STUDIES ON THE MICROBIAL METABOLISMS AT LOW TEMPERATURES

NOBUO KATO

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INTRODUCTION

Temperature may be an essential factor for the determination of the growth rate and growth yield, and for the control of the metabolism, including the physiology, of an organism. Each species of organism has an optimum temperature range for growth. On the basis of these temperature ranges, microorganisms are classified as thermophiles, mesophiles, or psychrophiles. Most microbiological research in the past and also in the present, has been concerned with mesophilic organisms which grow in the range of approximately 10 to 50°C. To a much lesser, yet considerable, extent, thermophilic organisms which grow at temperatures as high as 70 to 80°C have also been investigated.

In the early years of this century, the widespread distribution of psychrophiles, which grow well at near-zero temperature, was established (1,2). Nevertheless, the psychrophile had been somewhat neglected by microbiologists, until late in the 1950's. Recently, interest in low-temperature microbiology has increased tremendously, mainly because of the increasing use of refrigeration for preserving materials that are susceptible to spoilage by microorganisms. This awareness of the economic importance of low-temperature microbiology has brought with it a considerable increase in research into fundamental problems associated with the growth of microorganisms at low temperatures. Moreover, it is thought that low-temperature microorganisms or psychrophiles must be of paramount importance in the various cycles of matter in cold environments, including the earth, the oceans, the polar regions, and temperate zones during the winter season. Modern studies on psychrophiles touch upon intriguing problems of taxonomy, morphology, ecology, and physiology.

One widely accepted proposal for the definition of psychrophiles was made by Ingraham and Stokes (3), and Stokes (4). They suggested that psychrophiles be defined as those microorganisms which grow well at 0°C within one or two weeks. The psychrophiles are subdivided into obligate psychrophiles, which have an optimum temperature for growth below 20°C, and facultative psychrophiles, which have an optimum temperature above 20°C.

Psychrophilic microorganisms are very widely distributed in nature. They have been isolated in appreciable and, frequently, large numbers from air, water, soil, plants, animals, and many sorts of foods. They are present in the temperate and the polar regions of the earth and in the oceans. Their ability to grow at both low- and moderate-temperatures confers upon them an ecological and competitive advantage over mesophiles and this may be the reason, in part, for their wide distribution (5). Psychrophilic representatives occur in all of the major groups of microorganisms. Psychrophilic bacteria are usually members of the genera *Achromobacter, Alcaligenes, Flavobacterium, Proteus, Pseudomonas*, and *Serratia*, with the greatest representation in the genus *Pseudomonas* (3,6,7). The existence of obligate anaerobic psychrophilic-bacteria, all of which have been grouped in the genus *Clostridium* (8), has been reported, as well as the occurrence of spore formation and spore germination by psychrophilic strains of *Bacillus* at 0°C (9). The commonest psychrophilic yeasts are members of the genera *Candida* (10 - 12), *Cryptococcus* (13, 14), *Rhodotorula* (15), and *Torulopsis* (15). There have been occasional reports of the isolation of psychrophilic molds (16). Not so much is known about the distribution of the psychrophilic habitat among fungal genera as compared with bacteria.

Quite a number of theories have been put forward as to the physiological and biochemical basis for the three classes of microorganisms. The existence of extremely thermolabil enzymes (17 - 22), the

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Abbreviations: *n*-C_N, *n*-alkane with N carbon atoms; GSH, reduced glutathione; EDTA, ethylenediamine tetraacetic acid; *p*-CMB; *p*-chloromercuribenzoate; DFP, diisopropyl-fluorophosphate; STI, soybean trypsin inhibitor; TLCK, tosyl L-lysine chloromethyl-ketone; Z, benzyloxycarbonyl; Bz, benzoil; Ac, acetyl; Substitute amino acid substrates are abbreviated according to the Tentative Rules of the IUPAC-IUB commission on Biochemical Nomenclature.

INTRODUCTION

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Quite a number of theories have been put forward as to the physiological and biochemical basis for the three classes of microorganisms. The existence of extremely thermolabil enzymes (17 - 22), the

synthesis or accumulation of metabolic poison (23). Damage to or destruction of the metabolic control mechanism and the loss of permeability of the cells (24 - 28) have all been suggested as a biochemical basis for the relative low maximum temperature of psychrophiles.

Psychrophiles are distinguished from mesophiles by their ability to grow well at near-zero temperatures. Ingraham (24) found that the psychrophiles have a lower temperature characteristic for growth over the linear range as compared with the mesophiles, and suggested that the differences in the response of psychrophile and mesophile to low temperature might be due to differences in some aspects of the biochemical organization of the organisms rather than in the properties of individual enzymes. Baxter and Gibbons (25) reported that a psychrophilic strain of *Candida* respired endogenous reserves at a greater rate than a mesophile, *Candida lypolytica*, at all temperatures up to 30°C, and while the psychrophile oxidized glucose at an appreciable rate even at 0°C, virtually no exogenous substrates were oxidized by the mesophile below 5°C. They then suggested that the main factor determining the minimum temperature for growth of a mesophilic organism is the inactivation of the solute transport mechanism. Rose and Evison (27) obtained a similar conclusion from their experimental results with yeasts. By way of explaining the differences in the low-temperature response of the transport mechanism in psychrophiles and mesophiles, there is a notion that the carrier molecules in the membranes of the mesophiles are prevented from combining with the solute at a low temperature as a result of a change in the molecular architecture of the membrane. A change in composition, which is thought to be important in determining the ability of microorganisms to grow at low temperatures, is the increase in the proportion of unsaturated fatty acids in the cellular lipids as the temperature of incubation is lowered. Kates and Baxter (29) reported about a 50% increase in the content of double bonds in fatty acids from mesophilic and psychrophilic species of *Candida* grown at 10°C compared with 25°C, but detected no effect on the phospholipid composition of the yeasts. Similar results were reported by Marr and Ingraham (30) for a strain of *Escherichia coli*. Furthermore, many reports with respect to similar alterations in the fatty acid compositions of microbes related to their culture temperature have beem accumulated (31 - 33). It is known that an increase in the degree of unsaturation of the fatty acids in lipids causes a decrease in the melting point of the lipid (34). The physiological effect of the increased synthesis of unsaturated fatty acids at low temperatures is to maintain the liquid state at low temperatures thereby allowing membrane activity to continue.

