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ORIGINAL PAPER

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Metabolic analysis of acetate accumulation during xylose consumption by *Paenibacillus polymyxa*

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Abstract *Paenibacillus polymyxa* ATCC 12321 produced more acetic acid and less butanediol from xylose than from glucose. The product yields from xylose were ethanol (0.72 mol/mol sugar), *(R,R)*-2,3-butanediol (0.31 mol/mol sugar), and acetate (0.38 mol/mol sugar) while those from glucose were ethanol (0.74 mol/mol sugar), *(R,R)*-2,3-butanediol (0.46 mol/mol sugar), and acetate (0.05 mol/mol sugar). Higher acetate kinase activity and lower acetate uptake ability were found in xylose-grown cells than in glucose-grown cells. Furthermore, phosphoketolase activity was higher in xylose-grown cells than in glucose-grown cells. In fed-batch culture on xylose, glucose feeding raised the butanediol yield to 0.56 mol/mol sugar and reduced acetate accumulation to 0.04 mol/mol sugar.

Introduction

Paenibacillus polymyxa can produce optically active *(R,R)*-2,3-butanediol not only from glucose but also from xylose. Since the biomass contains cellulose and hemicellulose mainly consisting of xylose, in order to economically produce optically active *(R,R)*-2,3-butanediol, it is important to ferment all sugars present in the biomass during fermentation, with high yields and productivity. While the production of butanediol from xylose and other hemicellulosic components by *P. polymyxa* has been reported (de Mass et al. 1988; Laube et al. 1984) the xylose consumption rate is still much lower than that of glucose. Recently, we found that xylose consumption by *P. polymyxa* dramatically increased in fermentation at 39°C (Marwoto et al. 2002); i.e., 100 mM xylose was consumed within 24 h. However, acetate production was much higher on xylose than on glucose medium, leading to a low butanediol yield. Therefore, to increase butanediol production, the amount of acetate must be reduced. However, there is little knowledge regarding acetate production during xylose fermentation by *P. polymyxa*.

During mixed-acid fermentation of glucose and xylose by *P. polymyxa*, metabolites such as ethanol, acetate, lactate, and formate (Fig. 1) are produced. D-Glucose is mainly metabolized by the Embden-Meyerhoff-Pernas (EMP) pathway, and acetate is usually formed via acetyl phosphate through the action of acetate kinase. Upstream xylose metabolism is quite different than that of glucose. Xylose metabolism proceeds by the pentose-phosphate pathway (PPP), and xylose is usually converted to glyceraldehyde-3-phosphate (G3P). This reaction was described as being equivalent to the glycolysis of glucose to G3P by microorganisms carrying out mixed-acid fermentation (Jansen and Tsao 1983). Therefore, acetate accumulation during xylose fermentation can be explained by increased enzyme activity for acetate production, especially acetate kinase.

Nakashimada et al. (2000) reported that when acetate was added to the medium it was consumed together with glucose by *P. polymyxa*, and butanediol production increased dramatically. However, Laube et al. (1984), who studied the bioconversion of 1% xylose, observed no effect on butanediol production when an initial concentration of 0.5 or 1.0% (w/v) acetate was added to the culture medium. These results suggested that acetate accumulation on xylose medium is due to the difference in the uptake of acetate on glucose vs. xylose medium.

Some microorganisms convert pentoses to acetyl phosphate via xylulose-5-phosphate through the action of phosphoketolase (Fig. 1). Phosphoketolase activity has been found in lactobacilli, and in other bacteria and yeasts (Jeffries 1983; Marounek and Petr 1995; Ratledge and Holdsworth 1985). Although phosphoketolase has not been reported in *P. polymyxa*, its contribution to acetate production is expected.

