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고정화 균체에 의한 Pyridoxl Phosphate의 생산에 관한 연구

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Studies on the Formation of Pyridoxal Phosphate by Immobilized Cells

by

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Abstract

Studies were made of the continuous production of Pyridoxal 5′-phosphate (pyridoxal-p) on simultaneously immobilized cell column. Whole-cell of *Pseudomonas polycolor* having high activity of pyridoxine 5′-phosphate (pyridoxine-p) oxidase and *Kloeckera* sp. No. 2201[1] having high activity of catalase were used as the enzyme materials. The enzyme sources were entrapped in a polyacrylamide gel. Enzymatic properties of the simultaneously immobilized cells were investigated, comparing with those of the mixed whole-cells of the microorganisms. The simultaneously immobilized cells had higher enzyme activity than singly immobilized cells of *Pseudomonas polycolor*. From this result, the simultaneously immobilized pyridoxine-p oxidase-catalase system could be available to exert a protective effect upon the pyridoxine-p oxidase by destroying H_2O_2 which is a by-product of pyridoxine-p oxidation.

The optimum pH was 9.0 for the immobilized cells and the whole-cells. The optimum temperature was 45°C for the immobilized cells and 40°C for the whole-cells. The pyridoxine-p oxidase of the immobilized cells were activated by Hg^{++} and some SH-compounds.

Introduction

Vitamin B_6 is deeply connected with amino acid production as a co-enzyme of β-tyrosinase and tryptophanase.

Wada *et al*[2] demonstrated the conversion of pyridoxine-p into pyridoxal-p, obtaining a preparation from rabbit liver that catalysed this reaction. The pyridoxine-p oxidase was subsequently purifid from rabbit liver by Morisue *et al.* [3]. Although not in great detail, pyridoxine-p oxidase of microorganisms was studied by Henderson *et al.*[4], who obtained a

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preparation from *Escherichia coli.*

Yamamoto *et al.*[5] screened the pyridoxine-p oxidative activities among the cell-free extracts of various bacterias and the oxidase activities catalyzing pyridoxine-p oxidation were found mostly in the cell-free extracts of *Alcaligenes facalis, Micrococcus ureae, Pseudomonas fragi and Pseudomonas aeruginosa.*

Recently, enzymes have been immobilized successfully by many methodes and are being used in various fields.

Hydrogen peroxide is an inhibitor of pyridoxal-p production through pyridoxine-p oxidation by oxidase. This study on the purpose of the following experiments concerning pyridoxal-p production with the gel-entrapped microorgainsms, simultaneously immobilized *Pseudomonas polycolor* and *Kloeckera* sp. No.2201, was to examine some chracteristics of the immobilized cells and the mothod's applicability to a continuous reaction.

Materials and Methods

Chemical Compounds. Acrylamide monomer, N,N'-methylenebisacrylamide(BIS), ammonium persulfate were obtained from Wako Chemicals. Ltd., Kyoto, Japan, N,N, N', N'-tetramethylenediamine from Tokyo Kasei Kogyo Co., Ltd, Tokyo, Japan and others from commerical sources.

Microorganisms. The microorganisms used in this study were the strains preserved in the Laboratory of Applied Microbiology, Department of Agricultural Chemistry, Kyoto University.

Cultivation of Microorganisms. Whole-cell of *Pseudomonas polycolor* was used as pyridoxine-p oxidase material. The bacterial cell crop was harvested by incubating aerobically at 30°C for 24 hours from a medium (pH 7) containing peptone (1.5%), glucose (1.0%), yeast extract(0.2%), K_2HPO_4(0.5%), and KH_2PO_4 (0.1%). Whole-cell of *Kloeckera* sp. No. 2201 was used as catalase material. The yeast was cultured under the aerobic conditions at 30°C for 24 hours in a medium (pH 6.0) containing glucose (0.1 %), NH_4Cl(0.4%) K_2HPO_4(0.1%) $MgSO_4\ 7H_2O$(0.05 %), and yeast extract (0.2%). The cells wer erespectively separated by centrifugation at 10,000×g for 10 min, rinsed with Physiological saline and stored at 0-5°C.

Preparation of Immobilization. With slight modification was used the procedure of Chibata *et al.*[6].To washed cells of *Pseudomonas polycolor*(300mg) mixed with *Kloeckara* sp. No. 2201 (100mg) as dry weight in 10ml of physiological saline, acrylamide monomer (1.1g), N,N'-mentlylenebisacrylamide(60mg), 5% N, N, N', N'-tetramethylethylendiamine(0.5 ml) were added, and the mixture was well mixed with stirring at 0-5°C for 30 min, and incubated at 35°C for 10 min. The resultant gel was slightly crushed on 24 mesh shieve(0.7 mm) and kept at 0-5°C.