The synthesis and activity of enzymes in living cells are under the influence of a complex array of regulatory processes. Many of these regulatory processes are known to be abnormally sensitive to temperature below the optimum for growth of the organism, much more so than the majority of other metabolic processes in living cells. Halpern (35) reported that the synthesis of glutamate carboxylase in *E. coli* is inducible at 37°C but partly constitutive at 30°C. In addition, Ng and Gartner (36) showed that tryptophan induces the synthesis of tryptophanase in *E. coli* at 30°C but not at temperatures below 15°C. Repression of enzyme synthesis is also often sensitive to temperature. Gallant and Stapleton (37) isolated a mutant of *E. coli* B in which the extent of repression of alkaline phosphatase synthesis by inorganic phosphate decreases as the temperature is increased from 20 to 40°C. O'Donovan *et al.* (38) isolated a number of cold-sensitive mutants of *E. coli* which required histidine for growing at temperatures below 20°C. They showed that the requirement for histidine at low temperatures could be attributed to the synthesis of phosphoribosyl pyrophosphate - ATP pyrophosphorylase, which is 1,000 times more sensitive to feed back inhibition at 37°C by histidine than is the same enzyme from the parent (39).

It has been known that the effect of low temperatures on individual metabolic activities of microorganisms reflects differences in the synthesis or activities of individual enzymes at the various temperatures. Williams *et al.* (40) reported that synthesis of the red pigment, prodigiosin, by strains of *Serratia marcescens* is favoured between 20 and 25°C, although the optimum temperature for growth is near 37°C, and showed that the enzyme which catalyzes the last step in the biosynthesis is abnormally sensitive to temperature. Such an explanation has been proposed to explain the production of a red pigment by the silk-worm pathogen, *Bacillus cereus* var. *alesti*, at 15°C but not at 28°C (41). Many

Leuconostoc species and other lactic acid bacteria produced almost no dextran at 37°C, whereas, at 25°C, there is a remarkable accumulation of an extra-cellular dextran (42, 43). This effect has been attributed to the production by these bacteria of a dextransucrase that is very rapidly inactivated at temperatures above 30°C (42) and to the temperature-sensitive nature of the dextran-sucrase-synthesizing system (43). Nashif and Nelson (44) showed that *Pseudomonas fragi* produced a lipase preferentially at low temperatures and not at all at 30°C, although the optimum temperature for the activity of the enzyme was 40°C. Also, the synthesis of a proteolytic enzyme by *Pseudomonas fluorescens* was reported by Peterson and Gunderson (45) to be greater at 0°C than at higher temperatures.

It has frequently been observed with cultures of microorganisms that maximum cell crops are obtained at temperatures considerably below those at which the rate of growth is most rapid (46 - 49). Sinclair and Stokes (50) showed that these phenomena could be explained on the basis of the increased solubility of oxygen at lower temperatures. The solubility coefficient of oxygen in water at 5°C is about 2 times that at 30°C.

In the applied aspects of microbiology, the process using low-temperature cultivation is limited to brewery fermentation. The most favorable temperatures for alcoholic fermentation are around 30°C, but worts are fermented at a low temperature mainly because many of the volatile products of the fermentation, which contribute appreciably to the organoleptic qualities of the beer, are retained. However, few attempts have been made to positively employ low-temperature cultivation or psychrophilic organisms for industrial microbiological processes. This is because there are the following disadvantages in low-temperature cultivation: cultivation for a long period of time is required for sufficient growth at low temperatures, and higher cooling energy is necessary in the course of the cultivation as compared with the higher-temperature cultivation.

On the other hand, low-temperature cultivation is thought to possess the following merits for microbial production: a) the concentration of oxygen in the culture medium increases with a decrease in temperature, b) there is little fear of contamination by other microbes, c) low boiling substances are available for use as fermentation substrates, d) the alteration in metabolic activity by temperature is accessible for fermentative production, and e) the low-temperature cultivation is applicable to the screening of microorganisms producing a thermolabile substance, a novel substance including an antibiotic or an enzyme posessing unique properties.

In this thesis, taking into account the above merits, microbial activities at low temperatures are investigated from the standpoint of applied microbiology.

In this thesis, the term "low-temperature" refers to temperatures below 20°C.

CHAPTER I

AMINO ACID FORMATION BY A FACULTATIVE PSYCHROPHILIC BACTERIUM, *BREVIBACTERIUM* SP. P145[a,b)]

INTRODUCTION

Most researches on psychrophiles in the past and at present have been done in relatively limited fields, such as general microbiology, food hygiene and marine microbiology. In the field of applied microbiological processes that are conducted at low temperature, such as brewery fermentation. Konishi *et al.* (51) have investigated the microflora in yamahaimoto or sake making starter mush. They were able to stabilize the yamahaimoto by adding the nitrite forming psychrophilic bacteria.

Studies pertaining to relationships between temperature and microbial activity have been made by many researchers, concerning enzyme activity, cell permeability, cell component, nutritional requirement and so on. Mitchell and Houlahan (52) noted that the nutritional requirement of a mutant strain of *Neurospora* was affected by the incubation temperature. Similar studies on other microorganisms have been made with wild type strains (53, 54) as well as mutant ones (39, 55).

It has often been assumed that the rate of individual metabolic process decreases at the same rate as the growth rate, as the temperature decreases from the optimum. However, increased production of metabolites by microorganisms at suboptimum temperature are known in some cases as pigments (40, 41) and dextran production (42, 43). Owen and Johnson (58) reported with *Penicillium chrysogenum* that the best temperature for mycelium-production was at 30°C, while at 20°C the penicillin productivity was the best. Similar phenomenon was utilized to obtain high yields of citric acid by *Aspergillus niger* (59). Kosano and Yamada (60) reported that the ratio of two fermentation products, glutamic acid and lactic acid, was affected by the temperatures for seed-culture and main culture.

An attempt has been made to utilize microbial activities at low temperature. One psychrophilic bacterium forming glutamic acid, one of the most popular metabolic products, was isolated from soil. Some of the bacteriological characteristics and the relationships between temperature and growth or nutritional requirement and the conversion of fermentation products by incubation temperature is described. Further, the nature of this conversion is discussed on the basis of the results of cultural and enzymological experiments.

MATERIALS AND METHODS

Isolation of amino acid-forming psychrophilic bacteria

a) Medium for isolation. Peptone, 10 g; meat extract, 5 g; glucose, 5 g; NaCl, 5 g; agar, 20 g; in 1000 ml of tap water, pH 7.0.

b) Medium for amino acid formation. Glucose, 30 g; urea, 4 g; NaCl, 1 g; K_2HPO_4, 2 g KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$,

0.5 g: yeast extract, 2 g; phenol red, 2 mg; in 1000 ml of tap water, pH 7.4. The urea solution was fed not only to supply nitrogen but also to maintain the medium slightly alkaline.

c) Isolation method of psychrophilic bacteria. The suspension of soil, sewage, river and stream waters, *etc.*, were streaked on the nutrient agar plates described above. The plates were incubated at 5°C for 7 days and the organisms grown on the plates were transferred to the agar slants of the same composition. The slope cultures were carried out at 5°C for 7 days.

