

## Effects of Oxygen Supply and Mixed Sugar Concentration on D-Ribose Production by a Transketolase-Deficient *Bacillus subtilis* SPK1

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**D-Ribose is a value-added five-carbon sugar used for riboflavin production. To investigate the effects of oxygen supply and mixed sugar concentration on microbial production of D-ribose, a transketolase-deficient *Bacillus subtilis* SPK1 was cultured batch-wise using xylose and glucose. A change of agitation speed from 300 rpm to 600 rpm at 1 vvm of air supply increased both the xylose consumption rate and D-ribose production rate. Because the sum of the specific consumption rates for xylose and glucose was similar at all agitation speeds, metabolic preferences between xylose and glucose might depend on oxygen supply. Although *B. subtilis* SPK1 can take up xylose and glucose by the active transport mechanism, a high initial concentration of xylose and glucose was not beneficial for high D-ribose production.**

**Key words:** D-Ribose, xylose, *Bacillus subtilis*, oxygen supply, mixed sugar

A five-carbon sugar, D-ribose, has served as a starting material for the production of riboflavin (vitamin B<sub>2</sub>) with anticancer and antiviral effects as well as used for animal feed additives, cosmetics, and foods [4]. To produce D-ribose, enzymatic hydrolysis of yeast RNA and chemical synthesis using arabinose, glucose, and gluconic acid have been established, but these showed some disadvantages of low yield and complex processes for recovery and purification [5, 11]. Biological production of D-ribose at high titer has been achieved by fermentation of transketolase-deficient microbial strains. Transketolase is a metabolic enzyme connecting the pentose phosphate pathway and glycolysis. If transketolase loses its activity, D-ribose-5-phosphate, one of the substrates of transketolase, may accumulate

inside the cells and hence be dephosphorylated to D-ribose by nonspecific phosphatases [5, 8]. Among several bacterial strains lacking transketolase activity, *Bacillus subtilis* and *Bacillus pumilus* have gained interest as high D-ribose producers. For example, *B. subtilis* ATCC 21915 produced D-ribose from glucose with 48% yield [12]. Various carbon sources (glucose, mannose, sucrose, maltose, sorbitol, and mannitol) were used to produce D-ribose [6] and corn steep liquor was effective for a large-scale production [8]. Citrate enhanced the carbon fluxes in the pentose phosphate pathway and tricarboxylic acid cycle, and also reduced fluxes in glycolysis, yielding an 83.4 g/l D-ribose concentration and 59% (mol/mol) yield from glucose [11]. Supplementation of aromatic amino acids such as L-tryptophan, L-tyrosine, and L-phenylalanine increased D-ribose production yield and suppressed the formation of gluconic acid by-product [9]. When a D-ribose-producing mutant was grown on glucose with a second substrate (D-gluconate, D-xylose, L-arabinose, or xylitol), catabolite repression on utilization of the second substrate was not found [1, 5–6]. Increased concentrations of glucose, ammonium sulfate, and potassium ion increased cell mass and by-product formation, but reduced the D-ribose production yield by a transketolase-deficient *B. pumilus* strain [9]. Disruption of a transketolase gene (*tkt*) by a genetic method allowed a wild type of *B. subtilis* strain 168 to be able to produce D-ribose from glucose and xylose, a five-carbon sugar present in lignocellulosic biomass [4].

Previously, we screened a *B. subtilis* JY1 mutant lacking transketolase and the glucose-specific enzyme II of the phosphoenolpyruvate-mediated sugar transferase system (PTS) [6] and constructed a high D-ribose-producing *B. subtilis* SPK1 strain from the JY1 mutant. Among various fermentation conditions affecting D-ribose production, in this study, the two critical factors of oxygen supply and mixed sugar concentration were assessed in batch cultivation of *B. subtilis* SPK1 using xylose and glucose as carbon sources.