Composition of Standard Reaction Mixture. To estimate the pyridoxine-p oxidase activity, reactions were carried out in a total volume of 6 ml. The standard composition was: the simultaneously immobilized cell, 30 mg of *Pseudomonas polycolor* and 10 mg of *Kloeckera sp. No.* 2201; pyridoxine-p (0.5 μmoles), 1 ml; Tris-HCl buffer(0.5μmoles), 1 ml. The mixtures were incubated in water bath at 45°C, 1 hour with shaking. Reaction was terminated by the addition of 0.5 ml of 10% (w/v) trichloroacetic acid.

Measurement of Enzyme Activity. Phenylhydrazine procedure for the colorimetrc estimation of pyridoxal-p was followed according to the procedure developed by Wada and Snell[2].

Continuous Reactor of Pyridoxal-p Production. This reactor is shown in Fig, 1. The simultaneously imm-

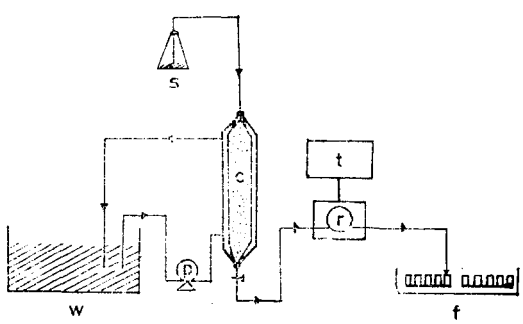

Fig.1. Diagram of continuous reactor
The feed solution was supplied at a constant flow rate from substrate reservoir controlled by the slide regulator. The elution rate is same as the flow rate (SV=0.25).
W: water bath, S: substrate, C: column
P: water pump, t: transformer,
r; slide regulator, f: fraction collector.

obilized cell (300mg of *Pseudomonas polycolor* and 100 mg of *Kloeckera sp. No.* 2201) was packed on the column(1.5×35cm) equipped with water jacket which was continuously supplied hot water (45°C). The substrate of pyridoxine-p was applied on the top of the column and 2ml of fraction was collected with fraction collector under the space velocity(0.25) controlled by regulator. Each tube was added 2ml of deionized water and 0.2ml of 2% phenylhydrazine, keeping at room temperature for 20min.The next was same as the above measurement of enzyme activity.

Results

Production of Pyridoxal Phspohate by Immobilized Cells of Several Microorganisms. For screening the most suitable microorganism of pyridoxal-p production, whole-cell immobilization for several microorganisms having pyridoxine-p oxidase activity was carried out by the polyacrylamide gel-entrapped method. Table 1 shows the enzyme activities of the whole-cells and immobilized cells of some microorganisms.

Enzyme activity differs from as microorganisms Pyridoxine-p oxidase of *Pseudomonas polycolor* was the most strong both native and immobilized cells among the microorganisms tested. Especially simultaneously immobilized cells of *Pseudomonas polycolor* and *Kloeckera sp. No.* 2201 had high activity,comparing with that of others. Howevere, the *Kloeckera* yeast has no activity of pyridoxine-p oxidase.

Table 1. Comparison of pyridoxine-p oxidase activities of several microorganisms

Microorganism	Enzyme activity (mμmoles/hr/ml)		Relative activity (%)
	Whole-cell	Immobilized-cell	Whole-cell/Immobilized-cell
Micrococcus ureae	30	12	40
Erwinia carotovora	47	35	74
Pseudomonas aeruginosa	80	38	48
Pseudomonas polycolor	96	60	63
Pseudomonas fragi	30	5	17
Pseudomonas rivofravina	32	10	31
#*Pseudomonas polycolor* *Kloeckera sp.* No, 2201	120	80	67

Each group-except #mixed group-contained 30mg cells as dry weight per one ml. of cell suspension. Mixed group had 30mg cells of *Pseudomonas polycolor* and 10mg of *Kloeckera* sp. No. 2201. Immobilization was performed as described in the text.