Cultivation

For the investigation of metabolites, one loopful cells grown on the nutrient agar slant was inoculated into 5 ml of the medium mentioned above. The cultivation was performed at 5°C for 5 to 7 days on a shaker (130 reciprocations per min). In the case of flask culture, the cultured broth of 5 ml in a test tube was inoculated into 100 ml of the medium in a 500 ml shaking flask. The incubation was carried out at various temperature on a reciprocal shaker (130 rpm). At intervals, a part of the cultured broth was withdrawn and centrifuged, and the supernatant solution was subjected to chemical analysis.

Determination of nutritional requirement

Composition of the basal medium was as follows: glucose, 20 g; NH_4Cl, 3 g; K_2HPO_4, 2 g; KH_2PO_4, 1g; $MgSO_4 \cdot 7H_2O$, 0.5 g; in 1000 ml of deionized water, pH 7.0. The concentrations of nutritional factors added to basal medium were as follows: thiamine HCl, 1 µg; riboflavin, 1 µg; pyridoxine HCl, 1 µg: nicotinic acid, 1 µg; Ca·pantothenate, 1 µg; *p*-aminobenzoic acid, 0.2 µg; folic acid, 0.01 µg; biotin, 0.01 µg: adenine, guanine, uracil and xanthine, 5.0 µg each; L-amino acids and glycine 100 µg each; DL-amino acids, 200 µg each were added in 1 ml of the medium.

The stock culture of the strain P145 was maintained on the nutrient agar slant at 5°C. Subcultures were made separately in the basal medium at 5° and 28°C for 3 and 1 day(s), respectively. After the subculture, the cells were harvested by centrifugation, washed three times with physiological saline and suspended in the saline. The cell concentration in the suspension was adjusted to give an optical density of 0.30 at 610 mµ, and then diluted to 10-fold.

The media of 2.5 ml each were dispensed in test tubes and inoculated with 0.1 ml of the diluted cell suspension mentioned above. The incubation was carried out on the reciprocal shaker at various temperatures. After the incubation for an appropriate period, 2.5 ml of saline was added per tube and the cell growth was estimated photometrically at 610 mµ. Duplicate tests for one sample were run concurrently.

Paper chromatography

Paper chromatography was employed for the detection of metabolites. Ascending paper chromatography was carried out on Toyo Roshi No. 53 filter paper with the following solvent system; *n*-butanol, acetic acid, water (4:1:1, v/v/v). After development, the paper chromatogram was treated with 0.5% ninhydrin in 75% ethanol. Quantitative analysis of amino acids was also made chromatographically according to the method of Katagiri *et al.* (56). After the treatment with ninhydrin at 50°C for 30 min, the colored zone was cut out and put in a test tube. The color was extracted with 5 ml of 75% ethanol containing 0.005% $CuSO_4 \cdot 5H_2O$. The intensity of the color of the extract was determined by measuring optical density at 500 mµ.

Analysis

The growth of the organism was routinely estimated by measuring the optical density at 610 mµ of 10-fold diluted broth. The dry weight of cells was calculated from the optical density, using a standard curve prepared with the culture grown exponentially at 5°C in a peptone-supplemented basal medium.

Protein was determined by the method of Lowry *et al.* (63).

Preparation of cell-free extract

The dialyzed cell-free extract was subjected to the assays of L-glutamic acid dehydrogenase and L-alanine dehydrogenase. After 4 and 2 days at 5° and 28°C, respectively, the cells were harvested by centrifugation. The cells were washed twice with 0.85% NaCl solution, then suspended in M/15 potassium phophate buffer, pH 7.8. The cell-free extract was prepared by exposing the suspension to a Kaijo Denki ultrasonic oscillator (19 kHz, for 30 min) and the cell debris was removed by centrifugation at 12,000 x *g* for 30 min. The extract thus obtained was dialyzed overnight against M/15 potassium phosphate buffer, pH 7.8, containing 0.05% 2-mercaptoethanol, at 5°C.

Enzyme assay

The enzyme activities were determined by measuring the change in optical density at 340 mμ according to the method reported by Olson and Anfinsen (64). The increase or decrease of the optical density was followed in a Hitachi Perkin-Elmer spectrophotometer Model 139. Specific activities of L-glutamic acid and L-alanine dehydrogenases were expressed as ΔE per gram of protein, where ΔE was the increase or decrease of optical density of NADH or NADPH at 5° and 28°C during the initical 1 min incubation.

RESULTS AND DISCUSSION

Isolation of psychrophilic bacteria

About 500 pure cultures capable of growing actively at 5°C, were isolated from soil, sewage, stream and river waters *etc.* The glutamic acid spot on the paper chromatogram was detected clearly in the cultured broth of about 15 strains of the tested bacteria. In most cases, the glutamic acid formation was accompanied by those of alanine and aspartic acid.

TABLE I. AMINO ACID FORMATION AT 5°C BY ISOLATED BACTERIA

Medium contained 30 g of glucose, 4 g of urea, 1g of NH_4Cl, 2 g of K_2HPO_4, 1g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$ and 2 g of yeast extract in 1000 ml of tap water (pH 7.4). Culture was carried out at 5°C for 7 days under aerobic condition.

Isolated strain	Glutamic acid (mg/ml)	Associated product
P20	0.85	Alanine, Aspartic acid
P31	0.60	Alanine, Aspartic acid
P54	0.50	Alanine
P142	0.72	Alanine, Aspartic acid, Valine
P145	1.02	Alanine
P472	0.63	Alanine, Aspartic acid
B32	0.88	Alanine, Aspartic acid
B47	0.70	Alanine

Table I shows the glutamic acid formation at 5°C by some isolated bacteria. The strain P145 which appeared in Table I accumulated a significant amount of glutamic acid upon cultivation at 5°C.

Growth temperature

The twenty strains among the isolates at 5°C were used for the determination of the growth temperature range. The inoculated media in the test tubes were incubated at 0° to 42°C. Eighteen cultures grew in the range of 0° to 37°C and 2 cultures at 5° to 42°C. Optimum growth temperature ranged from 15° to 20°C for the 18 cultures tested were the psychrophiles because of their ability to grow at 0°C, having relatively low optimum temperature, *e.g.*, 15° to 20°C.