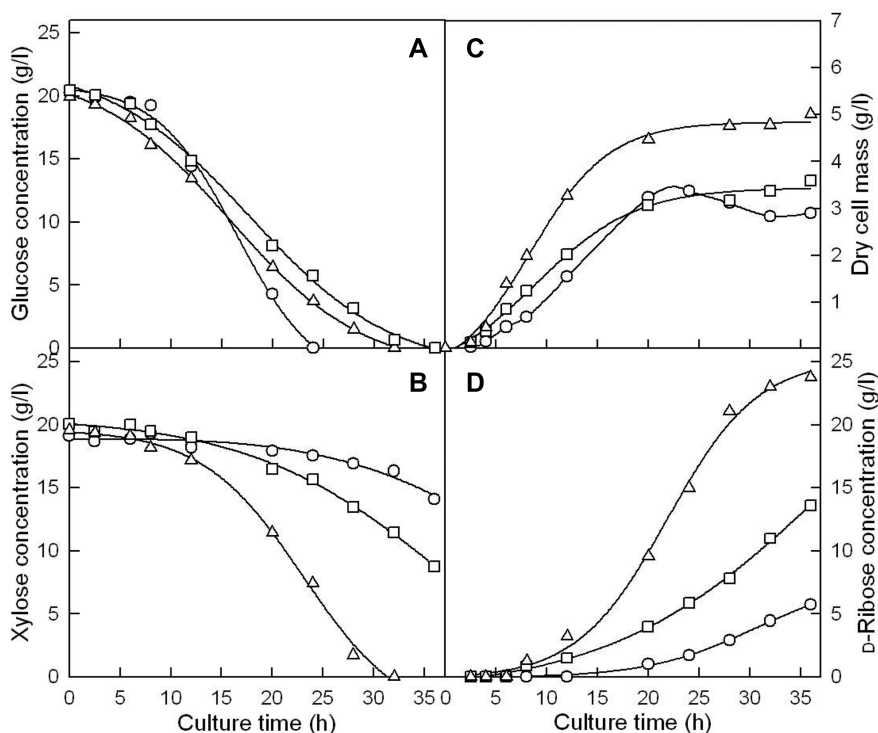
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Supplementation of oxygen into culture broth was crucial to produce D-ribose from xylose efficiently, because xylose consumption depended on ATP supply [6]. Batch cultivations with 20 g/l glucose and 20 g/l xylose were carried out to evaluate the effects of oxygen supply on sugar metabolism of *B. subtilis* SPK1. A D-ribose-producing *B. subtilis* SPK1 without transketolase activity was constructed previously by chemical mutagenesis [5]. Pre-culture in a 500-ml baffled flask (Nalgene 4110-0500; Nalge Nunc Int., Rochester, NY, USA) was carried out with 200 ml of culture medium consisting of 10 g/l yeast extract (BD, Franklin Lakes, NJ, USA), 5 g/l  $\text{KH}_2\text{PO}_4$ , 5 g/l  $\text{K}_2\text{HPO}_4$ , 1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  [5], and 10 g/l glucose at 37°C and 250 rpm in a shaking incubator (HK-S125C; Hankook Mechanics Co., Korea). Batch culture in a 3.7 L jar fermentor (Type ALF; Bioengineering AG, Switzerland) was performed at 37°C and 1 vvm of air flow rate. One liter of culture medium with various concentrations of xylose and glucose was used. Acidity was controlled at  $\text{pH } 7.0 \pm 0.05$  with 2 N hydrochloric acid and ammonia solution. To optimize the oxygen supply, agitation speeds were tested at 300, 450, and 600 rpm. Optical density at 600 nm was converted to dry cell mass by multiplication of the conversion factor 0.33 [5]. The concentrations of glucose, xylose, and D-ribose in culture broth were determined with an HPLC system (M930; Younglin Co., Korea) [2]. Diluted sample was injected into the carbohydrate analysis column

(Waters Co., Milford, MA, USA) at room temperature, in which 80% acetonitrile flowed at 1.5 ml/min. Detection was made with a reflective index detector (Knauer Co., Germany). As shown in Fig. 1A, glucose was consumed faster at a low agitation speed of 300 rpm than at 450 and 600 rpm. In all cases, the initially added glucose was consumed completely within 35 h. Compared with the glucose consumption profiles, xylose consumption was considerably affected by oxygen supply. Along with an increase in agitation speed, the xylose consumption rate was enhanced (Fig. 1B). Only 2.8 g/l xylose was consumed at 300 rpm in 32 h, whereas 20 g/l xylose was depleted at 600 rpm. Not only xylose consumption, but also both cell growth and D-ribose production were influenced. As shown in Fig. 1C, the highest dry cell mass of 5.0 g/l was obtained at 600 rpm, which was about 1.6 times higher than those at 300 and 450 rpm. The batch culture at 600 rpm resulted in the highest D-ribose concentration of 23.7 g/l, corresponding to 4.1 and 1.7 times higher values relative to those at 300 and 450 rpm, respectively (Fig. 1D). The yield of dry cell mass based on the consumed sugar concentration exhibited a similar value of 0.13 g cell/g sugar. However, yields of D-ribose production were dependent on the agitation speeds, and 0.19, 0.43, and 0.58 g D-ribose/g sugar were obtained at 300, 450, and 600 rpm, respectively. By-products such as acetoin and acetic acid were not detected throughout the cultivations. To elucidate the



**Fig. 1.** Profiles of glucose (A) and xylose (B) concentrations, dry cell mass (C), and D-ribose concentration (D) in batch fermentations of *B. subtilis* SPK1 with 20 g/l glucose and 20 g/l xylose at 300 rpm (○), 450 rpm (□), and 600 rpm (△), and 37°C and 1 vvm.