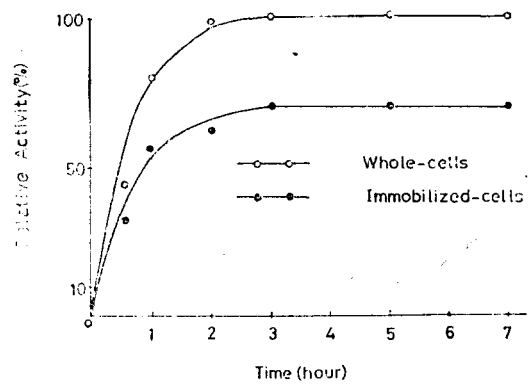

Fig. 2. Time course of pyridoxal-p production by the whole-cells and immobilized cells

Reaction mixture; 60mg of *Pseudomonas polycolor* and 20mg of *Kloeckera sp. No.* 2201, pyridoxine-p (0.1μmoles), 2ml; Tris-HCl buffer (1.0μmoles), 2ml. Reactions were carried out in a total volume of 12ml at 45°C with shaking in batch reactor.

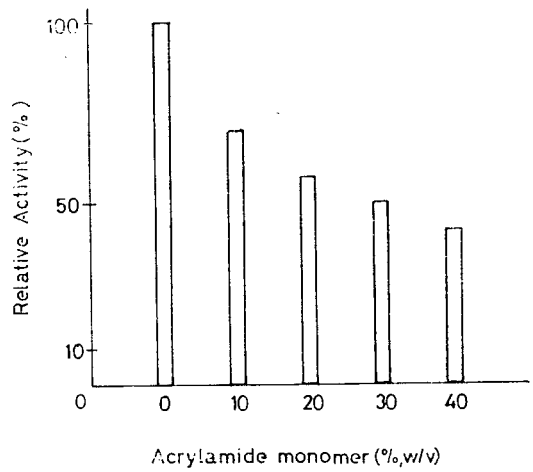

Fig. 3. Effect of Concentration of Acrylamide monomer on Pyridoxal-p production

The enzyme reaction was carried out under the standard conditions in the text except for varying concentration of acrylamide monomer. The highest activity of whole-cells was taken as 100%.

Time course of Pyridoxal-p Production by the hole-cell and the Simultaneously Immobilized cell in batch

reactor. The limit of pyridoxine-p oxidation by the whole-cell and the simultaneously immobilized cell was respectively examined at 45°C with shaking. Pyridoxal-p production curves are shown in Fig. 2, indicating that concentration of pyridoxal-p could show the limit value after 3 hours reaction.

Effect of Concentration of Acrylamide Monomer. The concentration of acrylamide as a monomer was changed and pyridoxine-p oxidase activities of the cells were compared(Fig. 3). The amount of acrylamide had a great influence upon the enzyme activity of simultaneously immobilized cells, and 10%-acrylamide group had the highest activity. On the other hand, polymerization was imperfect at less than 10% of acrylamide.

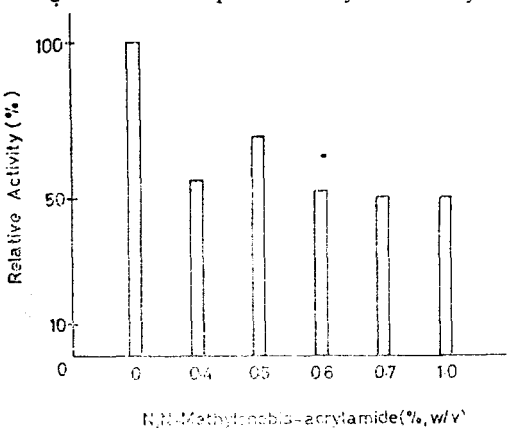

Fig. 4. Effect of concentration of BIS on pyridoxal-p production
The enzyme reaction was carried out under the standard conditions in the text except for the concentration of N,N'-methylenebisac rylamide. The highest activity of whole-cells was taken as 100%.

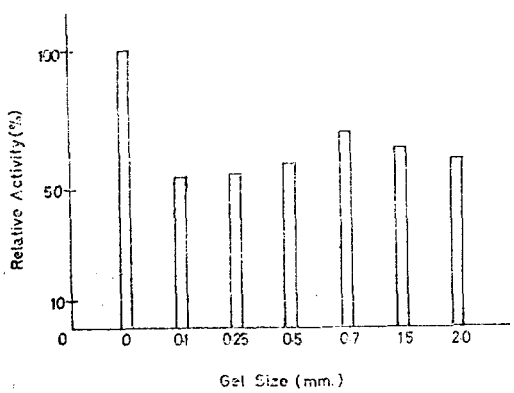

Fig. 5. Effect of gel size on pyridoxal-p production
The enzyme activity was carried out under the standard conditions in the text except for varying gel size. The highest activity of whole-cells was taken as 100%.

