⁻¹. Detection was made with a reflective index detector (Knauer Co., Germany).

Results

Batch fermentations of *B. subtilis* JY1 and SPK1

Batch fermentations were carried out with the transketolase-disrupted mutant *B. subtilis* SPK1 and its parental strain *B. subtilis* JY1 to compare their physiological properties of D-ribose production and sugar consumption. Figure 2a depicts the profiles of cell growth of *B. subtilis* JY1, xylose and glucose consumption, DO content, and pH. The maximum dry cell mass obtained was 3.28 g l⁻¹ in 28 h of cultivation. Xylose and glucose were consumed simultaneously until the end of fermentation, but 8.58 g l⁻¹ xylose and 10.7 g l⁻¹ glucose remained unused. D-Ribose was produced after the mid-exponential phase and a maximum concentration of 13.2 g l⁻¹ was obtained. An abrupt rise in DO content at 10 h likely indicated a decrease in the cellular activity of *B. subtilis* JY1. Another batch fermentation of mutant strain *B. subtilis* SPK1 was performed with 20 g l⁻¹ xylose and 20 g l⁻¹ glucose, as shown in Fig. 2b. A maximum dry cell mass of 4.78 g l⁻¹ was obtained at 32 h. Complete utilization of xylose and glucose by *B. subtilis* SPK1 resulted in D-ribose concentration of 23.0 g l⁻¹, which was higher than for *B. subtilis* JY1. A low DO content was measured during the period of D-ribose production. Acidity was monitored at pH 6.65 ± 0.13, which was similar to that for *B. subtilis* JY1

(pH 6.80 ± 0.19). As a high D-ribose producer, *B. subtilis* SPK1 can be used to optimize fed-batch fermentation strategies for maximizing D-ribose production.

Fed-batch processes of D-ribose biosynthesis

To increase the productivity of D-ribose biosynthesis, it is important to maintain the biological activity for xylose conversion to D-ribose after the batch stage. Bioconversion activity in D-ribose production from xylose was dependent on the cell growth phase, as shown in the batch fermentation (Fig. 2b). Fed-batch fermentations of *B. subtilis* SPK1 were designed to determine the optimal point among the late-exponential phase, early stationary phase, and mid-stationary phase for switching to fed-batch mode. The feeding solution was formulated on the basis of batch results of the simultaneous consumption of two carbon sources. Figure 3 presents a fed-batch fermentation with feeding at the late-exponential phase. After 20 h of batch culture, a mixture of 200 g l⁻¹ xylose and 200 g l⁻¹ glucose was supplemented continuously at a feed rate of 7.77 ml h⁻¹. The production rate of D-ribose was maintained constant during batch and fed-batch fermentation periods. A final dry cell mass of 6.0 g l⁻¹ and a concentration of 43.5 g l⁻¹ D-ribose were obtained during cultivation for 52 h. Addition of the sugar mixture at the early stationary phase or mid-stationary phase was carried out after 24 h or 28 h of the batch fermentation, with the same initial concentrations of xylose and glucose as for the late-exponential phase. A final dry cell mass of 5.15 g l⁻¹ and a concentration of 35.0 g l⁻¹ D-ribose were achieved for the early stationary phase; and a final cell dry mass of 5.28 g l⁻¹ and a concentration of 36.2 g l⁻¹ D-ribose were achieved for the mid-stationary phase. Comparison of the three fed-batch fermentations indicated that switching from batch mode to fed-batch mode at the late-exponential phase gave the best performance (0.91 g l⁻¹ h⁻¹ overall D-ribose productivity, 0.44 g g⁻¹ yield). In addition, a D-ribose productivity of 1.21 g l⁻¹ h⁻¹ was obtained in the fed-batch mode from 20 h to 48 h (Fig. 3), revealing a 1.3-fold increase compared with the overall D-ribose produc-

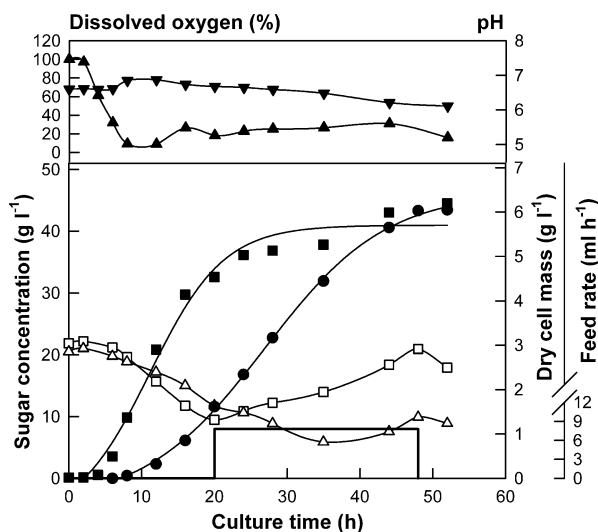


Fig. 3 Fed-batch fermentation of *B. subtilis* SPK1 by feeding a mixture of 200 g l⁻¹ xylose and 200 g l⁻¹ glucose at the late-exponential phase. Filled squares Dry cell mass, open squares glucose, open upright triangles xylose, filled circles D-ribose, filled upright triangles DO, filled inverted triangles pH, solid line feed rate

tion rate. It was suggested that the performance of D-ribose production could be improved by keeping the D-ribose production rate high during the fed-batch stage. Considering the final D-ribose concentration and production rate, the late-exponential phase was chosen as the best switching-point for feeding the xylose and glucose mixture in fed-batch fermentations.