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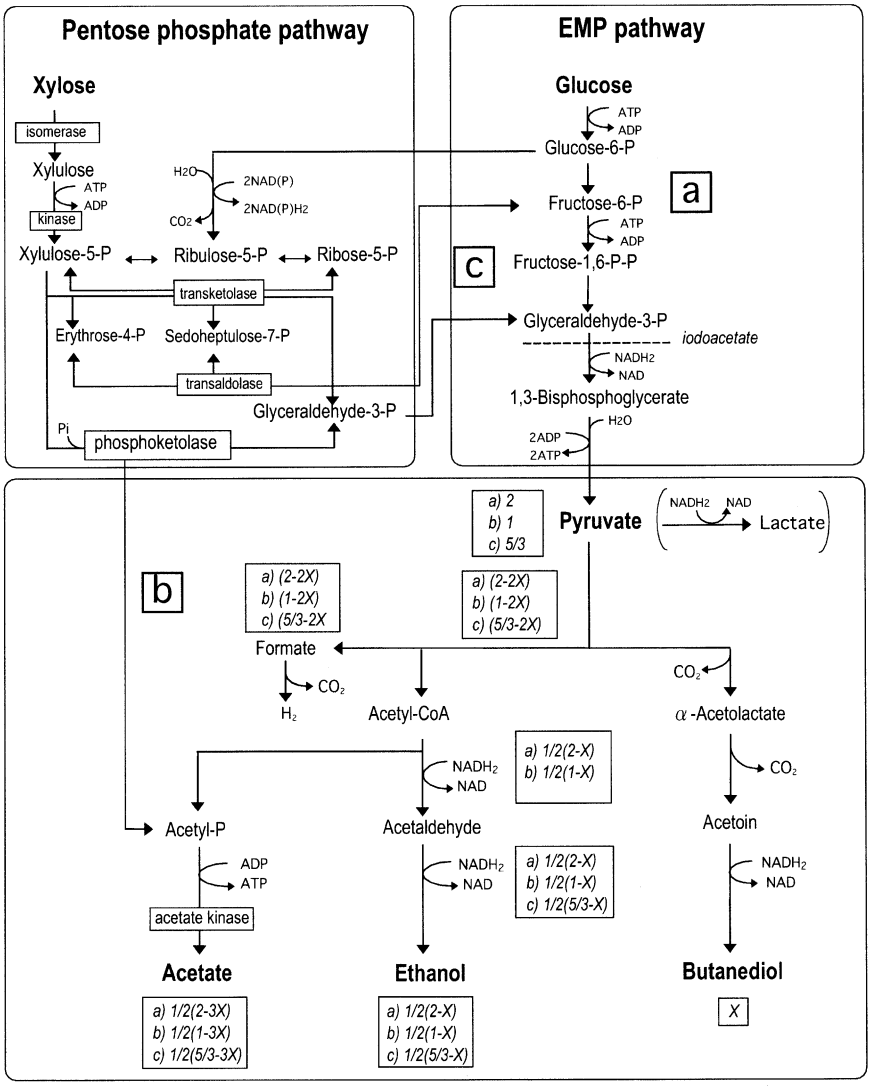

Fig. 1 Proposed pathways of metabolite formation from glucose and xylose by *Paenibacillus polymyxa*: **a** Embden-Meyerhoff-Pernas (EMP) pathway, **b** acetyl-P pathway, **c** pentose-phosphate (PP)-EMP pathway

The aim of this study was to explain the hyper-accumulation of acetate from xylose by testing the above hypotheses using fermentative, enzymatic, and stoichiometric analyses, and to increase production of butanediol by *P. polymyxa* cultured on glucose and xylose medium.

Materials and methods

Microorganism

Paenibacillus polymyxa (formerly *Bacillus polymyxa*) ATCC 12321 cultures were stored in 1-ml vials containing 60% glycerol protective medium in a low-temperature freezer at −80°C.

Medium composition

The basal medium used for experiments was described previously (Nakashimada et al. 1998). Xylose and glucose were sterilized and added separately to the medium in varied concentrations, as indicated in the results. A seed inoculum of *P. polymyxa* was prepared in the above medium containing 100 mM sugar and grown for 12 h at 39°C under agitation (120 rpm).

Batch culture

pH-uncontrolled batch culture in 125-ml bottles (working volume50 ml) and pH-controlled batch culture in a 3-l jar fermenter (working volume 2 l) were established as described previously (Marwoto et al. 2002). Fermentation was carried out at 39°C, pH 6.3, at an agitation speed of 200 rpm. Cell density, and the remaining sugar concentration and product concentration in the broth were monitored every 3 h.

Fed-batch culture

Fed-batch culture was done in a 3-l jar fermentor (working volume 2 l) in basal medium containing 1% yeast extract and 1% tryptone at 39°C. The pH was monitored by a pH electrode, and adjusted to 6.3 with a pH controller by the addition of 3 M KOH. The agitation speed was kept at 200 rpm. The culture was started in medium supplemented with xylose, and intermittent feedings of 2 M glucose and 2 M xylose solutions were started after 12 h of batch culture. When almost all the acetate in the medium was consumed, after 34 h, only xylose was fed into the medium at 36 h, thereafter both substrates were fed three more times.

Acetate uptake by growing cells

Cells grown on xylose and glucose medium were harvested after 12 h of culture by centrifugation (10,000 *g*, 10 min, 4 °C), washed twice with 100 mM phosphate buffer (pH 7.0), and resuspended in 50 ml basal medium containing sugar and acetate without yeast extract and tryptone.

Preparation of resting cells

Exponential-phase cells (12-h culture) were harvested by centrifugation (10,000*g*, 10 min, 4°C), washed twice with 100 mM phosphate buffer (pH 7.0), resuspended in 5 ml of the same buffer, and then inoculated into 20 ml of 100 mM phosphate buffer (pH 6.8) containing 100 mM xylose or glucose. The cultures were incubated at 39°C and 120 rpm.