The effect of temperature on the growth rate of strain P145 is illustrated in Fig. 1. The strain has ability to grow well at 0°C. The optimum growth was observed at 15°C but no growth occurred

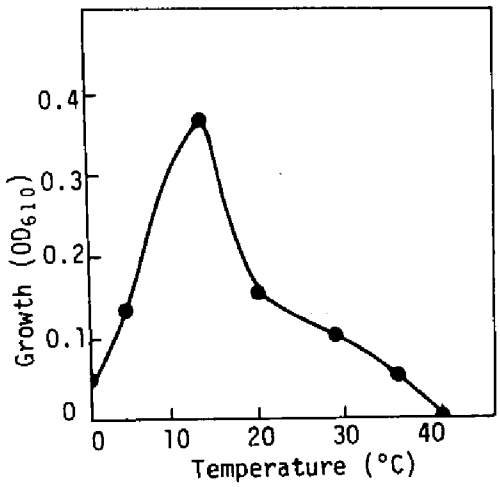

Fig. 1. Effect of Temperature on the Growth of Strain P145.

Medium contained 10 g of peptone and 5 g of NaCl in 1000 ml of tap water (pH 7.0). Cultures were carried out at different temperatures for 2 days under stationary condition.

above 37°C. According to the description by Stokes (4) based on optimum growth temperature, the organism belongs to obligate psychrophile. On the other hand, the strain belongs to facultative psychrophile by the definition of Hagen and Rose (57) which places stress on the maximum temperature. Because of its ability to grow at relatively high temperatures, it seems more practical to follow the latter definition.

Taxonomic studies on the isolated bacterium P145.

The results of taxonomic studies are summarized in Table II and Fig. 2. The strain P145 was unbranched rods, occurred as slightly short chains, non-motile, no acid-fast and with no gas from carbohydrates. Same results were obtained in all tests which were carried out at 5° and 28°C.

TABLE II. CHARACTERISTIC OF ISOLATED BACTERIUM

Morphological;

Cells were straight rods with rounded ends, 0.5 by 1.4 μ. Usually single, occasionally in pair V-shaped and irregular mass. Gram-positive. Non-motile, no flagellum and nonsporing.

Cultural;

1) Nutrient agar colony: Circular, smooth, entire, convex and milky-white to yellow
2) Nutrient agar slant: Moderate growth, filiform, glistening and milky-white to yellow. Medium was browned.
3) Nutrient broth: Moderate turbid, and pellicle was sometimes seen. Viscid yellow sediment, no odor. Amount of sediment was scanty.
4) Nutrient agar stab: Growth was scanty.
5) Gelatin stab: Growth occurred at top scantily. Line of puncture was villous, not liquefaction.

Physiological:

1) B.C.P. milk: Slightly acid production. Soft coagulation was formed. Coagulation by Lab enzyme was negative. No peptonization was observed after 7 days.
2) Nitrite: Produced from nitrate.
3) Indole: Not formed.
4) Acetyl methyl carbinol: Not formed.
5) Hydrogen sulfide: Produced.
6) Methyl red test: Negative.
7) Starch: Not liquefied.
8) Ureases: Present.
9) Catalase: Present.
10) Chromogenesis; Absent.
11) No acid-fast.
12) pH range: Optimum pH 6.0 to 8.5.
13) Relation to free oxygen: Aerobic.
14) Acid but no gas from arabinose, galactose, glucose and xylose.
15) Source: Isolation from soil.

Fig. 2. Electron Micrograph of Strain P145.

From these results, it was concluded that this strain belongs to genus *Brevibacterium*, making reference to the Bergey's Manual, 7th edition. From the comparative studies among the known species in the genus, the tested strain was similar to *Brevibacterium ammoniagenes* in physiological characteristics, except for the reaction to carbohydrates. The Bergey's Manual states that *B. ammoniagenes* does not produce acid and gas from carbohydrates. The tested bacterium, however, produced acid from arabinose, glucose, galactose and xylose. Thus it could be concluded that the strain P145 was a new species.

Nutritional requirement at 5° and 28°C

The nutritional requirement was determined at 5° and 28°C by the omission of amino acids, vitamins and bases from the complete medium. The results (Table III) show that at 28°C, in the basal medium the organism scarcely grew, but in the complete medium supplemented with amino acids, vitamins and bases, normal growth was observed. In the case of the omission of amino acids from the complete medium the growth occurred to the similar extent as in the basal medium. It appeared that the organism required some amino acids but not essentially required any vitamins or bases. On the other hand, at 5°C, there was very little difference of growth observed between basal and complete media, so the organism has no indispensable

TABLE III. NUTRITIONAL REQUIREMENT AT 5° AND 28°C

Complete medium contained bases, amino acids and vitamins in the basal medium. Base mixture contained 5 μg/ml each of adenine, guanine, uracil and xanthine. Amino acid mixture contained 2.5 mg/ml of casamino acid, 0.1 mg/ml of L-cysteine and 0.05 mg/ml of DL-tryptophan. Vitamine mixture contained 1 μg/ml each of thiamine HCl, riboflavin, pyridoxine HCl, nicotinic acid and Ca-pantothenate, and 0.2 μg/ml of *p*-aminobenzoic acid and 0.01 μg/ml each of folic acid and biotin.

Medium	Growth (OD at 610 mμ)	
	28°C (after 24 hr)	5°C (after 72 hr)
Basal medium	0.050	0.400
Complete medium	0.430	0.450
Complete medium - bases	0.425	0.432
Complete medium - amino acids	0.062	0.375
Complete medium - vitamins	0.450	0.470

TABLE IV. AMINO ACID REQUIRMENT AT 28°C

L-Form amino acids and glycine were added in concentration of 100 µg/ml and DL-form amino acids in 200 µg/ml.

Amino acid	Growth*(OD at 610 mµ) after 24 hr	
	Amino acid addition	Amino acid omission
(Basal medium)	0.055	
All amino acids tested	0.360	
DL-Aspartic acid	0.050	0.330
L-Glutamic acid	0.060	0.388
Glycine	0.050	0.355
L-Histidine	0.100	0.370
DL-Methionine	0.370	0.080
DL-Phenylalanine	0.045	0.360
DL-Valine	0.040	0.365

*Growth was expressed as OD at 610 mu of 2-fold dituted broth.

nutritional requirement.

