Journal of Life Science **2004**, Vol. 14. No. 4. 608~613 ©JLS

Optimization of Culture Conditions for the Production of Pyrimidine Nucleotide *N*-Ribosidase from *Pseudomonas oleovorans*

Tae Shick Yu*

Department of Microbiology, Keimyung University, Taegu 701-704, Korea

Received July 13, 2004 / Accepted August 4, 2004

Pyrimidine nucleotide *N*-ribosidase (pyrimidine 5'-nucleotide phosphoribo (deoxyribo)hydrolase/pyrimidine 5'-nucleotide nucleosidase, EC 3.2.2.10) directly catalyzes pyrimidine 5'-nucleotide to pyrimidine base and ribose (deoxyribo) 5-phosphate. In order to clarify the best nutritional conditions for the growth and the pyrimidine nucleotide *N*-ribosidase production of *Pseudomonas oleovorans* ATCC 8062 the effects of various nutrients such as different carbon and nitrogen sources were studied. For the both the growth and the enzyme production, 2% fumarate, 1.5% peptone, 5% corn steep liquor (CSL) and 1% ammonium chloride were excellent carbon and nitrogen sources, respectively. Optimum pH, temperature, and cultivation time for the enzyme production were 7.0, 28℃, and 48 h, respectively. The pyrimidine nucleotide *N*-ribosidase of *P. oleovorans* ATCC 8062 was not induced by UMP and its derivatives, and was constitutive enzyme.

Key words – Pyrimidine nucleotide *N*-ribosidase, *Pseudomonas oleovanans*, pyrimidine base

Generally, degradation of cytidine mono phosphate (CMP) in microorganisms is initiated by the elimination of phosphate to produce cytidine. Its degradation is performed through two pathways, one consists of the sequential catalysis of cytidine deaminase and nucleoside hydrolase (or nucleoside phosphorylase), and the other consists of the sequential catalysis of nucleoside hydrolase and cytosine deaminase.

Previously, Sakai et al. and Imada et al. demonstrated another CMP degrading pathway, in which cleavage of *N*-ribosyl linkage occurred without the liberation of phosphate, and CMP was directly degraded to ribose 5-phosphate and cytosine[3,4,8-11]. However, cytosine cannot be reused for biosynthesis of nucleotides, because microorganisms contain neither CMP : pyrophosphorylase nor cytidine phosphorylase[7]. Moreover, cytosine that cannot be a immediate precursor for on organisms is assumed to be converted to uracil, on the first step, and then reused for *Salvage* synthesis of cytosine nucleotide or degraded into urea and acetyl-CoA. Certain microorganisms (*i. e., Pseudomonas oleovorans*) have only this pathway for the direct degradation of CMP. The above authors also demonstrated the existence of a novel enzyme catalyzing this pathway, and called pyrimidine nucleotide *N*-ribosidase. Pyrimidine nucleotide *N*-ribosidase (pyrimidine 5'-nucleotide phosphoribo (deoxyribo) hydrolase/pyrimidine 5'-nucleotide nucleosidase, EC 3.2.2.10) catalyzes pyrimidine 5'-nucleotide to pyrimidine base and ribose (deoxyribo) 5-phosphate. However, the enzyme was not reported in detail.

In this paper, the effects of various nutrients such as different carbon and nitrogen sources were measured in order to clarify the best nutritional conditions on the growth and the enzyme production of *P. oleovorans* ATCC 8062.

***Corresponding author**
Tel : +82-53-580-5252, Fax : +82-53-580-5164
E-mail : tsyu@kmu.ac.kr

Materials and Methods

Chemicals

Peptone, corn steep liquor (CSL), meat extract were purchased from Difico Co. U.S.A. Cytidine mono phosphate (CMP), uridine mono phosphate (UMP), nucleotides, nucleosides, and pyrimidine base were obtained from Sigma Co. U.S.A. All other chemicals used were purchased to analytical reagent grade.

Microorganism and culture

Pseudomonas oleovorans ATCC 8062 was used throughout this study.