Effect of Concentraction of N,N'-Methylenebisacryl Amide. Fig. 4 shows that the concentration of N,N'-methylenbisacrylamide as a cross linking agent had also an effect on pyridoxine-p oxidase activity. The optimum concentration of N,N'-methylene bisacrylamide was 0.5%, showing 70% of relative activity.

Effect of Gel size. The effect of gel size on pyridoxine-p oxidase of the the simultaneously immobilized cells was also tested(Fig. 5). The result showed that the optimum gel size for pyridoxal-p production appeared to be around 0.7mm.

Effect of pH. Reaction mixtures were made up in the pH range 5—10 and incubated in at 45°C for 1hr. The pH activity curves of the whole-cells and the immobilized cells are given in Fig. 6, showing that apparent optimum pH of pyridoxine-p oxidation by the immobilized cells was around 9.0 as same as the whole cells. The activity of pyridoxine-p oxidase was very weak below pH 7.0 and above 9.5.

Effect of Temperature. The effect of temperature on the production of pyridoxal-p by the whole-cells and simultaneously entrapped-cells are presented in

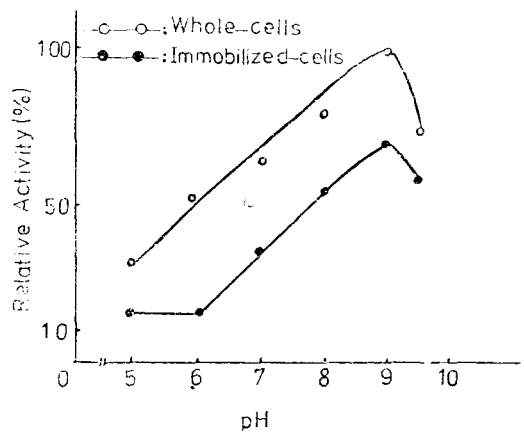

Fig. 6. Effect of pH on Pyridoxal-p Production
The enzyme reaction was carried out under the standard conditions except that the buffer used was 0.1M sodium acetate for pH 5~7 and 0.1M Tris-HCl for pH 7~10. The highest activity of whole-cells was taken as 100%.

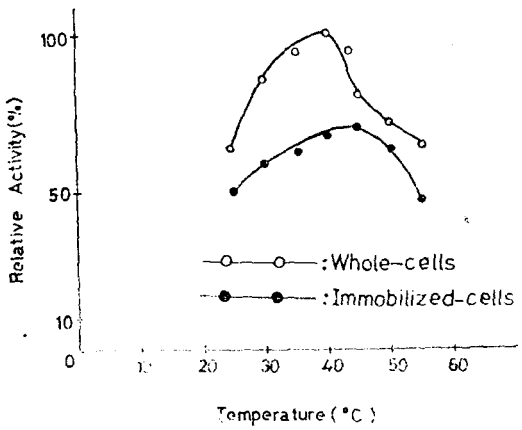

Fig. 7. Effect of Temperature on Pyridoxal-production

The enzyme reaction was carried out under the standard conditions except for varying temperature. The highest activity of whole-cells was taken as 100%.

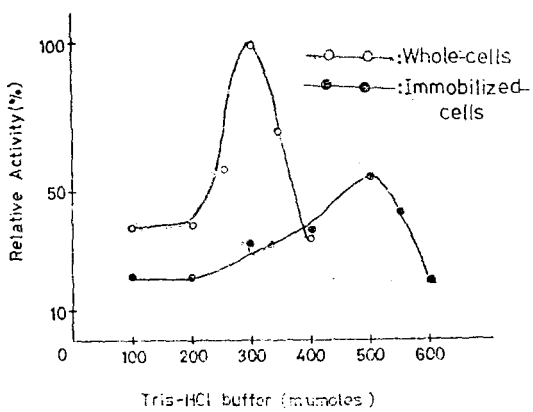

Fig. 8. Effect of the Buffer concentration on Pyridoxal-p production

The enzyme reaction was carried out under the standard conditions except for varying buffer concentration. The highest activity of whole-cells was taken as 100%.

The optimal temperature for pyridoxine-p oxidase was 40°C for the whole-cells, and 45°C for the simultaneously entrapped-cells. The quantity of pyridoxal-p was very poor below 30°C and above 50°C.