Optimized fed-batch fermentation

In the previous fed-batch fermentations, 10 g l⁻¹ xylose and 20 g l⁻¹ glucose in the culture broth remained unused. A large amount of xylose and glucose after the end of cultivation might be undesirable, due to the high substrate cost for fermentation and complicated purification steps. Complete utilization of the carbon sources is required for the development of an efficient fed-batch process. A fed-batch culture was attempted with a feeding solution containing a low concentration of glucose and a flexible adjustment of feed rate to control low levels of xylose and glucose (Fig. 4). After 22 h of batch fermentation, a mixture of 200 g l⁻¹ xylose and 50 g l⁻¹ glucose was fed into the fermentor at a feed rate of 7.4 ml h⁻¹, which was changed to 9.25 ml h⁻¹ at 28 h. Exponential growth of *B. subtilis* SPK1 at a specific growth rate of 0.26 h⁻¹ in batch culture yielded 5.91 g l⁻¹ dry cell mass. Cell mass was kept constant throughout the fed-batch mode. The optimized fed-batch process resulted in a concentration of 46.6 g l⁻¹ D-ribose, a yield of 0.43 g D-ribose g⁻¹ sugar and a D-ribose productivity of 0.88 g l⁻¹ h⁻¹. The levels of xylose and glucose were maintained below 1.5 g l⁻¹ and 7.0 g l⁻¹, respectively.

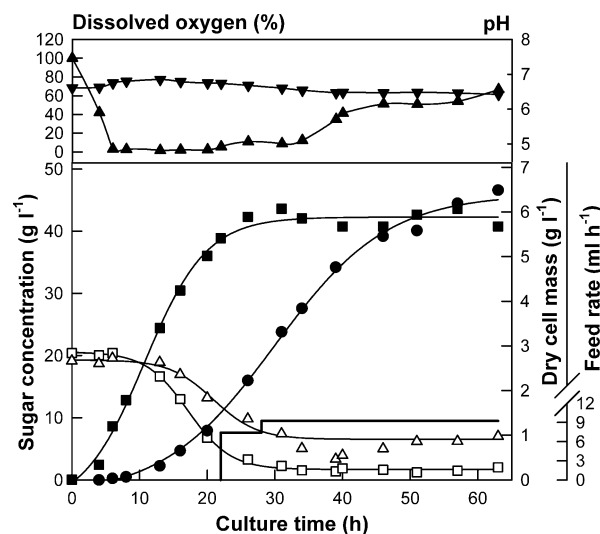


Fig. 4 Fed-batch fermentation of *B. subtilis* SPK1 by feeding a mixture of 200 g l⁻¹ xylose and 50 g l⁻¹ glucose at the late-exponential phase. Filled squares Dry cell mass, open squares glucose, open upright triangles xylose, filled circles D-ribose, filled upright triangles DO, filled inverted triangles pH, solid line feed rate

Discussion

A transketolase-deficient *B. subtilis* JY1 was able to produce D-ribose from xylose and glucose (Park and Seo 2004). A large amount of the carbon sources, however, remained unused in the culture medium. The high D-ribose-producing strain *B. subtilis* SPK1 was developed by chemical mutagenesis with NTG. Several batch and fed-batch fermentations by the mutant *B. subtilis* SPK1 resulted in improved fermentation parameters of D-ribose biosynthesis, as summarized in Table 1. The D-ribose concentration and productivity of *B. subtilis* SPK1 in batch culture were 1.7- and 1.5-fold higher than those of *B. subtilis* JY1, because of an increase in xylose and glucose consumption. But *B. subtilis* SPK1 synthesized D-ribose at a lower conversion yield (based on the consumed xylose and glucose) than the parental strain, *B. subtilis* JY1. Assuming that consumed xylose was converted into D-ribose at 100% yield by blocking the non-oxidative PP pathway (Fig. 1), *B. subtilis* SPK1 produced a 15% yield of D-ribose from glucose, whereas JY1 yielded 19%. The difference in yield seemed to be due to the fact that more glucose was used for cell growth by *B. subtilis* SPK1. Differing from the preferential consumption of glucose over xylose by wild-type *B. subtilis*, the simultaneous consumption of xylose and glucose was observed for both *B. subtilis* strains JY1 and SPK1. Other transketolase-negative mutants of a *Bacillus* sp. had a decreased ability to consume glucose, which was ascribed to an indirect relation between transketolase activity and enzyme II in the phosphoenolpyruvate:sugar transferase system (PTS; De Wulf and Vandamme 1997). A previous report for batch production of D-ribose suggested that *B. subtilis* JY1 was a PTS-negative strain in several batch fermentations with various carbon sources (Park and Seo 2004). Glucose