The microorganism was inoculated into a 500 ml flask containing 100 ml of medium containing 2% disodium fumarate, 1.5% peptone, 5% CSL, 1% ammonium chloride, 0.03% $MgSO_4 \cdot 7H_2O$, 0.01% KH_2PO_4, and 0.01% K_2HPO_4, at pH 7.0. The subculture was carried out at 28℃ for 24 h under reciprocal shaking. Each 500 ml of this subculture was

inoculated into a 20 L jar fermentor containing 10 L of the medium and cultivation was carried out for 48 h at 28℃ under aeration (10 L/min). The cells were harvested by centrifuge, and washed three times with 0.9% saline.

Enzyme preparation

All subsequent procedures were performed below 5℃ unless noted.

The cell paste (400 g) obtained from 100 L of the medium was suspended in 0.05 M phosphate buffer (pH 6.5) to give a suspension of about 0.1 g/ml The suspension was divided into 500 ml portions and each portion was subjected to ultrasonic oscillation (20 kHz) for 30 min. The cell debris was removed by centrifugation at 19,000×g for 30 min. The supernatant solution was dialyzed for 24 h against 10 L of 0.01 M phosphate buffer containing 5×10^{-4} M EDTA (pH 6.5).

Enzyme assay

Enzyme activity was assayed by measuring the amount of ribose 5-phosphate (R-5-P) formed from UMP.

For the determination of R-5-P, the following procedure was used. The reaction mixture contained 10 μm UMP, 100 μm Tris-HCl buffer (pH 7.0), and various amounts of crude enzyme solution. The total reaction volume was 1.0 ml The reaction was carried out at 37℃ for 30 min with gentle shaking and was stopped by boiling for 3 min. After cooling, the precipitate formed was removed by centrifuge and the supernatant was submitted to analysis. The amount of R-5-P was determined according to Somogyi's methods[12].

Ribose, ribose 1-phosphate, and ribose-5-phosphate were detected by Agarwol's methods[1].

In the enzyme assays, reaction mixtures without substrate were used as the control. One unit of enzyme activity was defined as the amount of enzyme catalyzing the degradation of 1 μm of UMP per hour under the assay conditions described.

Specific activity is expressed as unit per milligram enzyme protein.

Protein determination

The protein concentration was determined according to the method of Lowry et. al.[6].

Results and Discussion

To establish optimum conditions for the production of the pyrimidine nucleotide *N*-ribosidase (Fig. 1) from *P. oleovorans* the following investigation was carried out.

Effect of carbon source

Various carbon sources such as 2 sugars, 1 alcohol, and 5 organic acids were examined. Of those tested, citrate and fumarate were found to be the most favorable for both growth and enzyme production, as shown in Table 1. Fumaric acid is a analogue of aspartic acid which is initial

