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Production of Pyridoxal Phosphate by a Mutant Strain of *Schizosaccharomyces pombe*

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Conditions for extracellular production of vitamin B_6 compounds (B_6), especially pyridoxal 5′-phosphate (PLP) by *Schizosaccharomyces pombe leu1* strain were examined. The productivity was dependent on concentration of L-leucine in the culture medium: 30 mg/l gave the highest concentrations of total B_6 and PLP. The viable cells harvested at different growth phases showed different productivity: middle and late exponential phase cells showed the highest productivity of total B_6 and PLP, respectively. D-Glucose (1%, w/v) among other sugars gave the best productivity. Supplementation of air and ammonium sulfate significantly increased extracellular production of PLP. Superoxide anion producers, menadione and plumbagin, and H_2O_2 increased the productivity of PLP. Cycloheximide inhibited the increase of PLP by the oxidative stress and, in contrast, increased pyridoxine.

Key words: pyridoxal phosphate-production; oxidative stress; yeast; *Schizosaccharomyces pombe*

Pyridoxal 5′-phosphate (PLP) is the coenzyme form of vitamin B_6 and plays an essential role in more than 200 vitamin B_6-dependent enzymes mainly involved in metabolic conversions of amino acids. PLP has a potential market value in clinical and pharmacological areas, *e.g.*, preservation of whole blood or supplementing of zinc.[1] PLP is currently produced chemically from pyridoxine (PN), which is also produced chemically and has wide market value.

Screening for PN-excreting microorganisms has been intensified because a clean ecological production is preferable.[2] Several microorganisms, such as *Flavobacterium* strain 238-7,[3] *Pichia guilliermondii* Wickerham NK-2,[4] and *Rhizobium meliloti* IFO 14782,[5] have been reported as overproducers of PN. In contrast to the screening for PN producers, that for PLP-producing microorganisms has not been done.

Several yeasts have been shown to accumulate vitamin B_6 compounds in their culture media. *P. guilliermondii* Wickerham NK-2,[4] *Saccharomyces carlsbergensis* (now designated as *S. cerevisiae*),[6] *Schizosaccharomyces pombe*,[7] and a mutant strain of *Saccharomyces marxianus*[8] accumulate almost all forms of vitamin B_6 compounds during their cultivation. Relative amounts of the forms varied depending on the strains and culture conditions such as carbon and nitrogen sources. The striking feature of vitamin B_6 accumulation by yeasts is the existence of fairly high amount of PLP. If appropriate conditions to increase productivity of PLP could be found, the yeast cells could be used as a bioreactor for the production of PLP.

In this paper we have found that a *leu1* strain of *S. pombe* produced large amounts of vitamin B_6 compounds, including PLP, in a culture medium. Conditions for high productivity of vitamin B_6 compounds, especially PLP, were examined. It was found that air was required for extracellular production of PLP and that oxidative stress further increased the productivity.

Materials and Methods

Yeast strain and growth conditions. *S. pombe*, a L-leucine-requiring mutant strain (ATCC 38399, h⁻ *leu1-32*), was obtained from the American Type Culture Collection. The mutant cells were maintained on 2% agar slants containing YES[9] and stored at 4°C. A basic medium for growth of cells was a medium (YNBGL) composed of 1% D-glucose, incomplete yeast nitrogen base, 10 nM PN and 30 mg/l of L-leucine, pH 4.5. The incomplete yeast nitrogen base was yeast nitrogen base that was prepared without any amino acids, thiamine, or PN.

Preparation of the yeast cells. The mutant cells were aerobically cultivated in YNBGL at 30°C for

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Abbreviations: PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; PL, pyridoxal; PM, pyridoxamine; PN, pyridoxine; HPLC, high-pressure liquid chromatography

36 h. The culture broth was centrifuged to harvest cells, and the cells were washed twice with 0.9% sodium chloride and then with distilled water. The resting cells were stored for no longer than 24 h at 0–4°C during the course of an experiment.

For preparation of the yeast cells at different growth phases, cells were harvested at 4-h intervals. Growth of cells was measured from absorbance at 600 nm.