Table 1 Summarized results of D-ribose biosynthesis in batch and fed-batch cultures of transketolase-disrupted *B. subtilis* mutants. Parameters are given as overall values

Culture mode (<i>B. subtilis</i> strain)	D-Ribose concentration (g l ⁻¹)	Yield (g D-ribose g ⁻¹ sugar)	D-Ribose productivity (g l ⁻¹ h ⁻¹)	Xylose consumption rate (g l ⁻¹ h ⁻¹)
Batch (JY1)	13.2	0.64	0.47	0.40
Batch (SPK1)	23.0	0.58	0.72	0.61
Fed-batch (SPK1, optimized)	46.6	0.43	0.88 (1.16) ^a	1.43 (2.05) ^a
Batch (ATCC21951) ^b	60.0	0.30	0.55	1.67

^aD-Ribose productivity and xylose consumption rate in parentheses were acquired in the fed-batch stage

^bBatch results of *B. subtilis* ATCC21951 using 100 g l⁻¹ xylose and 100 g l⁻¹ glucose were cited in De Wulf et al. (1996)

seemed to be delivered into *B. subtilis* SPK1 cells by a PTS-independent glucose-uptake system, such as GlcP or GlcU (Paulsen et al. 1998). The DO content in the culture broth was kept low during D-ribose production, indicating that maintenance of cellular activity was important to maximize D-ribose biosynthesis. In *Debrayomyces hanse-nii*, levels of xylose metabolic enzymes inside cells were related to the oxygen transfer rate (Nobre et al. 2002).

An endeavor to increase the D-ribose titer was made by designing the fed-batch process with a feed of sugar mixtures. Three fed-batch fermentations with a feed of 200 g l⁻¹ xylose and 200 g l⁻¹ glucose resulted in higher D-ribose concentration than did the batch culture. But a feed rate higher than the sugar consumption rates led to an accumulation of xylose and glucose (over 10 g l⁻¹) in the culture broth. The optimized fed-batch fermentation, when a mixture of 200 g l⁻¹ xylose and 50 g l⁻¹ glucose was fed stepwise after the late-exponential phase, resulted in a 2.0- and 1.2-fold increase in D-ribose concentration and overall productivity, respectively, compared with those of the batch fermentation (Table 1). Especially, D-ribose productivity in the fed-batch stage was 1.6-fold higher than in the batch fermentation. A significant improvement in D-ribose biosynthesis seemed to be attributable to the considerable increase in the xylose consumption rate (3.4-fold) in the fed-batch mode. Although a similar observation of xylose flux elevation in a fed-batch fermentation was not reported, an *E. coli* mutant strain showing a PTS-negative phenotype exhibited an increased ability to consume xylose (Lindsay et al. 1995). In addition, housekeeping enzymes involved in the glucose catabolism of *B. subtilis* showed similar activities or expression levels through cultivation (Blencke et al. 2003; Freese et al. 1970). The reason for an increase in xylose flux might be that PTS-dependent catabolite repression of xylose metabolism did not work and a low glucose level during the D-ribose production stage reduced the glucose effect (Stülke and Hillen 2000). In a comparison of four fed-batch cultures, the optimized fermentation reduced the final contents of xylose and glucose in the culture broth to less than 7.0 g l⁻¹, although similar D-ribose concentrations were obtained.

To compare our data with those obtained for *B. subtilis* ATCC21951, which is known as a good producer, batch results for *B. subtilis* ATCC21951 with 100 g l⁻¹ xylose and 100 g l⁻¹ glucose for 110 h are summarized in Table 1

(De Wulf et al. 1996). Experimental data from a fed-batch fermentation for D-ribose production have not been reported to date. In comparison with the optimized fed-batch fermentation results, D-ribose yield and productivity for *B. subtilis* SPK1 were 1.4- and 1.6-fold higher than the corresponding values of *B. subtilis* ATCC21951. More D-ribose was produced by *B. subtilis* ATCC21951. The sugar consumption rates of *B. subtilis* ATCC21951 were higher than those of *B. subtilis* SPK1, but *B. subtilis* SPK1 converted xylose to D-ribose more rapidly during the fed-batch stage than *B. subtilis* ATCC21951. *B. subtilis* SPK1 did not tolerate high osmotic pressure (data not shown). More research is in progress to characterize the metabolic pathways of D-ribose biosynthesis at the enzyme level between the mutant strain *B. subtilis* SPK1 and its parental strain JY1.

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