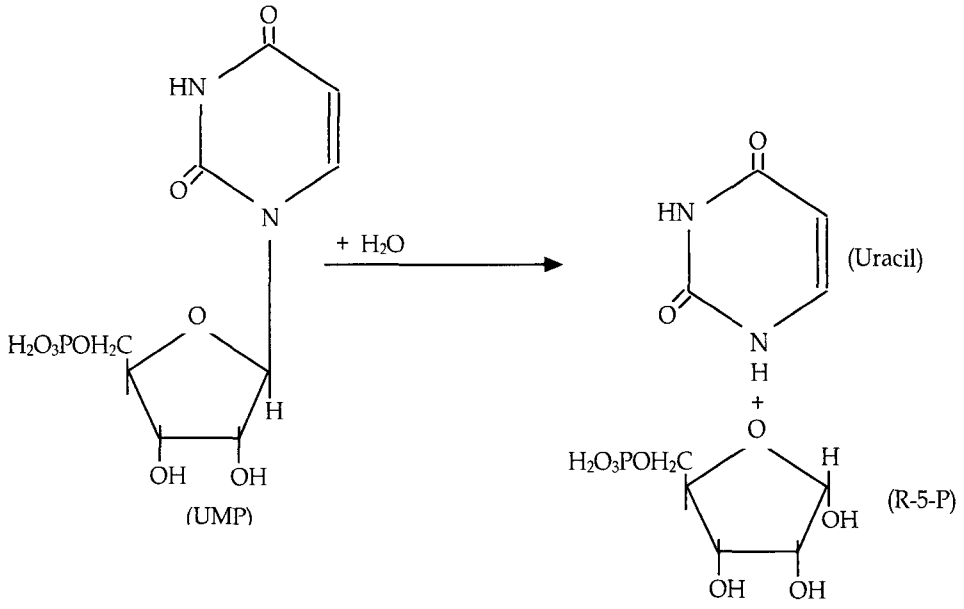

Fig. 1. Hydrolysis of UMP by the action of pyrimidine nucleotide. *N*-ribosidase from *P. oleovorans*.

Table 1. Effect of carbon sources on the enzyme production

Addition	(%)	Growth (mg/ml)	Enzyme activity (units/mg cell)
Glucose	2.00	3.02	0.18
Sucrose	1.90	1.60	0.13
Glycerin	2.05	2.07	0.13
Citrate-2Na	2.87	2.93	0.25
Tartrate-2Na	2.23	1.00	0.11
Acetate-Na	2.73	2.60	0.20
Succinate-2Na	4.49	2.00	0.16
Fumarate-2Na	1.95	3.55	0.28
None	-	1.70	0.18

In addition to the carbon source stated above, each medium contained 1.0% casein hydrolysate, 0.2% meat extract, 0.01% K_2HPO_4, 0.01% KH_2PO_4 and 0.03% $MgSO_4 \cdot 7H_2O$. Initial pH was adjusted to 7.0. Concentration of the carbon sources were equalized to 2% glucose on carbon atom basis.

substance of pyrimidine nucleotide synthesis. Fumaric acid is easily converted to aspartic acid under conditions of existence of ammonia. This fumaric acid was to be most favorable carbon source on cytosine deaminase production of *P. aureofaciens*[14]. Author took an interest in this point. As shown in Table 1, fumarate ultilized more easily than glucose as a carbon source and in fumarate medium the pH of broth was not changed, but after 24 h of incubation time changed sharply to alkalic pH as shown in Fig. 2. Fumarate is not only to be carbon source, but also to keep up steady pH level of broth. Fumarate was chosen as the main carbon source for the subsequent experiments.

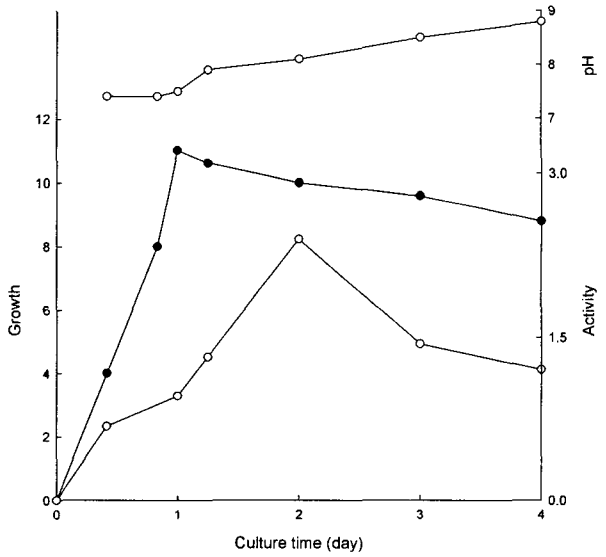

Fig. 2. Culture time and the enzyme production. (○), Activity (units/ml medium) ; (●), growth (OD at 660 nM) ; (○), pH.

As shown in Table 2, 2% of fumarate (as disodium salt) was found to be the best concentration for the enzyme production. Concentrations higher than 2% fumarate stimulated growth, but not the enzyme production.

Effect of nitrogen source

As shown in Table 3, the bacterial growth and the enzyme production were affected by the nitrogen source employed. Peptone, CSL, yeast extract, and meat extract were the good nitrogen sources for the growth. But ammonium chloride, urea, peptone, and CSL were the good effective on the enzyme production. Moreover, it was observed that ammonium chloride and urea were effective stimulators of the

Table 2. Effect of concentration of fumarate on the enzyme production

Conc. of fumarate-2Na (%)	Growth (mg/ml)	Enzyme activity (units/mg cell)
0	1.71	0.07
1.0	2.80	0.25
1.5	3.65	0.40
2.0	4.60	0.52
2.5	5.13	0.34
3.0	5.10	0.29

Beside fumarate, all media contained 0.2% meat extract, 0.01% K_2HPO_4 and 0.03% $MgSO_4 \cdot 7H_2O$. Initial pH was adjusted to 7.0.