Production of vitamin B_6 by the yeast cells. The collected cells (1%, w/v) were suspended in 2 ml of a reaction mixture composed of 1% (w/v) D-glucose and incomplete yeast nitrogen base without any amino acids or vitamins. The reaction mixture was incubated with shaking at 30°C for 6 h in the dark. A suspension (1 ml) of the reaction mixture was centrifuged to separate cells and supernatant. The supernatant was boiled for 10 min. After this cooled, 33.3 μl of 9 M perchloric acid was added to it, and the solution was left on ice for 30 min. Then the solution was filtered with a filter unit (DISMIC13 cp, Toyo Roshi, Tokyo) with a cellulose acetate membrane (pore size, 0.2 μm). The filtrates were put through HPLC to measure extracellular vitamin B_6 compounds.

When the effects of chemicals such as menadione on the productivity were measured, the chemicals were added to the reaction mixture.

The intracellular concentration of vitamin B_6 compounds was measured with the precipitated cells by the method described previously.[7]

Effects of air and nitrogen on the production. Nitrogen gas or air was pumped at 80 ml/min into 10 ml of the reaction mixture under vigorous mixing with a stirrer.

Analytical methods. Vitamin B_6 compounds were identified and measured by reversed-phase isocratic HPLC equipped with a fluorescence detector as reported previously.[10] The viability of cells was measured by a microdilution plate assay.[11] Cell suspension, which was incubated in the reaction mixture for 6 h, was diluted by fresh reaction mixture and plated on YES agar plates to obtain viable cell counts. Acid phosphatase activity was measured by the method with *p*-nitrophenyl phosphate.[12]

Statistical analysis. The experiment was repeated three times and indicated values as mean ± S.E. A multiple comparison test (PLSD method of Fisher) was used to compare means.

Results and Discussion

Effects of L-leucine concentration on growth of cells and concentration of vitamin B_6 compounds in the culture medium

The accumulation of vitamin B_6 compounds in culture medium and growth of cells at various concentrations of L-leucine, using the mutant cells, were investigated, as shown in Fig. 1. The growth of cells was dependent on the concentration of L-leucine in the medium and the maximum growth rate was obtained at higher than 60 mg/l of L-leucine under our conditions (Fig. 1, A). The growth became stationary after 48 h of cultivation. In contrast to the growth of cells, the highest total amount of vitamin B_6 compound was found in the medium containing 30 mg/l of L-leucine (Fig. 1, B). A higher concentration than this decreased the amount of vitamin B_6, showing that L-leucine itself or unknown metabolite(s) of L-leucine may inhibit the synthesis or excretion of vitamin B_6. The accumulation of total vitamin B_6 compounds did not show saturation of productivity. Thus, the cells in stationary phase continued to synthesize and released vitamin B_6.

The amounts of different forms of vitamin B_6 compounds showed various responses to the concentrations of L-leucine (Fig. 1, C–G). Concentrations of pyridoxamine (PM) and PN, and pyridoxamine 5′-phosphate (PMP) were the highest in the medium containing 10 mg/l and 60 mg/l of L-leucine, respectively. On the other hand, concentration of pyridoxal (PL), PLP and total vitamin B_6 compounds were the highest in the medium containing 30 mg/l of L-leucine. The production of PLP became saturated at this concentration. These results showed that the medium containing 30 mg/l of L-leucine was appropriate for the yeast cells to synthesize vitamin B_6 compounds, especially PLP.

Growth phase and productivity of extracellular vitamin B_6 compounds

To investigate the relationship between growth phase and the productivity of extracellular vitamin B_6 compounds by the yeast cells, the yeast cells were harvested at various growth times, then the washed cells were incubated in the reaction mixture for 6 h as described in Materials and Methods. During the incubation, no growth of the yeast was observed, because the reaction mixture did not contain L-leucine, which is essential for growth of the mutant yeast cells. The cells harvested at 28 h (middle of exponential phase) produced the maximum amount of PL in the reaction mixture (Fig. 2). PLP production was the highest with the cells cultivated at 36 h (late exponential phase). In contrast, PMP, PM, and PN were produced abundantly in the early exponential phase. The results showed that excretion and inter-conversion of each of the forms of vitamin B_6 compounds were dependent on the growth phase.