**Table 1.** Effect of agitation speeds on specific rates for sugar consumption and D-ribose production by *B. subtilis* SPK1.

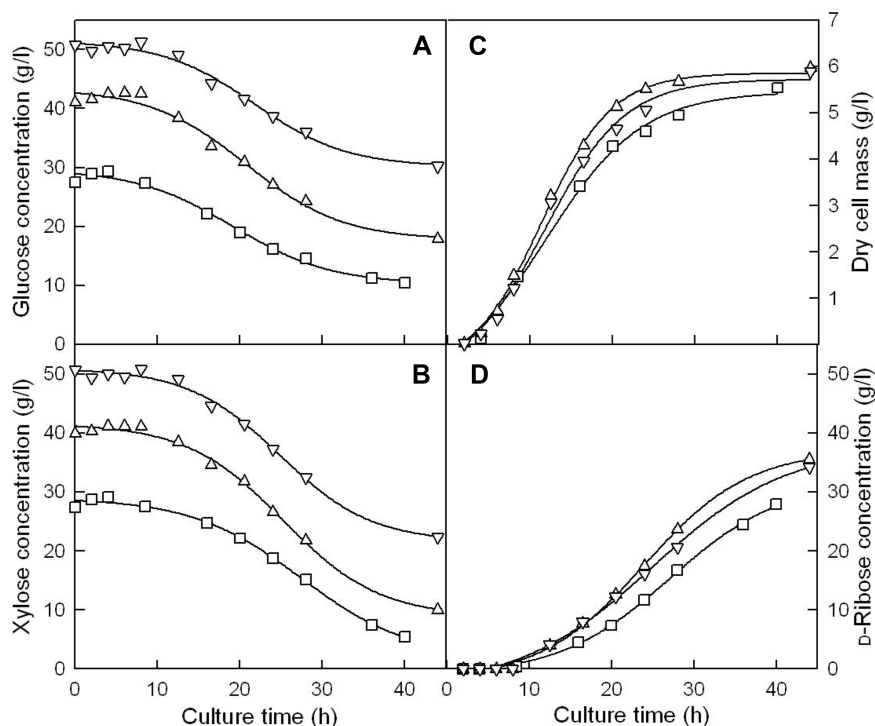
Agitation speed (rpm)	Specific rates (1/h)		
	Glucose consumption	Xylose consumption	D-Ribose production
300	0.37 ± 0.00	0.04 ± 0.01	0.07 ± 0.03
450	0.22 ± 0.03	0.16 ± 0.02	0.17 ± 0.04
600	0.21 ± 0.13	0.20 ± 0.04	0.24 ± 0.05

All values were calculated at the pseudo-steady state from 20 to 32 h of cultivation described in Fig. 1.

relationship between oxygen supply and sugar metabolism in *B. subtilis* SPK1, specific rates for sugar consumption and D-ribose production were calculated at the pseudo steady-state by assuming that dry cell mass of *B. subtilis* SPK1 did not change, and glucose and xylose were metabolized at constant rates (Table 1). A change of agitation speed from 300 rpm to 600 rpm allowed 5.0- and 3.4-fold increases in specific xylose consumption rate and specific D-ribose production rate, respectively. Specific glucose consumption rate, however, decreased by about 57% in accordance with an increase in agitation speed. The dissolved oxygen (DO) content considerably affected the flux distribution between glucose and D-ribose. A transketolase-deficient *B. pumilis* produced a higher concentration of D-ribose using

200 g/l glucose at 40% DO than using 100 g/l glucose at 5% DO, which suggested that the glucose flux through the pentose phosphate pathway was not only dependent on DO and glucose concentration, but also depended on the glucose/oxygen ratio [8]. Pyruvate production by *Torulopsis glabrata* was highly affected by agitation speed. High agitation speed led to high pyruvate yield but decreased the glucose consumption rate, whereas low agitation speed yielded a high glucose consumption rate, which seemed to be caused by different regeneration pathways for NADH produced via glycolysis [3]. The exact evidence to elucidate the variation of the glucose and xylose consumption rates upon aeration was unclear. However, a sum of the specific consumption rates for xylose and glucose was similar at about 2.4 mmol/g cell-h in each case. It is suggested that the expression or reaction of metabolic enzymes involved in the xylose metabolism seems to be regulated by the oxygen content, and that the total capacity of carbon uptake or consumption in *B. subtilis* SPK1 is probably controlled accordingly. Subsequent fermentations to produce D-ribose were carried out at 600 rpm.