Effect of Buffer Concentration. Investigation of optimal concentration of buffer solution on the enzyme activity was also performed. As shown in Fig. 8, the whole-cells had the highest activity in the concentration of 30 mμmoles of Tris-HCl buffer and the immobilized cells had the highest activity in 500 mμmoles.

Effect of Metal Ion on Pyridoxal-p Production The effect of various metal ions on pyridoxine-p oxidase was also tested at the final concentration of 10^{-3} M(Table 2). Zn^{2+}, Ba^{2+}, Ag^{2+}, Li^{2+}, Cu^{2+}, Mg^{2+}, and Al^{3+} had

Table 2. Effect of Ions on Pyridoxal-p Production

Metal Ions (10^{-3}M)	Relative Activity(%)	
	Whole-cells	Immobilized-cells
None	100	100
Be^{2+}	123	84
CN^-	100	95
$MoO^{2}{}_{4}{}^{+}$	126	63
Zn^{2+}	108	34
Hg^{2+}	200	210
Ni^{2+}	123	63
Ag^{2+}	105	89
Li^{+}	114	89
Cu^{2+}	108	79
F^-	114	110
Mg^{2+}	103	110
Al^{3+}	116	63
CO^{2+}	120	59

The enzyme activity was measured under the standard conditions, The final concentration of metal ions was 10^{-3}M, The activities of control cells were taken as 100%.

not an effect on the oxidase activity in batch reaction. $Hg^{\#}$, Be^{2+}, MoO_4^{2-}, Ni^{2+} and CO^{2+} accelarated by Hg^{2+} and inhibited by $MoO^{25+}{}_{4}$, Zn^{2+}, Ni^{2+}, Al^{3+} and CO^{2+} Effect *of SH-Compounds, Amino Acids and Other Compounds on Pyridoxal-p Production.* The effect of SH-compounds, amino acids and other compounds on the enzyme activity of the cells was also investigated.

Table 3 shows that dithiothreitol, mercaptoethanol and ascorbic acid had an effect on the enzyme activity of the immobilized cells and mercaptoethanol, ascorbic acid and sodium citrate activated the whole-cells.

Continuous Pyridoxal-p Production by the Simultaneously Immobilized Cells. Continuous pyridoxine-p oxidation by the immobilized cells on a column was examined (Fig. 9). Reaction was carried out continuously for 30 hours on the column. In the column in which

Table 3. Effect of SH-compounds, amino acids and other compounds on pyridoxal-p production

Compounds	Relative Activity(%)	
	Whole-cells	Immobilized cells
None	100	100
Dithiothreitol	106	300
Mercaptoethanol	120	260
Ascorbic acid	120	200
Glutamic acid	113	90
Cysteine	100	95
Leucine	113	100
Sodium citrate	120	105
Sodium azide	100	105
Thiourea	100	100

The enzyme activity was measured under the standard conditions. The final concentration of dithiothreitol and mercaptoethanol in reaction mixture were 0.1% and others 1×10^{-3}M, The activity of control was taken as 100%

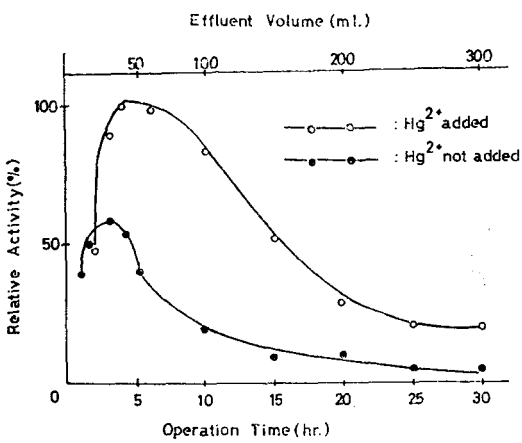

Fig. 9. Continuous production of pyridoxal-p by the simultaneously immobilized cells

The substrate solution dissolved in Tris-HCl buffer (0.5mμmoles) contained 0.5μmoles of pyridoxine-p per one ml. and 10^{-3}M Hg^{2+}. The pyridoxine solution was passed through a column(1.5×35cm) packed with the simultameously immobilized cells at the flow rate of SV=0.25 at 45°C. The highest activity of Hg^{2+} added group was taken as 100%.

Hg^{2+} was absent, enzyme activity was the greatest after 3 hours; subsequent activity decreased as operation time increased. In the presence of Hg^{2+}, enzyme activity was the grestest after 4 hours, remaining relatively constant until the sixth hour, thereafter decreasing with time. Enzyme activity was far greater in the presence of Hg^{2+}. Pyridoxal-p quantities in the volume shows the total production quantities of pyridoxal-p.