The cells after 6 h of incubation in the reaction mixture showed 99% viability, showing that vitamin B_6 compounds in the reaction mixture was released

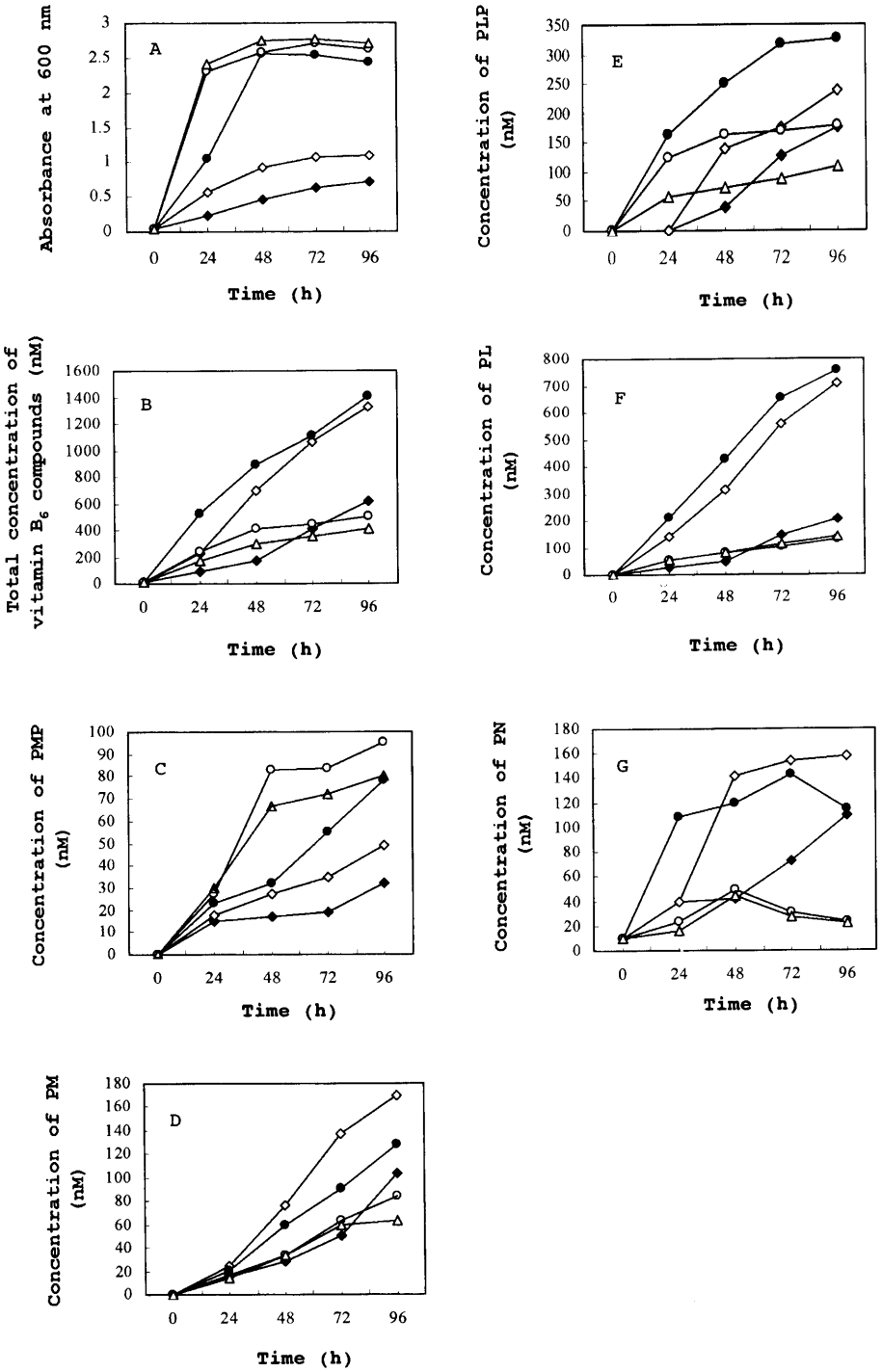

Fig. 1. Accumulation of Vitamin B_6 Compounds in Culture Medium and Growth of *leu1* Mutant Cells at Various Concentration of L-Leucine.