From the results described above, it was recognized that the organism required some amino acids only at 28°C. Then, the amino acid requirement of the organism at 28°C was examined. It was observed that the growth was stimulated by the addition of DL-methionine to the basal medium and was depressed with the omission of the amino acid from the complete medium. Therefore, the organism indispensably required methionine at 28°C (Table IV).

TABLE V. GROWTH PROMOTING EFFECT OF VITAMINS AT 28°C

Concentration of vitamins added was the same as described in Table III.

Vitamin	Growth* (OD at 610 mµ)		
	Methionine no addition (24 hr)	Methionine addition (12 hr)	Methionine addition (24 hr)
(Basal medium)	0.000	0.200	0.460
Thiamine	0.000	0.370	0.460
Riboflavin	0.000	0.180	0.440
Pyridoxine	0.000	0.210	0.460
Nicotinic acid	0.000	0.205	0.457
Pantothenate	0.000	0.200	0.440
p-Aminobenzoic acid	0.000	0.198	0.420
Folic acid	0.000	0.230	0.430
Biotin	0.000	0.290	0.440

*Growth was expressed as OD at 610 mµ of 2-fold diluted broth.

The growth promoting effect of vitamins in the DL-methionine-supplemented synthetic medium was investigated at 28°C (Table V). Observation of growth after 12 hr revealed that the addition of thiamine and biotin stimulated the initial growth rate but that after 24 hr no difference in growth extent occurred in any media.

Effect of temperature on methionine requirement

The relationship between methionine requirement and cultural temperature was investigated in detail, employing the pulp-disc method. The agar used was throughly washed with water in order to prevent the contamination by organic and inorganic materials. As shown in Table VI, the methionine requirement was observed at temperatures higher than 28°C, whereas below 20°C the organism grew moderately without methionine.

The results presented thus far infer that a biochemical blocking occurred at certain step of the methionine biosynthesis at elevated temperatures above 28°C. The organism was precultured in the basal

TABLE VI. RELATIONSHIP BETWEEN METHIONINE REQUIREMENT AND TEMPERATURE

In order to test the growth response for DL-methionine, the pulp-disc method was employed. The pulp-disc soaked 2 mg/ml DL-methionine solution was placed on the basal agar plate which was smeared the washed cell suspension on the plate. In place of DL-methionine solution, deionized water was used as the control.

	Temperature (°C)					
	5	10	15	20	28	37
Control	+	+	+	+	-	-
Growth response for methionine	+	+	+	+	+	+

TABLE VII. EFFECT OF PRECULTURE CONDITIONS ON METHIONINE REQUIREMENT AT 5° AND 28°C

Cells grown in the precultured broth was washed three times with saline and suspended in the saline. One tenth ml of the cell suspension was inoculated to the assay medium. The preculture conditions were as follows; a) cultured at 28°C for 3 days, b) cultured at 28°C for 10 days, c) cultured at 28°C for 3 days and then maintained at 5°C for 7 days, d) cultured at 5°C for 10 days in the basal medium. Growth was measured after 72 hr with 5°C culture and after 24 hr with 28°C culture.

Culture temp.	Assay medium	Growth (OD at 610 mu) Precultural condition			
		(a)	(b)	(c)	(d)
5°C	Basal medium	.185	.120	.200	.280
	Methionine supplemented	.210	.180	.230	.310
28°C	Basal medium	.000	.000	.000	.062
	Methionine supplemented	.300	.210	.285	.320

medium at 5° and 28°C for various periods, and the washed cell suspenstions were inoculated into the fresh basal and methionine-added media. When the basal medium inoculated with the cells grown at 28°C was incubated, no growth occurred at 28°C. However, the incubation at 5°C revealed the normal growth even by using the 10 days culture at 28°C as the inoculum. While, employing cells grown at 5°C as the inoculum, the scanty growth was observed in the culture in basal medium at 28°C (Table VII). Therefore, it is suggested that the block of methionine biosynthesis at 28°C may be off at 5°C. These facts could be accounted as follows; certain enzymes for methionine biosynthesis may be reversibly inactivated at 28°C.

Identification of L-glutamic acid and L-alanine

L-Glutamic acid and L-alanine accumulated in the cultured broth were identified as described below. For the identification of L-glutamic acid, the manometric method using L-glutamic acid decarboxylase prepared from *Escherichia coli* (65) was

TABLE VIII. IDENTIFICATION OF L-GLUTAMIC ACID AND L-ALANINE

The L-amino acids were determined by paper chromatographic and biological methods. The biological methods were the manometric method using L-glutamic acid decarboxylase of *Escherichia coli* for L-glutamic acid and the microbiological method using *Leuconostoc citrovorum*, for L-alanine.

Sample	Assay method			
	Chromatographic		Biological	
	Glutamic acid (mg/ml)	Alanine (mg/ml)	L-Glutamic acid (mg/ml)	L-Alanine (mg/ml)
1*	5.50	0.72	5.20	1.00
2**	0.07	2.54	0.09	2.25

*Broth cultured at 5°C for 10 days.
**Broth cultured at 28°C for 4 days.

employed. For L-alanine, microbiological analysis using *Leuconostoc citrovorum* (66) was carried out. Results were summarized in Table VIII. The values obtained enzymatically or biologically coincided to those measured by paper chromatography.

Cultural condition

Some cultural conditions favoring the accumulation of L-glutamic acid at 5°C were examined.

1) Growth substance. Various growth promoting substances shown in Table IX were added to the basal medium. It was demonstrated that peptone and meat extract gave a considerable stimulative effect on both

TABLE IX. EFFECT OF GROWTH SUBSTANCE ON GLUTAMIC ACID FORMATION AT 5°C

Cultures were performed with addition of each growth substance (0.2%) to basal medium at 5°C for 8 days.

Growth substance	Growth (mg/ml)	Glutamic acid (mg/ml)
None	2.0	0.72
Peptone	3.8	3.90
Yeast extract	2.7	1.14
Meat extract	4.3	3.78
Casamino acid	2.8	1.88

growth and L-glutamic acid accumulation, whereas yeast extract and casamino acid did not show any noticeable effect.

2) Glucose concentration. The effect of glucose concentration in the medium on the amino acid accumulation and the growth was investigated. The results are shown in Table X. It was observed that the optimum concentration of glucose was 3% and

TABLE X. EFFECT OF GLUCOSE CONCENTRATION ON GLUTAMIC ACID FORMATION AT 5°C

Cultures were performed with the medium supplemented with 0.2% peptone at 5°C for 8 days.

Glucose (%)	Growth (mg/ml)	Glutamic acid (mg/ml)
3.0	4.3	5.88
5.0	3.8	4.08
7.0	1.2	0.86
10.0	1.4	1.77

that the higher concentration caused the lower yield of L-glutamic acid as well as the poor growth.