Discussion

It has been reported that the activity of immobilized enzymes is less than that of free enzymes.

Generally, the rate of activivity of the immobilized enzyme decreases to approximately 20—80% of that of the free enzyme.

In these experiments, We found that pyridoxine-p oxidase of singly immobilized cell of *Pseudomonas polycolor* retains 60% activity

In the conversion of pyridoxine-p to pyridoxal-p, hydrogen peroxide is a by-product which inhibits the production of pyridoxal-p. Therefore, the destroduction of hydrogen peroxide removes this inhibiting factor and provides oxygen for the reaction. The kernel of this metabolism is presented to the following pathway.

$$\text{Pyridoxine phosphate} \xrightarrow[\text{pyridoxine-p oxidase}]{-2H} \text{pyridoxal phosphate} + H_2O_2 \cdots\cdots (1)$$

$$H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2 \cdots\cdots (2)$$

To realize these, *Pseudomonas polycolor* and *kloeckera sp. No.*2201 having catalase activity were simultaneously immobilized, resulting in a retention of 67% activity It means in equivalent of 83% of whole-cell activity of *Pseudomonas polycolor*. The formation of acrylamide gel was made, under the cond itions of this experiment, above 10% of the monomer. The maximum enzyme activity was shown at 10% of the monomer probably because the less the monomer quantity, the larger the net work of the gel and substrate penetration was easy. Also, it was considered that the less the gel size, the better was the enzyme activation, but the optimum size was around 0.7mm

This is probably because, as gel particles are crushed on the shieve, in other words, the less the gel size, there is a high possibility of the native cell entrapped in the gel leaking out. The optimum temperature for the immobilized cells was observ-

ed to be 45°C which was 5°C higher than that of the whole-cells and the pyridoxine-p oxidase was inactivated by the heat treatment for 20 minutes at 60°C.

Enzymes immobilized may bring a change in the state of electron of its protein and, influenced by the electric charge on the surface of the packing material, optimum pH of enzyme reactions may vary. There are cases[7,8,9] reported where the optimum temperature of insoluble enzymes is higher than native enzymes.

S. Yamamoto *et al*[10]. suggested that the reaction product, pyridoxal-p, inhibites the oxidation and that Tris-HCl buffer partially overcomes this inhibition through the formation of a less inhibitory Schiff's base[2]. In this experments, potassium phosphate and Tris-HCl buffer had similar effect on the oxidation in batch reaction. However, in continuous column reaction, the yield of pyridoxal-p was more rich in Tris-HCl buffer than potassium phosphate.

Of the metal ions examined, Hg^{2+} was required to activate for the pyridoxine-p oxdase of the simultaneously immobilized cells.

In general, SH-enzymes are stable in SH-compound and inactivated in Hg^{2+}, SH-inhibitor, however, the enzyme in this experiment has been activated in both of them. This is considered a characteristic of the enzyme, and more studies should be made of this characteristic.

K. Yamamoto *et al.*[11] reported that L-histidine ammonialyase activity of the immobilized cell column was stable over 40 days in the presence of Mg^{2+}.

In the continuous column reaction, the decrease in activity may be due to the instability of the enzyme and/or the change in enzyme properties and leakage of cells from the entrapping gel. Further research is required for these imagination with the method of cell immobilization for continuous production of pyridoxal -p.

要 約

固定化 菌體를 利用한 pyridoxal 5'-phosphate(pyridoxal-p)의 連續生産에 關해實驗하였다.

pyridoxine 5'-phosphate (pyridoxine-p) oxidase活性을 갖는 *Pseudomonas polycolor* 菌體와 Catalase 活性을 갖는 *Kloeckera sp. No.* 2201菌體를 酵素源으로 使用하였다. 菌體는 Polyacrylamide gel에 不溶化 시켰으며 同時固定菌體의 活性이 *Pseudomonas Polycoler*單獨固定菌體의 活性보다 强하였다.

이 結果는 同時固定菌體의 Pyridoxine-p oxidase-catalase system은 Pyridoxine-p 酸化의 副産物인 H_2O_2를 分解하므로서 保護效果를 얻을 수 있음을 意味한다. 同時固定菌體와 生菌體의 酵素活性에 미치는 最適pH는 9.0이었고 同時固定菌體의 最適溫度는 45°C로 生菌體보다 5°C높았다. 固定菌體의 Pyridoxine-p oxidase는 Hg^{2+}와 몇몇 SH-化合物에 依하여 活性化되었다.

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