Table 3. Effect of nitrogen sources on the enzyme production

Addition	(%)	Growth (mg/ml)	Enzyme activity (units/mg cell)
Ammonium carbonate	0.53	0.10	-
Ammonium sulfate	0.62	0.35	0.26
Ammonium chloride	0.50	0.26	0.47
Ammonium phosphate (dibasic)	0.62	0.45	0.25
Ammonium phosphate (monobasic)	1.07	0.57	0.29
Ammonium nitrate	0.75	1.71	0.14
Sodium nitrate	0.28	1.65	0.20
Urea	0.28	1.65	0.43
Peptone	0.90	3.34	0.37
CSL*	2.20	3.28	0.39
Yeast extract	0.45	2.20	0.12
Meat extract	7.10	3.80	0.32

In addition to the nitrogen source stated above, each medium contained 2.0% fumarate-2Na, 0.01% K_2HPO_4, 0.01% KH_2PO_4 and 0.03% $MgSO_4 \cdot 7H_2O$. Initial pH was adjusted to 7.0. Concentration of the nitrogen sources were equalized to 1.0% casein hydrolysate on nitrogen atom basis.
*CSL; Corn steep liquor.

enzyme production and had less effect on bacterial growth than yeast extract and meat extract did.

The optimum concentration of peptone for both growth and the enzyme production were found to be approximately 1.5%, as shown in Table 4. When the concentration was increased over that, the growth and the enzyme production were somewhat inhibited.

As described above results, ammonium chloride was effective in the enzyme production. The effect of concentrations of ammonium chloride as an auxiliary nitrogen source in the presence of 1.5% peptone is given in Table 5. The addition of 1% ammonium chloride was found to be best favorable for the enzyme production and growth.

Effect of complex nutrients

A series of complex nutrients, such as peptone and CSL tested for effect on the enzyme production and growth.

Table 4. Effect of concentration of peptone on the enzyme production

Conc. of peptone (%)	Growth (mg/ml)	Enzyme activity (units/mg cell)
0.01	0.10	-
0.05	0.26	-
0.10	0.44	-
0.50	2.03	0.04
0.75	2.60	0.16
1.00	5.26	0.22
1.25	4.80	0.30
1.50	4.70	0.56
1.75	4.81	0.32
2.00	3.20	0.16

Beside peptone, all media contained 2.0% fumarate-2Na, 0.01% K_2HPO_4, 0.01% KH_2PO_4 and 0.03% $MgSO_4 \cdot 7H_2O$. Initial pH was adjusted to 7.0.

Table 5. Effect of concentration of NH_4Cl on the enzyme production

Conc. of NH_4Cl	Growth (mg/ml)	Enzyme activity (units/mg cell)
0.00	3.75	0.47
0.50	3.89	0.44
1.00	4.18	0.67
1.25	4.20	0.50
1.50	4.90	0.45
3.00	6.00	0.42
5.00	6.22	0.38
6.00	6.45	0.27

See footnote of Table 4.

When the concentration of peptone was increased, the growth and enzyme production were stimulated. An concentration limit of 5% CSL stimulated the growth and enzyme production, although did not stimulate the growth. The enzyme production was best obtained with the addition of 1.5% peptone and 5% CSL, as shown in Table 6.

Effect of initial pH

From the results of the above experiments, a favorable medium for the production of the enzyme was devised as followed; 2% fumarate, 1.5% peptone, 5% CSL, 1% NH_4Cl, 0.03% $MgSO_4 \cdot 7H_2O$, 0.01% KH_2PO_4, and 0.01% K_2HPO_4. When this bacteria was incubated in the devised medium, the pH of the medium increased in direct proportional to cultural time. The initial optimum pH was found to be near 7.0 (data not shown), and this pH value was used in the followed experiments.