Effect of temperature

Five milliliters of the broth cultured in the basal medium for 3 days at 5°C was inoculated into 100 ml of the medium in a 500-ml flask. The culture was incubated at 5°, 15° or 28°C. As shown in Table XI, the highest extent of growth was obtained at 15°C. Although the maximum growth extent reached most rapidly at 28°C (after 2 days), the cell yields were lower than those at other temperatures. As regard to the amino acid accumulation

TABLE XI. EFFECT OF TEMPERATURE ON AMINO ACID FORMATION

Cultures were performed with the medium supplemented with 0.2% peptone.

Incubation temperature	Maximum cell yield (mg/ml)	(day)	Amino acid Glu* (mg/ml)	Ala** (mg/ml)	after (day)
5°C	5.2	4	5.88	0.38	8
15	5.7	3	1.88	1.00	6
28	4.0	2	0.21	2.54	4

*Glutamic acid, **alanine.

at 5°C, L-glutamic acid was mainly accumulated and L-alanine was only slightly, whereas at 28°C, the main product was L-alanine but not L-glutamic acid. At 15°C, both amino acids were accumulated in the same extent which was almost the mean of those observed at 5° and 28°C.

The time courses of amino acid accumulation at 5° and 28°C are presented in Fig. 3.

Effect of the addition of methionine, thiamine and biotin

As described previously, *Brevibacterium* sp. P145 requires methionine essentially, and thiamine and biotin accessorily.

Relationship between productivity of amino acid and nutritional requirement of the organism was investigated. In the experiment, the inoculum was prepared from the washed cells grown fully in the basal medium at 5°C. When DL-methionine (200 μg/ml) was added in the basal medium the growth was rather lower. Although the yields of the amino acids were lower compared with the control, main products were consistently L-glutamic acid at 5°C and L-alanine at 28°C. Biotin did not affect the amino acid formation. On the other hand, the L-alanine accumulation at 28°C seemed to be influenced in some extent by the concentration of thiamine added. If the excess thiamine, (5.0 μg/ml) was added to the basal medium supplemented with methionine, the rate of L-alanine accumulation decreased. In the case of thiamine deficiency, L-alanine was the main product of the culture at 28°C (Table XII). In the alanine accumulation, thiamine might play an important role. Tanaka and Kinoshita (67) reported with *Bacillus lentus* that alanine production increased with the suboptimum concentration of thiamine even if the biotin level was high. The fact that alanine was accumulated at 28°C by the strain P145 seemed to agree with the accessory requirement of thiamine at 28°C as the case of *B. lentus*.

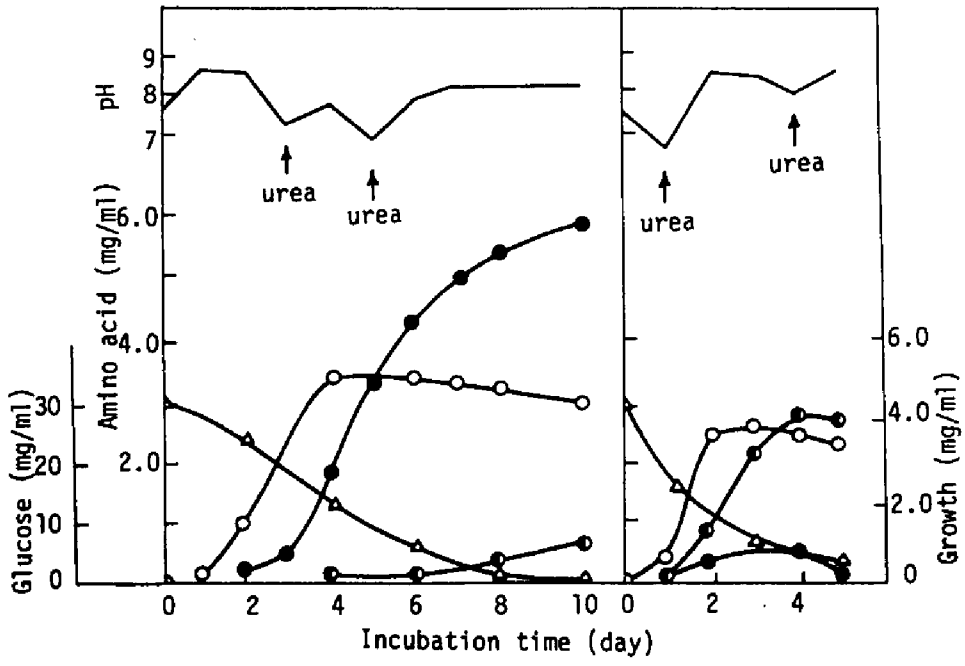

Fig. 3. Time Course of Amino Acid Formation at 5° and 28°C.

Medium contained 30 g of glucose, 4 g of urea, 1 g of K_2HPO_4, 2 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 2 g of peptone and 2 mg of phenol red in 1000 ml of tap water. (△); glucose, (●) glutamic acid, (◐); alanine, (○); growth.

TABLE XII. EFFECT OF METHIONINE, BIOTIN AND THIAMINE ON AMINO ACID FORMATION

The basal medium mentioned in Materials and Methods was symbolized by GU and the basal medium supplemented with DL-methionine was symbolyzed by GUM.

Basal medium	Addition	(μg/ml)	Culture condition: at 28°C for 4 days, Glutacic acid (mg/ml)	at 28°C for 4 days, Alanine (mg/ml)	at 5°C for 10 days, Glutamic acid (mg/ml)	at 5°C for 10 days, Alanine (mg/ml)
GU	(Basal medium)		(no growth)		0.70	tr
GU	DL-Methionine	20	tr	0.68	0.62	tr
GU	DL-Methionine	200	tr	0.67	0.90	tr
GU	DL-Methionine	2000	tr	0.72	0.80	tr
GUM	Biotin	0.005	tr	0.60	0.88	tr
GUM	Biotin	0.05	tr	0.72	0.60	tr
GUM	Biotin	0.5	tr	0.80	0.70	tr
GUM	Thiamine HCl	0.5	tr	0.60	0.98	tr
GUM	Thiamine HCl	1.0	tr	0.60	0.90	tr
GUM	Thiamine HCl	5.0	tr	tr	1.00	tr
GUM	Peptone	2000	0.60	2.34	4.61	0.70

tr: trace

Effect of abrupt temperature shift on amino acid formation

This series of experiments were made to establish whether, after temperature shifted, the amino acid accumulation by the organism was changed or not. *Brevibacterium* sp. P145 was cultured aerobically in the peptone-supplemented medium at 5° and 28°C. After these cultures reached