Growth curve (A), total concentration of vitamin B_6 compounds (B), concentration of PMP (C), PM (D), PLP (E), PL (F) and PN (G) were shown. The mutant cells were grown in YNBGL medium containing various amounts of L-leucine; ◆, 5 mg/l; ◇, 10 mg/l; ●, 30 mg/l; ○, 60 mg/l and △, 100 mg/l.

from living cells.

Since the yeast cells released acid phosphatase in the reaction mixture (0.086 U/ml), we have examined the effects of an inhibitor of the enzyme, sodium fluoride, on the relative amount of PLP and PL, and that of PMP and PM. In the presence of 5 mM sodium fluoride, which inhibited 89% of the acid phosphatase activity in the reaction mixture, the PLP/PL and PMP/PM ratios were increased by 27% and 24%, respectively. The result indicated that a part of PLP and PMP was hydrolyzed in the reaction mixture by the enzyme. Sodium fluoride, however, significantly decreased production of the extracellular vitamin B_6 compounds, probably because it inhibits

glycolysis. Thus, hereafter, we have measured the productivity in the absence of sodium fluoride.

Effects of sugars and ammonium sulfate on the extracellular production of vitamin B_6 compounds

The production of vitamin B_6 by the yeast cells was measured with different kinds of sugars (1%, w/v, each) as shown in Table 1. The highest extracellular concentration of PLP and total vitamin B_6 were obtained when D-glucose was used. Other sugars used as a carbon source for the cells also supported the production. Therefore, a supply of energy and carbon skeleton was necessary for synthesis and excretion of PLP, PL, and PN. The PLP/PL ratio was decreased when disaccharides were used: 45% with maltose and 44% with sucrose compared to 51% with D-glucose. In contrast to the extracellular concentration, the intracellular concentration of vitamin B_6 compounds was mostly kept constant with different sugars.

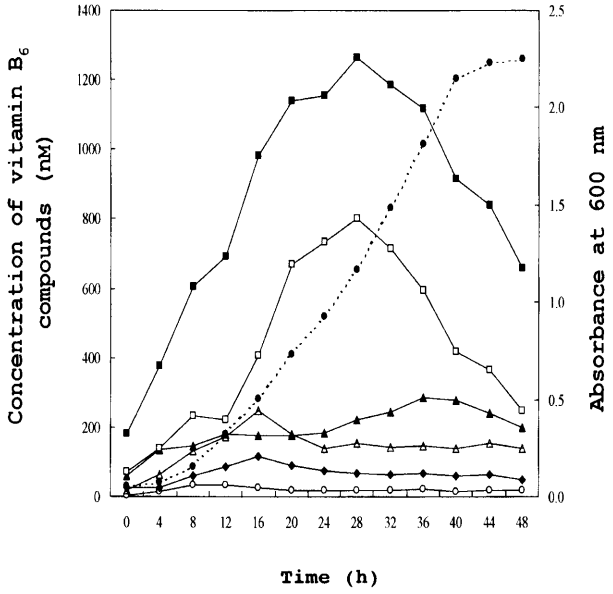

Fig. 2. Relationship between Growth Phase and Extracellular Production of Vitamin B_6 Compounds.

Cells were grown in medium containing 30 mg/l of L-leucine and harvested at various growth times. The resting cells were incubated in the reaction mixture for 6 h and vitamin B_6 compounds: PMP (○), PM (◆), PLP (▲), PL (□), PN (△) and total vitamin B_6 compounds (■), in the reaction mixture were measured by HPLC. Dashed line shows a growth curve, and closed circles (●) show the growth curve when cells were harvested at intervals.

The productivity of extracellular vitamin B_6 compounds was not increased when the concentration of D-glucose was increased to 2% (w/v), 5%, or 10%.