Enzyme assays

Cells grown in bottles at 39°C on 150 mM glucose or xylose medium were harvested at mid-exponential phase (14 h) by centrifugation (10,000*g*, 10 min, 4°C). The cells were washed four times with 100 mM phosphate buffer (pH 7.0), and resuspended into 5 ml of the same buffer containing 20 mM $MgSO_4$, 0.01 mM EDTA, and 0.2 mM dithiothreitol. The cell suspension was kept on ice and disrupted by sonication (5 min, pulse 3 s on and 1 s off, and amplitude 30%; Sonics Vibracell VCX500, Newtown, Conn., USA). Cell debris was removed by centrifugation (10,000*g*, 10 min, 4°C). The cell-free extract was stored at −80°C until needed.

Acetate kinase activity was measured by the method of Rose et al. (1954), with slight modifications. The final incubation volume of 1 ml contained 0.3 ml substrate (3.2 M potassium acetate, 1.0 M Tris-HCl buffer, pH 7.4, 1.0 M $MgCl_2$in a volume ratio 25:5:1), 0.35 ml 14% neutral hydroxylamine (mixing equal volumes of 28% hydroxylamine-HCl and 4.0 M KOH), 0.1 ml 0.2 M ATP, and 0.25 ml cell-free extract. The samples were incubated at 39°C, and then 1 ml of 10% trichloroacetic acid was added to terminate the reaction. After centrifugation to remove any precipitates, 4.0 ml 5% $FeCl_3.6H_2O$ in 0.1 N HCl was added. The amount of ferric chloride-hydroxamic acid complex formed was measured at 540 nm. Acetate kinase activity was calculated as the amount of acetyl phosphate liberated in 1 min.

Phosphoketolase activity was determined using a modification of the method of Marounek and Petr (1995). Although this method is based on the conversion of xylulose-5-P to acetyl phosphate, we were unable to purchase xylulose-5-P from commercial sources, and therefore used xylulose as the substrate in this experiment. The reaction mixture contained: 70 ml 1 M phosphate buffer (pH 6.5), 50 ml 0.05 M $MgCl_2$, 50 ml 3.6 mM thiamine pyrophosphate, 50 ml 33 mM xylulose, 50 ml 100 mM ATP, and 250 ml cell extract. The reaction mixture was incubated at 39°C for 1 h, and H_2SO_4(10.8% w/v, final concentration) was added to terminate the reaction. The samples were centrifuged and the acetate formed was determined by HPLC. Phosphoketolase activity was calculated as the amount of acetate liberated in 1 min.

Analyses

Cell growth was monitored by measuring the optical density (OD) at 660 nm, and a 1-OD unit was estimated to be equivalent to 0.47 g dry cell mass l^{-1} . The sugar concentration (glucose, xylose) and the products of fermentation (butanediol, ethanol, acetate, acetoin, formate, succinate and lactate) were determined using by HPLC (Aminex HPX-87H Bio-rad column, CO-985, RI-930, PU-980, Jasco, Japan). The gas products (CO_2, H_2) were analyzed by gas chromatograph (GC-8A TCD, Shimadzu, Kyoto, Japan).

Table 1 Products diversity of *Paenibacillus polymyxa* during fermentation on glucose and xylose in pH-uncontrolled batch culture. Culture conditions: 24 h at 39°C and initial pH 6.3 in 200 mM glucose or xylose medium. Values represent averages of at least triplicate determinations

	Xylose medium		Glucose medium	
	Yield		Yield	
	(mM)	(mol/mol)	(mM)	(mol/mol)
Sugar consumed	142±4.7		181±2.8	
(R,R)-2,3-butanediol	59±1.3	0.42	101±0.1	0.56
Ethanol	89±2.8	0.63	124±0.4	0.69
Acetate	41±0.6	0.29	2±0.01	0.01
Acetoin	4±0.1	0.02	3±0.1	0.02
Lactate	2±0.3	0.01	20±1.4	0.11
Formate	7±0.2	0.05	49±0.01	0.27
Succinate	1.6±2.7	0.01	20±1	0.11
H_2	73±5	0.51	77±6	0.42
CO_2	208±9	1.47	247±12	1.36
Cell (g/l)	1.7±0.03		1.8±0.02	
Carbon recovery (%)	104		102	
Electron recovery (%)[b]	102		103	

[a]Carbon recovery was calculated independent of cells produced

[b]Electron recovery was calculated using equivalent of available electrons (Erickson et al. 1979) in products and substrates listed in the table

The protein concentrations of the cell-free extract were determined by using the bicinchoninic acid protein assay reagent kit (Pierce, Rockford, Ill., USA) and measuring the results spectrophotometrically at 562 nm (Smith et al. 1985). All assays were carried out in triplicate using bovine serum albumin as the standard protein with appropriate controls; the differences in the replicate were less than 10%.