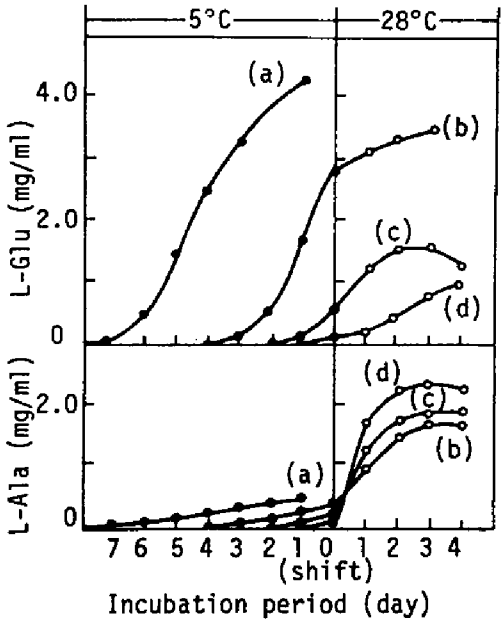

Fig. 4. Effect of Abrupt Temperature Shift from 5° to 28°C on the Formation of Amino Acid.

Medium was the same as described in Fig.3.
(a): Culture was performed at 5° for 8 days.
(b): Incubation temperature was shifted after 4 days culture at 5°C.
(c): Incubation temperature was shifted after 2 days culture at 5°C.
(d): Incubation temperature was shifted after 1 day culture at 5°C.

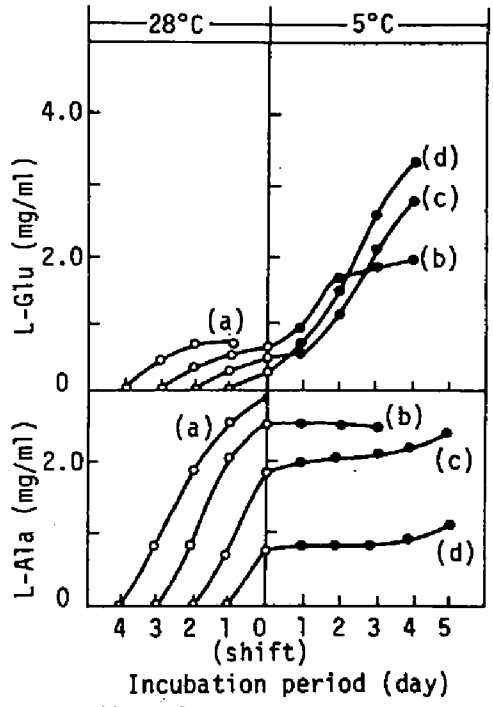

Fig. 5. Effect of Abrupt Temperature Shift from 28° to 5°C on the Formation of Amino Acid.

Medium was the same as described in Fig.3.
(a): Culture was performed at 28°C for 4 days.
(b): Incubation temperature was shifted after 3 days culture at 28°C.
(c): Incubation temperature was shifted after 2 days culture at 28°C.
(d): Incubation temperature was shifted after 1 day culture at 28°C.

the certain stages, the temperature was rapidly changed to 28° and 5°C respectively. Figures 4 and 5 represent the effect of the temperature shift from 5° to 28°C and 28° to 5°C on the accumulations of L-glutamic acid and L-alanine. Although L-alanine was scantily produced at 5°C, after the shift from 5° to 28°C the production of L-alanine occurred abruptly. As to L-glutamic acid, the accumulation continued even after the shift to 28°C, although the rate of the formation was considerably depressed comparing with the culture at 5°C. In the case of shift down (from 28° to 5°C) the formation of L-alanine stopped completely and L-glutamic acid was produced progressively. From these results, it is assumed that the conversion of main products by changing the incubation temperature reflects the substantial difference of metabolic processes at each temperature. Such alteration by incubation temperature is remarkable especially in L-alanine formation. It is presumed that the organism has a cold-sesitive step in the L-alanine biosynthesis.

Effect of temperature on L-glutamic acid and L-alanine dehydrogenases activities

In order to explain enzymatically the conversion of fermentation by incubation temperature, the presence and activity of L-glutamic acid and L-alanine dehydrogenases in the cells which might be the last steps of biosynthesis of L-glutamic acid and L-alanine, were examined. As shown

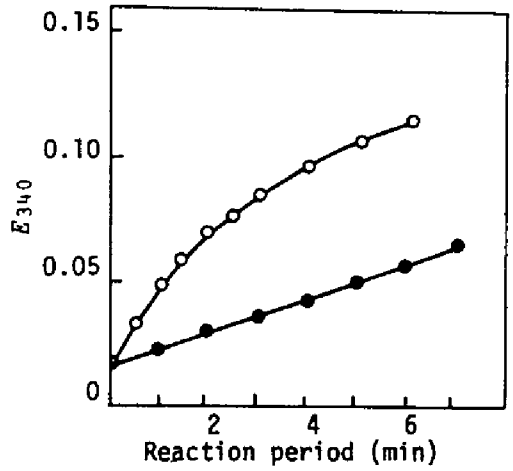

Fig. 6. L-Glutamic Acid Dehydrogenase Activity at 5° and 28°C.

Reaction mixture contained 50 μmoles of L-glutamic acid, 1 μmole of NADP, 200 μmoles of phosphate buffer (pH 8.3) and 3 mg (as protein) of enzyme in total volume of 3.5 ml. The enzyme was prepared from cells grown at 5°C.

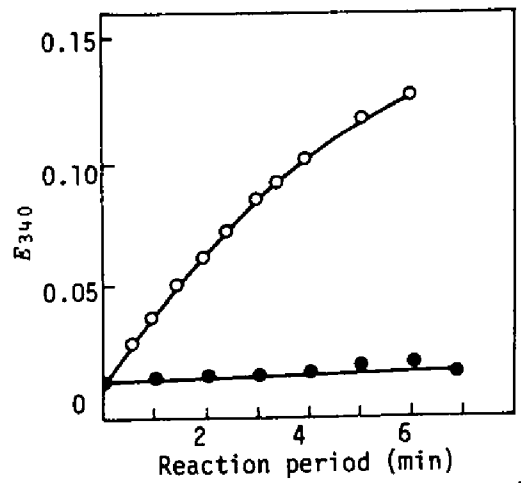

Fig. 7. L-Alanine Dehydrogenase Activity at 5° and 28°C.