When ammonium sulfate was removed from the standard reaction mixture, the extracellular concentration of PLP became about 50% of the standard value. Neither concentration of total extracellular vitamin B_6 compounds nor that of intracellular PLP changed in the absence of ammonium sulfate.

Requirement of air for extracellular production of vitamin B_6 compounds

The extracellular production of vitamin B_6 compounds by the yeast cells were examined under aerobic and anaerobic conditions (Table 2). The yeast cells excreted significantly higher concentrations of PL, PLP, PN, and PM under the aerobic conditions, but the concentration of PMP did not increase. The viability of the cells under aerobic and anaerobic conditions was 99% and 98%, respectively. The intracellular concentrations of vitamin B_6 compounds in the cells were not affected by the aeration. Thus, the yeast cells released PLP, PL, PN and PM under aerobic conditions rather than accumulated them, sug-

Table 1. Effects of Sugars on Vitamin B_6 Production in a Reaction Mixture with Resting Cells of a *S. pombe* Mutant Strain

Sugars	Concentration of extracellular (nM) and intracellular (nmol/g, wet weight) vitamin B_6 compounds					
	PMP	PM	PLP	PL	PN	Total
Extracellular						
Control	7 ± 0.3^a	15 ± 0.3^a	nd	nd	nd	22 ± 0.5^a
D-Glucose	57 ± 2.0^b	75 ± 1.0^b	206 ± 1.3^a	407 ± 1.3^a	162 ± 2.0^a	907 ± 3.0^b
D-Fructose	57 ± 1.5^b	85 ± 2.0^c	195 ± 1.6^b	411 ± 1.2^a	151 ± 2.0^b	899 ± 1.0^c
D-Mannose	58 ± 1.4^b	99 ± 2.0^d	154 ± 2.0^c	327 ± 1.7^b	135 ± 1.6^c	773 ± 3.3^d
Maltose	47 ± 2.3^c	94 ± 1.8^d	165 ± 1.7^d	367 ± 1.7^c	141 ± 2.0^d	814 ± 4.3^e
Sucrose	59 ± 1.2^b	99 ± 1.6^d	180 ± 1.3^c	408 ± 1.5^a	147 ± 1.3^c	893 ± 3.4^f
Intracellular						
Control	24 ± 1.3^a	5 ± 0.6^a	13 ± 0.6^a	6 ± 0.3^a	1 ± 0.3^a	49 ± 0.6^a
D-Glucose	21 ± 0.5^a	5 ± 0.5^a	15 ± 0.3^a	7 ± 0.8^a	1 ± 0.3^a	49 ± 0.6^a
D-Fructose	17 ± 0.6^b	4 ± 0.8^a	14 ± 1.0^a	6 ± 0.6^a	2 ± 0.5^a	43 ± 1.6^b
D-Mannose	18 ± 0.5^b	5 ± 0.3^a	14 ± 1.3^a	6 ± 0.0^a	2 ± 0.6^a	45 ± 1.6^{bc}
Maltose	18 ± 0.8^b	5 ± 0.8^a	15 ± 0.3^a	7 ± 0.3^a	2 ± 0.5^a	47 ± 1.3^{ac}
Sucrose	18 ± 0.5^b	5 ± 0.3^a	15 ± 0.5^a	8 ± 1.0^a	2 ± 0.3^a	48 ± 1.3^{ac}

Yeast cells were grown in YNBGL for 36 h and washed as described under "Materials and Methods". In the control experiment, the reaction mixture without sugar was used. nd: None detectable. Values with the same letters are not significantly different for $P \leq 0.05$.

Table 2. Effects of Aerobic and Anaerobic Conditions on Vitamin B_6 Production in a Reaction Mixture with Resting Cells of a *S. pombe* Mutant Strain[a]

	Concentration of extracellular (nM) and intracellular (nmol/g, wet weight) vitamin B_6 compounds					
	PMP	PM	PLP	PL	PN	Total
Extracellular						
Nitrogen	27±2.5	53±2.4	43±3.7	273±1.8	139±3.8	535±3.1
Air	29±1.8	63±1.2*	192±1.2*	486±2.9*	147±3.1*	917±2.9*
Intracellular						
Nitrogen	24±1.6	5±0.4	9±0.7	5±0.7	1±0.1	44±1.5
Air	21±1.3	4±0.6	11±0.8	6±0.7	1±0.1	43±1.9

[a] Air or Nitrogen gas was sent into a reaction vessel containing 10 ml of the reaction mixture under stirring at a flow rate of 80 ml/min.
* Significantly different between two conditions for $P \leq 0.05$.