*B. subtilis* usually transports glucose into the cell via PTS and takes up xylose by a non-specific sugar transporter [10]. The mother strain of *B. subtilis* SPK1, *B. subtilis* JY1, lacks an element of PTS and delivers glucose via the non-PTS (facilitated diffusion or active transport) [6]. The

**Fig. 2.** Profiles of glucose (A) and xylose (B) concentrations, dry cell mass (C), and D-ribose concentration (D) in batch fermentations of *B. subtilis* SPK1 with various mixtures of xylose and glucose at 37°C, 600 rpm, and 1 vvm.

□, 28 g/l glucose + 28 g/l xylose; △, 40 g/l glucose + 41 g/l xylose; ▽, 51 g/l glucose + 51 g/l xylose.

**Table 2.** Fermentation parameters of D-ribose biosynthesis and sugar consumption using various compositions of glucose and xylose.

Sugar composition	28 g/l glucose + 28 g/l xylose	41 g/l glucose + 40 g/l xylose	51 g/l glucose + 51 g/l xylose
D-Ribose concentration (g/l)	27.9	35.6	34.3
Yield of D-ribose (g ribose/g sugar)	0.72	0.67	0.68
D-Ribose productivity (g/l-h)	0.70	0.81	0.78
Glucose consumption rate (g/l-h)	0.43	0.53	0.48
Xylose consumption rate (g/l-h)	0.55	0.68	0.66

The fermentation was carried out in duplicate, and the average values with lower than 5% standard deviations are shown.

mechanisms of facilitated diffusion and active transport have been known to follow an equation analogous to Michaelis–Menten kinetics depending on the extracellular and/or intracellular sugar concentration [7]. The effects of sugar concentration on cell growth, sugar consumption, and D-ribose production were investigated in batch fermentations with various mixtures of xylose and glucose. The batch fermentation was independently repeated two times and the average values are displayed in Fig. 2 and Table 2. As shown in Fig. 2C, the cells grew exponentially at a similar specific growth rate between 0.24 and 0.30 h<sup>-1</sup> and cell mass was not changed after 24 h. Batch culture with 51 g/l glucose and 51 g/l xylose provided the highest dry cell mass of 5.90 g/l, similar to that with 41 g/l glucose and 40 g/l xylose. In D-ribose production described in Fig. 2D, high concentrations of glucose and xylose led to an increase in D-ribose concentration, of which 35.6 g/l was obtained from 41 g/l glucose and 40 g/l xylose. Consumption profiles of glucose and xylose are illustrated in Figs. 2A and 2B, respectively. In all conditions, xylose and glucose were consumed simultaneously, confirming that PTS for glucose transport in *B. subtilis* SPK1 was defective. A large amount of sugars was left unused in culture broth and high values of DO content above 50% were monitored after 30 h cultivation (data not shown). It indicated that over 60 g/l sugar concentration and reduced cell viability might cause the deactivation of the sugar metabolism and prevent further enhancement of D-ribose production by *B. subtilis* SPK1. Fermentation parameters of yield and productivity for D-ribose, and consumption rates for glucose and xylose are summarized in Table 2. Whereas a mixture of 41 g/l glucose and 40 g/l xylose gave the highest productivity of 0.81 g/l-h, the highest yield of 0.72 g D-ribose/g sugar was obtained in the cultivation with 28 g/l glucose and 28 g/l xylose. Remarkable differences in yield and productivity were not observed among the four cultivations, however. Overall consumption rates of total carbon sources were achieved at around 1.2 g/l-h, except for the case of 28 g/l glucose and 28 g/l xylose. Additionally, specific rates for glucose consumption, xylose consumption, and D-ribose production at the pseudo steady-state were similar in all cases, of which average values were estimated to be 0.15 ± 0.01 h<sup>-1</sup>, 0.21 ± 0.02 h<sup>-1</sup>, and 0.24 ± 0.02 h<sup>-1</sup>, respectively.

Considering all kinetic parameters, a higher initial sugar concentration was not beneficial for efficient production of D-ribose from xylose and glucose by *B. subtilis* SPK1.

In conclusion, a transketolase-deficient mutant of *B. subtilis* SPK1 was characterized to lose one component of the PTS specific for glucose and to metabolize both xylose and glucose to D-ribose in our previous report. To investigate the effects of oxygen supply on D-ribose production, batch cultures using mixed sugars were carried out in a bioreactor at three agitation speeds. More oxygen supply boosted the metabolic ability of *B. subtilis* SPK1 for D-ribose production from xylose. On the basis of active transportation of xylose and glucose into the *B. subtilis* SPK1 cells, a higher sugar concentration was expected to elevate the D-ribose production ability. Against expectation, a high initial sugar concentration was not beneficial for high production of D-ribose, and high amounts of xylose and glucose still remained in the bioreactor. To solve this problem and hence increase the D-ribose titer, various fed-batch strategies should be devised by fine control of the xylose and glucose concentrations, and for the maintenance of cell viability throughout the cultivation.

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