Effect of culture temperature

The optimum culture temperature for the enzyme production was found in 28℃ to 30℃ (data not shown). When the strain was cultured at 37℃, the growth and the enzyme production were strongly inhibited.

Effect of UMP and its related compounds

The effect of UMP and its related compounds was examined under these optimum cultural conditions. As shown

Table 6. Effect of peptone and CSL mixture on the enzyme production

Addition		Growth (mg/ml)	Enzyme activity (units/mg cell)
peptone (%)	CSL (%)		
0.0	1.0	1.57	0.07
0.5	1.0	3.83	0.18
1.0	1.0	4.13	0.24
1.5	1.0	4.47	0.30
0.0	3.0	2.88	0.16
0.5	3.0	5.25	0.28
1.0	3.0	4.94	0.32
1.5	3.0	2.30	0.42
0.0	5.0	4.94	0.16
0.5	5.0	4.91	0.35
1.0	5.0	5.43	0.40
1.5	5.0	6.22	0.74
0.0	6.0	4.71	0.35
0.5	6.0	2.31	0.54
1.0	6.0	4.25	0.51
1.5	6.0	6.45	0.47

See footnote of Table 4.

Table 7. Effect of UMP and its derivatives on the enzyme production

Addition	(%)	Growth (mg/ml)	Enzyme activity (units/mg cell)
UMP	0.1	5.80	0.38
	0.05	5.75	0.39
Uridine	0.1	5.50	0.37
	0.05	5.50	0.37
Uracil	0.1	7.05	0.36
	0.36	6.65	0.39
None	-	6.00	0.38

Besides UMP or its derivatives, each medium contained 2.0% fumarate-2Na, 1.5% peptone, 5% CSL, 0.01% KH_2PO_4, 0.01% K_2HPO_4, 5% NH_4Cl, and 0.03% $MgSO_4 \cdot 7H_2O$. Initial pH was adjusted to 7.0.

in Table 7, the compounds tested in this study did not affect the enzyme production. Furthermore, UMP and its related compounds did not also produce any noticeable effect. Furthermore, the systhesis of cytosine deaminase in *Salmonella typhimurium* is repressed by both a uridine and a cytidine compounds[13]. The growth of *P. aureofaciens* was stimulated by cytosine, but cytosine was not induced the cytosine deaminase from this strain[14]. *Hydrogenomonas facilis* utilizes thymine, cytosine, and uracil as nitrogen sources; *Hydrogenomonas* H 16, however, only cytosine. Cytosine is completely metabolised by *H. facilis,* but is only deaminated by *H.* H 16 and the resulting uracil accumulates in the culture medium. The cytosine deaminase activity of *H.* H 16 cells were increased an 18-fold during the incubation in a cytosine containing medium[5].

The time course of the enzyme production

As shown in Fig. 2, pyrimidine nucleotide *N*-ribosidase from *P. oleovorans* at different cultural periods showed that the maximum production of the enzyme was obtained after 48 hr of cultivation and gradually declined thereafter. Moreover, the level of pyrimidine nucleotide *N*-ribosidase of *P. oleovorans* varies depending on culture conditions, but the enzyme activity do not effect by many natural substrances, such as nucleic acid related compounds.

From those results, I assume that in cell of *P. oleovorans* the pyrimidine nucleotide *N*-ribosidase level may be regulated by means of production or degradation of the enzyme itself.

The pyrimidine nucleotide *N*-ribosidase of *P. oleovorans* ATCC 8062 was not induced by UMP and its derivatives, and constitutive enzyme.

However, a clear elucidation of the regulation system has not been obtained. More detailed investigations for those to be elucidated are now in progress.