Results

Batch culture on glucose and xylose medium

The diverse fermentation products of *P. polymyxa* on xylose and glucose medium after 24 h of pH-uncontrolled batch culture are listed in Table 1. On glucose medium, only a small amount of acetate was produced, giving an acetate yield of 0.01 mol/mol sugar and a butanediol yield of 0.56 mol/mol sugar. On xylose medium, the acetate yield increased to 0.29 mol/mol, and the butanediol yield decreased to 0.42 mol/mol. The lactate yield on glucose medium was much higher than that on xylose medium. The yields of ethanol were almost the same on the two sugars.

The fermentation products of *P. polymyxa* on xylose and glucose medium after 24-h of pH-controlled batch culture at pH 6.3 were similar to obtained with pH-uncontrolled batch culture (Table 2).

Table 2 Products diversity of *P. polymyxa* during fermentation on glucose and xylose in pH-controlled batch culture. Culture conditions: 24 h at 39°C under pH control at 6.3 in 300 mM glucose or xylose medium. *ND* Not detected. Values represent averages of at least duplicate determinations

	Xylose medium		Glucose medium	
	Yield		Yield	
	(mM)	(mol/mol)	(mM)	(mol/mol)
Sugar consumed	265±8		316±6	
$\mu_{max}(h^{-1})$	0.37±0.1		0.51±0.1	
(R,R)-2,3-butanediol	82±9	0.31	146±9	0.46
Ethanol	191±11	0.72	233±11	0.74
Acetate	101±8	0.38	17±2	0.05
Acetoin	2±1	0.01	ND	0.00
Lactate	15±3	0.06	52±5	0.17
Formate	7±7	0.06	27±3	0.09
Succinate	8±0.4	0.03	2±0.2	0.01
H_2	291±15	1.09	249±15	0.79
CO_2	441±9	1.67	515±12	1.63
Cell (g/l)	2.4±0.2		2.5±0.1	
Carbon recovery (%)	117		100	
Electron recovery (%)	110		102	

Acetate kinase activity

Acetate production has to be reduced in order to increase the butanediol yield on xylose medium. However, the reason for such high acetate accumulation was obscure. Therefore, the mechanism of acetate accumulation on xylose medium was investigated. Because acetate kinase is the key enzyme in acetate production, its activity was measured in cell-free extracts from xylose- and glucose-grown cells. The activity of the enzyme in xylose- and glucose-grown cells was 80±5.1 mmol/mg protein/min and 15±1.7 mmol/mg protein/min, respectively, suggesting that the five-fold higher acetate kinase activity in xylose-grown cells would result in increased acetate accumulation.

Acetate uptake on glucose and xylose medium

Xylose-grown cells produced more acetate than glucose-grown cells on glucose medium. When acetate was added to the glucose medium, glucose-grown cells consumed acetate and increased butanediol yields (Table 3). In contrast, acetate consumption by the xylose-grown cells was quite poor on glucose medium to which acetate had been added. This result corresponded to experiments reported previously (Laube et al. 1984), in which 0.5 or 1.0% (w/v) acetate was added to the medium. Following acetate addition to xylose medium, neither glucose-grown cells nor xylose-grown cells consumed acetate (Table 4). These results clearly indicated that *P. polymyxa* does not take up acetate together with xylose.

Alternative pathway for acetate production

In order to test whether acetate was produced via the EMP pathway on xylose medium, iodoacetate was added to the medium. Since iodoacetate inhibits the oxidation of G3P to 1,3-bisphosphoglycerate by G3P dehydrogenase (Senac and Hahn-Hagerdal 1990), if all of the end-products were formed via the EMP pathway, their production would be stopped by the addition of iodoacetate (Fig. 1). When xylose-grown cells were incubated on glucose with iodoacetate, no acetate was produced, while on xylose, acetate was still being produced and yielded 0.7 mol/mol sugar (Fig. 2). By contrast, cells grown on glucose hardly produced metabolites when iodoacetate was added with glucose or xylose. These results suggest that an alternative pathway plays a role in producing acetate. One possibility

Table 3 Effect of acetate addition on product diversity of glucose- and xylose-grown cells in glucose medium. Culture conditions: 24 h at 39°C under pH control at 6.3 in 100 mM glucose medium with or without acetate (100 mM).*ND* Not detected. Values represent averages of at least duplicate determinations