Table 3. Effects of Oxidative Stress on Vitamin B_6 Production in a Reaction Mixture with Resting Cells of a *S. pombe* Mutant Strain

Oxidative sources	Concentration of extracellular (nM) and intracellular (nmol/g, wet weight) vitamin B_6 compounds					
	PMP	PM	PLP	PL	PN	Total
Extracellular						
Control	45±2.3[a]	61±1.7[a]	207±1.5[a]	399±1.5[a]	179±1.7[a]	891±2.3[a]
0.09 mM Menadione	58±1.7[b]	68±1.5[b]	350±2.1[b]	503±2.8[b]	175±1.4[ac]	1,154±2.6[b]
0.05 mM Plumbagin	51±1.8[a]	74±0.8[c]	311±1.5[c]	437±2.4[c]	189±1.7[bd]	1,062±2.8[c]
1 mM H_2O_2	51±2.1[a]	75±1.1[c]	270±1.2[d]	411±1.8[d]	183±0.8[ae]	990±2.6[d]
Intracellular						
Control	26±0.8[a]	5±0.3[a]	10±0.3[a]	4±0.6[a]	1±0.3[a]	46±2.3[a]
0.09 mM Menadione	27±0.8[a]	4±0.6[a]	8±0.3[b]	4±0.5[a]	2±0.3[a]	45±1.1[a]
0.05 mM Plumbagin	34±1.2[bc]	6±0.8[a]	8±0.3[b]	3±0.3[a]	2±0.3[a]	53±1.0[b]
1 mM H_2O_2	29±1.2[ac]	5±0.3[a]	9±0.8[a]	3±0.5[a]	1±0.5[a]	47±1.2[a]

Yeast cells were grown in YNBGL for 36 h and washed as described under "Materials and Methods". In the control experiment, the reaction mixture contained 1% (w/v) D-glucose. Values with the same letters are not significantly different for $P \leq 0.05$.

gesting that cells under aerobic conditions increased the vitamin B_6 synthesis and excretion at the same time.

Oxidative stress and the productivity of vitamin B_6 compounds

Requirement of the aerobic conditions for extracellular production of PLP, PL, PN, and PM suggested that oxidative stress also may increase their productions. Table 3 showed that superoxide anion producers menadione and plumbagin[13] or H_2O_2 promoted accumulation of extracellular concentration of vitamin B_6 compounds, particularly that of PLP. The oxidative compounds did not decrease viability of the yeast cells under these concentrations: the viability of cells were higher than 98%. Menadione increased extracellular concentration of PMP, PM, PLP, and PL. The production of PM, PLP, PL, and PN was increased by plumbagin. The effect of H_2O_2 on PLP production was less than those of menadione and plumbagin.

The intracellular concentration of PLP in the reaction mixture containing menadione and plumbagin slightly decreased, but PLP concentration in that containing H_2O_2 did not change. As a whole the intracellular concentrations of vitamin B_6 compounds were kept fairly constant under the oxidative stress.

The increase in extracellular production of PLP by oxidative stress may be caused by at least three processes: (a) acceleration of PN synthesis, (b) induction of enzyme(s), which convert PN to PLP, and (c) acceleration of efflux system of PLP, PL and PN. The first process may be dependent on an induced expression of the *SOR1* homologous gene, which may be involved in the oxidative stress response controlled by *PAP1*.[14] The second process may involve induction or activation of pyridoxal kinase, which phosphorylates PL, PM and PN.[15] We have little information on the third process. Although pioneering work on budding yeast by Shane and Snell,[6] a recent work,[8] and this study showed that PLP is released from yeast cells without lysing of the cells, the mechanism of the efflux of PLP has not been studied. PLP may be released by a system resembling that for ATP efflux[16] or the ABC family transporter.[17] Recently, an efflux system for PN has been reported:[18] PLP could not efflux through this system.