References

1. Agarwal, D. P., G. G. Sanwal and P. S. Krishnan. 1963. Improved paper-chromatographic separation of sugar phosphates by using borate-impregnated paper. *Analyst.* **88**, 969-970.
2. Bankel, L., G. Lindstedt and S. Lindstedt. 1976. On the metabolic role of thymidine 2'-hydroxylase (thymidine, 2-oxoglutarate : oxygen oxidoreductase, EC 1.14.11.3) in *Nurospora crassa. FEBS Lett.* **71**, 147-153.
3. Imada, A., M. Kuno and S. Igarasi. 1967. Degradation of pyrimidine nucleotides by enzyme systems of *Streptomyces.* I. Ribose-5-phosphate formation from pyrimidine nucleotides. *J. Gen. Appl. Microbiol.* **13**, 255-265.
4. Imada, A. 1967. Degradation of pyrimidine nucleotides by enzyme systems of *Streptomyces.* II. Pyrimidine 5'-nucleotide phosphoribo (deoxyribo) hydrolase of *Streptomyces virginiae. J. Gen. Appl. Microbiol.* **13**, 267-278.
5. Kaltwasser, H. and J. Kramer. 1968. Verwertung von cytosine und uracil durch *Hydrogenomonas facilis* und *Hydrogenomonas* H 16. *Archiv. fur Mikrobiolgie.* **60**, 172-181.
6. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. 1951. Protein measurement with the follin phenol reagent. *J. Biol. Chem.* **193**, 265-275.
7. Neuhard, J. 1968. Pyrimidine nucleotide methabolism and pathways of thymidine triphosphate biosynthesis in *Salmonella typhimurium. J. Bacteriol.* **96**, 1519-1527.
8. Sakai, T., T. Watanabe and I. Chibata. 1968. Metabolism of pyrimidine nucleotides in bacteria. *J. Ferm. Technol.* **46**, 202-213.
9. Sakai, T., T. Watanabe and I. Chibata. 1971. Metabolism of pyrimidine nucleotides in bacteria, (II) Studies on the regulation system of the degradation of nucleotides in *Pseudomonas oleovorans. J. Ferm. Technol.* **49**, 488-498.
10. Sakai, T., T. Watanabe and I. Chibata. 1971. Metabolism of pyrimidine nucleotides in bacteria, (III) enzymatic production of ribose-5-phosphate from uridine-5-monophosphate by *Pseudomonas oleovorans. Appl. Microbiol.* **22**, 1085-1090.
11. Sakai, T., T. Yu and S. Omata. 1976. Distribution of enzymes related to cytidine degradation in bacteria. *Agric. Biol. Chem.* **40**, 1893-1895.
12. Somogyi, M. 1952. Notes on sugar determination. *J. Biol. Chem.* **195**, 19-23.
13. West, T. P. and G. A. O'Dpnovan. 1982. Repression of cytosine deaminase by pyrimidines in *Salmonella typimurium. J. Bacteriol.* **149**, 1171-1174.
14. Yu, Tae Shick. 1976. Nutritional and cultural characteristics of cytosine deaminase from *Pseudomonase aureofaciens. J. Inst. Nat. Sci. Keimyung university.* **3**, 97-106.

초록 : *Pseudomonas oleovorans*의 pyrimidine nucleotide N-ribosidase의 생성 최적조건

유 대 식*
(계명대학교 미생물학과)

Pyrimidine nucleotide *N*-ribosidase (pyrimidine 5'-nucleotide phosphoribo (deoxyribo) hydrolase/ pyrimidine 5'-nucleotide nucleosidase, EC 3.2.2.10)는 CMP와 UMP를 직접 분해하여 cytosine과 uracil를 생성하는 가수분해효소이다. 본 연구에서는 *Pseudomonas oleovorans* ATCC 8062의 생육과 pyrimidine nucleotide *N*-ribosidase 생성에 미치는 탄소원과 질소원의 영향 및 생육인자 등에 대하여 검토했다. 효소생성의 최적배양조건은 2% fumarate, 1.5% peptone, 5% corn steep liquor (CSL)과 1% ammonium chloride의 배지조성(초기 pH 7.0)으로 28℃, 48시간 진탕 배양이 양호했다. 효소의 활성은 생육이 최대에 도달하는 정지기 후기에 최대에 도달하며, 그 이후부터 급속히 불활성화 되었다. *P. oleovorans*의 pyrimidine nucleotide *N*-ribosidase는 UMP에 의하여 유도생성되지 않으므로 구성효소이며, 내생효소였다.