	Cells grown on xylose		Cells grown on glucose	
	Glucose medium		Glucose medium	
	Without acetate	With acetate (100 mM)	Without acetate	With acetate (100 mM)
Glucose consumed (mM)	107.0	94.0	105.0	118.0
(R,R)-2,3-butanediol (mM)	50.4	46.1	56.0	87.0
Ethanol (mM)	78.7	54.6	75.7	62.0
Acetate (mM)	4.8	−5.6[a]	0.9	−54.1[a]
Acetoin (mM)	2.2	2.0	0.7	1.3
Lactate (mM)	4.6	11.9	2.4	14.6
Formate (mM)	4.0	7.1	10.9	15.9
Succinate (mM)	ND	9.9	ND	11.8
H2 (mM)	98.5	54.6	108.4	62.0
CO2 (mM)	231.4	138.1	231.0	168.6
Cell (g/l)	1.6	1.7	1.6	2.4
Carbon recovery (%)	96.0	92.2	99.7	106.1
Electron recovery (%)	94.8	93.2	97.9	106.8

[a] (−) Acetate consumption

Table 4 Effect of acetate addition on product diversity of glucose and xylose grown cells in xylose medium. Culture conditions: 24 h at 39°C in 100 mM xylose medium (minimal) with or without acetate (100 mM). Values represent averages of at least duplicate determinations

	Cells grown on xylose		Cells grown on glucose	
	Xylose medium		Xylose medium	
	Without acetate	With acetate (100 mm)	Without acetate	With acetate (100 nm)
Xylose consumed (mM)	100	100	100	100
(R,R)-2,3-butanediol (mM)	46	53.7	45.8	53.3
Ethanol (mM)	50.3	60.1	47.1	65.3
Acetate (mM)	5.2	4.1	8.3	3
Acetoin (mM)	0.7	1.7	1.6	2.5
Lactate (mM)	21.8	5.3	18.7	5.3
Formate (mM)	18.5	8.8	12.1	8.3
H2 (mM)	37	53	43	60
CO_2(mM)	130	164	230	156
Cell (g/l)	1.5	1.5	2.3	2.4
Carbon recovery (%)	100.3	102.5	98.1	108.5
Electron recovery (%)	102.4	105.3	101.3	108.1

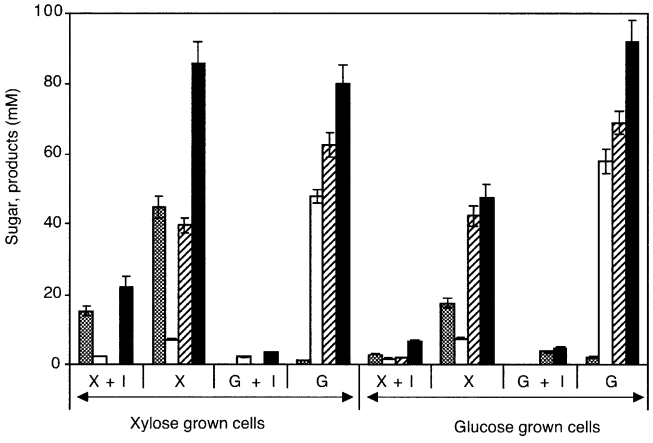

Fig. 2 Effect of iodoacetate addition on metabolite production by *P. polymyxa* in resting-cell experiments using xylose- and glucose-grown cells. *Shaded columns* Acetate, *open columns* butanediol, *hatched columns* ethanol, *filled columns* sugar consumed. Incubation: 24 h at 39°C in 100 mM xylose (*X*) or glucose (*G*) in 100 mM phosphate buffer, pH 6.8, with iodoacetate (+*I*, 3 mM) or without iodoacetate

is acetate formation via phosphoketolase, which cleaves xylulose-5-phosphate to G3P plus acetyl phosphate.

To test this hypothesis, phosphoketolase activity was determined in xylose- and glucose-grown cells. The enzyme activity in xylose-grown cells was 139±23 nmol/mg protein/min, which was 25-fold higher than in glucose-grown cells (6±1 nmol/mg protein/min). Phosphoketolase activity did not change by the addition of iodoacetate (data not shown), indicating that some of the acetate was formed via not only the EMP pathway, but also the phosphoketolase pathway.

Contributions of phosphoketolase to xylose catabolism

Figure 1 shows the most probable sequence of biochemical reactions, and their enzymes, that result in the conversion of sugar to the typical products (alcohols, acids, and gases) of butanediol and mixed-acid fermentation. The equation to calculate the contribution of phosphoketolase for xylose catabolism was derived on the basis of existing information on fermentation biochemistry, and the NADH and carbon balances (see Appendix). It describes the interrelations among the fermentation products and the consumed substrate (sugar), and was tested using experimental data obtained from the batch fermentations. Calculation of the products based on the EMP and pentose metabolism pathways did not explain the differences in product formation on the glucose vs. the xylose substrates. When the acetyl-P (phosphoketolase) pathway was included in the calculations, good results were obtained. For example, using the experiment data from Table 1, the percentage of xylose catabolized via phosphoketolase could be estimated by Eqs. 1, 2, and 3, presented in the Appendix, as follows:

Since the butanediol yield (x)=0.42:

$$\begin{aligned}\text{Acetate yield} &= (9\alpha + 5\beta - 3.78)/6 \\ &= (9\alpha + 5(1-\alpha) - 3.78)/6 \\ &= (4\alpha + 1.22)/6\end{aligned}$$

Since the experimental yield of acetate was 0.29 mol/mol, acetate yield=0.29=(4α+1.22)/6, therefore, α=0.15 and β=0.85 This means that 15% of the xylose consumed was catabolized through phosphoketolase in the pH-uncontrolled batch culture. The ethanol yield from the pH-uncontrolled batch culture at 39°C was also calculated as 0.57 mol/mol sugar, which is 8.9% lower than the experimental value (0.63 mol/mol sugar).

Fed-batch cultures on xylose and glucose at 39°C

To test whether the acetate that accumulated in xylose medium was consumed when glucose was supplemented, fed-batch culture was carried out. As shown in Fig. 3, the cells grew rapidly after cultivation in medium supplemented with xylose, and 22 mM acetate accumulated after 12 h. However, when glucose together with xylose was fed into the culture at 12 and 24 h, acetate was consumed gradually together with glucose consumption. Since acetate concentration decreased to 6 mM after 34 h, glucose addition was stopped. Subsequently, acetate production was increased due to xylose consumption. After glucose addition was resumed at 48 h, although acetate consumption was relatively low, its concentration was maintained at 20 mM. Finally, 240 mM xylose and 260 mM glucose were consumed after 108 h, and 278 mM butanediol, 293 mM ethanol, 22 mM acetate, 24 mM formate and 63 mM lactate were produced, corresponding to an overall butanediol yield of 0.56 mol/mol sugars. Carbon recovery and electron recovery were 102% and 98% respectively.

Discussion

Both in the previous study (Marwoto et al. 2002) and in this study, we found that the amount of *(R,R)*-2,3-butanediol produced by *P. polymyxa* cells grown on xylose medium decreased, while acetate yield dramatically increased (Table 1). It is important to reduce by-product formation to increase the yield of the intended chemical. Since our goal was to increase the butanediol yield, we investigated both why *P. polymyxa* accumulated acetate on the xylose medium and the effect of glucose feeding together with xylose on the reduction of acetate accumulation in fed-batch culture.

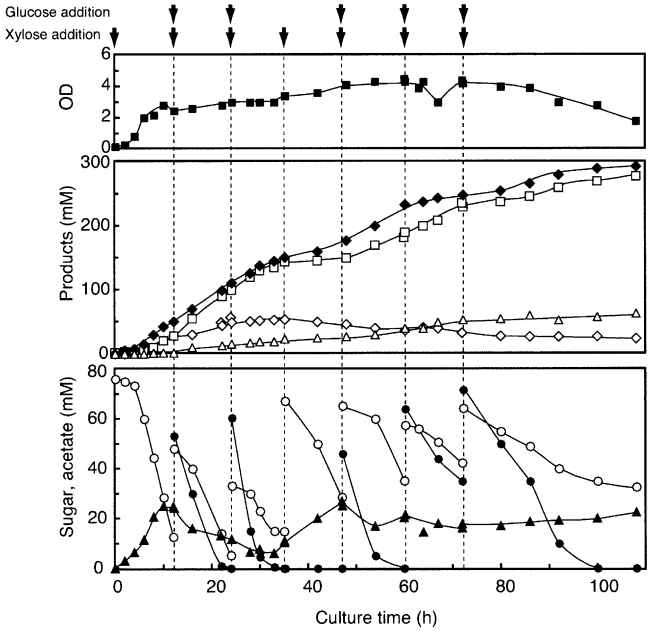

Fig. 3 Fed-batch culture of *P. polymyxa* on glucose and xylose at 39°C and pH 6.3. ■ OD, ◇ formate, △ lactate, □ butanediol, ◆ ethanol, ▲ acetate, ○ xylose, ● glucose

Since acetate is usually formed from acetylphosphate, the difference in acetate formation by xylose- and glucose-grown cells may have been due to acetate kinase activity. We confirmed this hypothesis by measuring the enzyme activity in cell-free extracts prepared from xylose- and glucose-grown cells. Acetate kinase was five-fold higher in the former. These results indicated that acetate kinase activity in xylose-grown cells might be responsible for acetate accumulation.

In addition, xylose-grown cells were poor consumers of acetate in the presence of glucose medium supplemented with acetate (Table 3), although the amount of uptake of acetate together with glucose by glucose-grown cells was very high. However, during further culture in xylose medium no acetate was consumed by either xylose-grown cells or glucose-grown cells (Table 4). These results suggested that low acetate uptake might also be the reason why acetate accumulated on xylose medium.