Effects of cycloheximide on the function of menadione

Table 4. Effects of Cycloheximide on Function of Menadione (Vitamin B_6 Production)

	Concentration of extracellular (nM) and intracellular (nmol/g, wet weight) vitamin B_6 compounds					
	PMP	PM	PLP	PL	PN	Total
Extracellular						
Control	47 ± 0.5^a	56 ± 0.6^a	215 ± 1.5^a	347 ± 2.1^a	195 ± 1.2^a	860 ± 4.2^a
0.17 mM Cycloheximide (C)	65 ± 0.7^b	73 ± 2.4^b	212 ± 1.3^a	364 ± 1.9^b	228 ± 2.2^b	942 ± 2.3^b
0.09 mM Menadione (M)	60 ± 1.3^c	89 ± 1.9^c	346 ± 1.0^b	489 ± 2.2^c	219 ± 2.0^c	$1,203 \pm 1.2^c$
C + M	73 ± 2.0^d	90 ± 2.1^c	202 ± 3.6^c	406 ± 1.9^d	309 ± 1.6^d	$1,080 \pm 3.4^d$
Intracellular						
Control	26 ± 1.8^a	9 ± 0.5^a	14 ± 0.5^a	6 ± 0.5^a	3 ± 0.3^a	58 ± 2.2^a
0.17 mM Cycloheximide (C)	18 ± 0.9^b	6 ± 0.3^{bc}	14 ± 0.6^a	7 ± 0.3^a	3 ± 0.3^a	48 ± 2.1^b
0.09 mM Menadione (M)	24 ± 0.5^a	8 ± 0.6^{ac}	16 ± 1.2^a	9 ± 0.3^b	3 ± 0.0^a	60 ± 1.4^a
C + M	21 ± 1.2^c	7 ± 0.9^{ac}	14 ± 0.5^a	7 ± 0.3^a	3 ± 0.3^a	52 ± 1.6^b

Yeast cells were grown in YNBGL for 36 h and washed as described under "Materials and Methods". In the control experiment, the reaction mixture contained 1% (w/v) D-glucose. Values with the same letters are not significantly different for $P \leq 0.05$.

Cycloheximide *per se* slightly increased the extracellular concentration of PMP, PM, PL, and PN (Table 4). The effects of cycloheximide on function of menadione appeared to significantly increase PN and PMP, but concentration of PLP was decreased. The intracellular concentration of PMP and PM slightly decreased in cycloheximide. The result coincided with the increase in the extracellular concentrations of the vitamin B_6 compounds and indicated that cycloheximide may accelerate release of vitamin B_6. In extracellular fluid, cycloheximide inhibited the increased of PLP by the oxidative stress and increased PN under the same conditions. This suggests that PN is the first form of vitamin B_6 compounds in *de novo* synthesis, which is induced by the oxidative stress, and it is converted to PLP by the enzyme, which is induced by the oxidative stress. In *E. coli*, the first form of vitamin B_6 compounds is pyridoxine 5′-phosphate (PNP).[19] Throughout this study, we could not detect PNP in all samples from extracellular and intracellular preparations. Thus, it is plausible that the first product in yeast is PN rather than PNP because PLP and PMP could be measured. If PNP synthesized was hydrolyzed by a phosphatase, which show generally low substrate specificity,[20] PLP and PMP also should be hydrolyzed. The possibility that a vitamin B_6 compound other than PNP and PN is the first product can not be ruled out because of distinct origin of the nitrogen atom of PN synthesized in yeasts[21] and presence of different kind of gene involved in synthesis of vitamin B_6 in yeasts. More studies are needed to determine the first product.

In this study, we have found that viable *S. pombe leu1* cells produce PLP in the reaction mixture under aerobic (oxidative) conditions. Although the productivity is not high to be used for an industrial purpose, our results are useful for further studies on microbiological production of vitamin B_6, such as screening for yeast strains that produce much higher concentrations of PLP.

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