In the fermentation of xylose, the conversion to G3P from xylose through the pentose-phosphate pathway is thought to be equivalent to the glycolysis of glucose to G3P by microorganisms carrying out mixed-acid fermentation (Jansen and Tsao 1983). Finally, we found that acetate production from xylulose-5-phosphate proceeds via the phosphoketolase pathway. This was confirmed by inhibition of the EMP pathway using iodoacetate and subsequent measurement of enzyme activity. On addition of 3 mM iodoacetate, glucose grown cells were not able to ferment xylose, while cells grown on xylose still produced acetate (yield 0.69 mol/mol xylose). The experiment using cell-free extracts prepared from glucose- and xylose-grown cells also supported acetate production via phosphoketolase on the xylose medium (data not shown). In additional experiments, phosphoketolase-mediated acetate production by cells grown on xylose medium was evaluated. Based on the results shown in Fig. 2, if all of the acetate was formed via phosphoketolase when iodoacetate was added, then it can be calculated that 34% of the acetate produced is formed through phosphoketolase in the absence of iodoacetate.

The contribution of phosphoketolase to acetate production can also be evaluated by stoichiometric calculations. Using the equations for the calculation of phosphoketolase contribution to acetate formation shown in the Appendix, it was found that 15% of the xylose was consumed via the phosphoketolase pathway (Table 1). However, by the same calculation but using data from the pH-controlled batch culture (Table 2), only 2% of the acetate was determined to be produced through the phosphoketolase pathway. Using previous experimental data from pH-uncontrolled batch culture at 39°C (Marwoto et al. 2002), it was calculated that 26% of the xylose was consumed through the phosphoketolase pathway. These calculations suggest that the contribution of phosphoketolase to acetate production is highly dependent on the culture pH.

In conclusion, the higher production of acetate on xylose is due to a combination of at least three factors: (1) higher acetate kinase activity, (2) lower acetate uptake ability, and (3) higher expression of phosphoketolase by xylose-grown cells than by glucose-grown cells. In cultures on xylose medium, although acetate production was significantly stimulated, it was decreased or reduced when glucose was added together with xylose, as shown Fig. 3. This phenomenon could be explained by catabolite repression of xylose metabolism by glucose. The fed-batch experiment indicated that the addition of glucose during xylose consumption effectively reduced acetate accumulation. The final butanediol yield was 0.56 mol/mol sugar after fed-batch culture for 108 h. This value was same as the maximum theoretical yield from xylose (0.56 mol/mol). Therefore, the effective feeding of glucose during fermentation on xylose may enhance butanediol production.

Appendix

Equations for calculating the ratio of the contribution of phosphoketolase to xylose consumption were derived as follows:

Embden-Meyerhoff-Pernas pathway

For the production of pyruvate from glucose through the EMP pathway (Fig. 1):

$$\text{Glucose} \rightarrow 2\ \text{NADH}_2 + 2\ \text{Pyruvate} + 2\ \text{ATP}$$

Based on the NADH and carbon balances from EMP (Fig. 1a), product formation, including ATP formation, can be represented by:

$$\begin{aligned}&\text{Glucose}\\ &\rightarrow x\text{BD} + (2-3x)/2\ \text{HAC} + (2-x)/2\ \text{Et-OH}\\ &\quad + 2\ \text{CO}_2 + (2-2x)\text{H}_2 + (2 + (2-3x/2)\ \text{ATP}\end{aligned}$$

where x represents the yield of 2,3-butanediol (BD) for molar glucose, and HAC, and Et-OH represent 2,3-butanediol, acetate, and ethanol, respectively. In this formula, lactate formation was omitted due to the low production of lactate in xylose medium.

Acetyl-P pathway (phosphoketolase)

The formation of acetate and pyruvate through the acetyl-P pathway can be represented by:

$$\text{Xylose} \rightarrow \text{Acetate} + \text{NADH}_2 + 2\ \text{ATP} + \text{Pyruvate}$$

Based on the NADH and carbon balances (Fig. 1b), product formation can be represented by:

$$\begin{aligned}&\text{Xylose}\\ &\rightarrow x\text{BD} + (1-x)/2\ \text{Et-OH} + (1-3x)/2\\ &\quad \text{HAC} + \text{HAC} + \text{CO}_2\\ &\rightarrow x\text{BD} + (1-x)/2\ \text{Et-OH} + (3-3x)/2\ \text{HAC}\\ &\quad + \text{CO}_2 + (1-2x)\ \text{H}_2 + (2 + (1-3x)/2)\ \text{ATP}\end{aligned}$$

Pentose-phosphate–EMP pathway

Pyruvate production from xylose via the pentose-phosphate (PP) and EMP pathways is through the following reaction:

$$\text{Xylose} \rightarrow 5/3\ \text{ATP} + 5/3\ \text{NADH}_2 + 5/3\ \text{Pyruvate}$$

Based on the NADH and carbon balances (Fig. 1c), product formation can be represented by:

$$\begin{aligned}&\text{Xylose}\\ &\rightarrow x\text{BD} + (5-3x)/6\ \text{Et-OH} + (5-9x)/6\ \text{HAC}\\ &\quad + 5/3\ \text{CO}_2 + (5/3-2x)\ \text{H}_2 + (5/3 + (5-9x)/6)\\ &\quad \{\text{ATP}\end{aligned}$$

Product formation from xylose through the acetyl-P pathway can be simplified by the following equation:

$$\begin{aligned}&\alpha\ \text{Xylose}\\ &\rightarrow \alpha\ x\text{BD} + \alpha(1-x)/2\ \text{Et-OH}\\ &\quad + \alpha(3-3x)/2\ \text{HAC} + \alpha\text{CO}_2 + \alpha(1-2x)\text{H}_2\end{aligned} \tag{1}$$

For xylose metabolism via the PP–EMP pathway:

$$\begin{aligned}&\beta\ \text{Xylose}\\ &\rightarrow \beta x\text{BD} + \beta(5-3x)/6\ \text{Et-OH}\\ &\quad + \beta(5-9x)/6\ \text{HAC} + \beta 5/3\ \text{CO}_2\\ &\quad + \beta(5/3-2x)\text{H}_2\end{aligned} \tag{2}$$

where α and β represent the ratio of xylose consumed via the acetyl-P and PP-EMP pathway, respectively. Therefore:

$$\alpha + \beta = 1 \tag{3}$$

Using Eqs. 1, 2, and 3:

$$\text{BD yield} = \alpha x + \beta x$$

$$\begin{aligned}\text{Acetate yield} &= \alpha(3-3x)/2 + \beta(5-9x)/6 \\ &= (9\alpha + 5\beta - 9x)/6\end{aligned}$$

$$\begin{aligned}\text{Ethanol yield} &= \alpha(1-x)/2 + \beta(5-3x)/6 \\ &= (3\alpha + 5\beta - 3x)/6\end{aligned}$$

If the BD and acetate or ethanol yields are given, the percentage of xylose catabolized through the acetyl-P pathway can be calculated.

References

de Mass C, Jansen NB,Tsao T (1988) Production of optically active 2,3-butanediol by *Bacillus polymyxa*. Biotechnol Bioeng 31:366–377

Erickson LE, Minkevich IG,Eroshin VK (1979) Utilization of mass-energy balance regularities in the analysis of continuous-culture data. Biotechnol Bioeng 21:575–591

Jansen NB,Tsao GT (1983) Bioconversion of pentose to 2,3-butanediol by *Klebsiella pneumoniae*. Adv Biochem Eng Biotechnol 27:85–99

Jeffries TW (1983) Utilization of xylose by bacteria, yeast and fungi. Adv Biochem Eng Biotechnol 27:1-32

Laube VM, Groleau D,Martin SM (1984) 2,3-Butanediol production from xylose and other hemicellulose components by *Bacillus polymyxa*. Biotechnol Lett 6:257

Marounek M, Petr O (1995) Fermentation of glucose and xylose in ruminal strains of Butyrivibrio fibrisolvens. Lett in Appl Microbiol 21:272–276

Marwoto B, Nakashimada Y, Kakizono T,Nishio N (2002) Enhancement of (R,R)-2,3-butanediol production from xylose by *Paenibacillus polymyxa* at elevated temperatures. Biotechnol Lett 24:109–114

Nakashimada Y, Kanai K,Nishio N (1998) Optimization of dilution rate, pH and oxygen supply on optical purity of 2,3-butanediol produced by *Paenibacillus polymyxa* in chemostat culture. Biotechnol Lett 20:1133–1138

Nakashimada Y, Marwoto B, Kakizono T, Nishio N (2000) Enhanced 2,3-butanediol production by addition of acetic acid in *Paenibacillus polymyxa*. J Biosci Bioeng 90:661–664

Ratledge C,Holdsworth JE (1985) Properties of a pentulose-5-phosphate phosphoketolase from yeasts grown on xylose. Appl Microbiol Biotechnol 22:217–221

Rose IA, G-Manago M, Korey SR,Ochoa S (1954) Enzymatic phosphorylation of acetate. J Biol Chem 21:737–756

Senac T,Hahn-Hagerdal B (1990) Intermediary metabolite concentration in xyluloseand glucose-fermenting *Saccharomyces cerevisiae* cells. Appl Environ Microbiol 56:120–126

Smith PK, Krohn RI, Hermanson GT, Malia AK, Goeke NM, Olson BJ,Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150:76–85