Reaction mixture contained 50 μmoles of L-alanine, 1 μmole of NADP, 200 μmoles of phosphate buffer (pH 8.3) and 3 mg (as protein) of enzyme in total volume of 3.5 ml. The enzyme was prepared from cells grown at 5°C.

in Fig. 6, the dehydrogenase activity for L-glutamic acid was observed at both 5° and 28°C. On the other hand, L-alanine dehydrogenase activity was not apparent at 5°C (Fig. 7).

The stoichiometry of the reverse reactions in shown in Table XIII which demonstrates that the amount of reduced NADP is approximately equivalent to the amount of L-glutamic acid and L-alanine formed.

Using the cell-free extracts prepared from cells grown at 5° and 28°C, the spe-

TABLE XIII. STOICHIOMETRY OF L-GLUTAMIC ACID AND L-ALANINE DEHYDROGENASES

Oxidation of NADPH was measured by a decrease in OD at 340 mμ. Glutamic acid and alanine formation were determined by paper chromatographic method. Reaction mixture contained 50 μmoles of α-ketoglutarate or Na-pyruvate, 120 μmoles of $(NH_4)_2SO_4$, 0.5 μmole of NADPH, 200 μmoles of phosphate buffer (pH 7.8) and 3 mg (as protein) of enzyme in total volume of 3.5 ml. The reaction was carried out at 28°C for 10 min.

	Dehydrogenation of	
	L-Glutamic acid μmole	L-Alanine μmole
NADPH oxidized	0.40	0.32
Amino acid formed	0.36*	0.30**

*Glutamic acid, **alanine.

TABLE XIV. SPECIFIC ACTIVITIES OF L-GLUTAMIC ACID AND L-ALANINE DEHYDROGENASES AT 5° AND 28°C

Reaction mixtures were the same as described in Figs. 3 and 4.

Reaction temp. (°C)	Substrate	Enzyme from cells grown at 5°C coenzyme		Enzyme from cells grown at 28°C coenzyme	
		NAD	NADP	NAD	NADP
5	L-Glutamate	0.00	8.33	0.00	8.33
	L-Alanine	0.00	0.00	0.00	0.00
28	L-Glutamate	0.00	41.6	0.00	36.7
	L-Alanine	0.00	30.0	0.00	22.1

cific activities of both dehydrogenases were determined at 5° and 28°C. As for co-enzyme specificity, the enzymes were found to be specific for NADP in the dehydrogenations of both amino acids, but NADP could not be replaced by NAD (Table XIV).

As shown in Table XIV, regardless of the temperature of the cultivation, the cell-free extract showed the dehydrogenase activities at 28°C for both amino acids but at 5°C, L-alanine dehydrogenase activity was not observed. The extract from cells grown at 5°C showed L-alanine dehydrogenase activity in the reaction at 28°C but not at 5°C. It follows from these results that the organism is able to synthesize the enzyme at 5°C but the enzyme is not active at the low temperature. The results obtained at enzyme level are in accord with the information from cultural experiments (Fig. 4,5).

SUMMARY

An attempt has been made to isolate the bacteria capable of accumulating amino acids during the growth at low temperatures from various natural sources. A psychrophilic strain P145 forming glutamic acid at 5°C was obtained and identified as a *Brevibacterium* sp. P145. The bacterium grew in the range of 0° to 37°C and exhibited the optimum growth at 15°C. The bacterium was defined as a facultative psychrophile.

The strain strictly required methionine only above 28°C; below this temperature it grew normally without the amino acid. When methionine was added thiamine and biotin stimulated the growth of this strain at 28°C.

With the bacterium, the effect of incubation temperature on the extracellular amino acid accumulation has been examined from cultural and enzymological point of view. The strain was found to accumulate L-glutamic acid up to 5.88 mg/ml and L-alanine 0.38 mg/ml at 5°C, whereas it formed 0.21 mg/ml of L-glutamic acid and 2.54 mg/ml of L-alanine at 28°C.

The accumulation of L-alanine in the medium at 28°C seemed to be related to the thiamine requirement of the organism. In the case of thiamine deficiency, L-alanine was the main product in the culture at 28°C. When the incubation temperature was abruptly shifted from 28° to 5°C or from 5° to 28°C, the amino acid accumulation was also changed to that of the final temperature. L-Alanine dehydrogenase existed even in the cells grown at 5°C but was not active at this low temperature. These results were in accord with the informations obtained from cultural experiments.

CHAPTER II

ORGANIC ACID FORMATION BY A PSYCHROPHILIC BACTERIUM *PSEUDOMONAS* SP. B-71[c)]

INTRODUCTION

In previous chapter, the author has described on the formation of amino acids by a psychrophilic bacterium. During the course of the isolation of psychrophiles, a bacterium which was able to produce succinic acid at a low temperature was obtained. Further, it has been found that the organism was able to produce a different organic acid at higher temperatures. The author has therefore investigated the accumulation of organic acids at various temperatures by this psychrophilic bacterium and some factors contributing to the alteration in the products.

MATERIALS AND METHODS

Microorganism. The organism used principally in these experiments was a bacterium, strain B-71, which was isolated from the water of Lake Biwa.

Medium and cultivation. The medium for organic acid formation consisted of 7.0 g glucose, 0.4 g $(NH_4)_2SO_4$, 0.2 g K_2HPO_4, 0.1 g KH_2PO_4, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.2 g yeast extract, and 2.0 g $CaCO_3$ in 100 ml of tap water, pH 7.0. Fifty milliliters of the liquid medium was placed in a 500 ml shaking flask and inoculated with 2 ml of broth which had been sub-cultured in the same medium for 3 days at 10°C. Incubation was carried out on a reciprocal shaker (130 cycles per min) at the desired temperature.

Determination of growth. In order to dissolve the precipitate of $CaCO_3$ in 0.1 ml of 1 N HCl was added to 5 ml of culture broth and then the optical desity at 610 mμ was measured.

Paper chromatography. A small amount of Dowex 50 (H^+) was added to culture broth from which the cells had been previously removed by centrifugation, and then an aliquot of acidified supernatant was spotted on a piece of Toyo filter paper No. 53. Chromatographies were developed one dimentionally with the following solvent system: *n*-butanol-acetic acid-water (4:1:1, v/v), *n*-butanol-formic acid-water (4:1.5:1, v/v), ethanol-methanol-water (9:9:2, v/v), and *n*-butanol-pyridine-water (6:4:4, v/v) (68). The first two solvents were used for the identification of general organic acids, and the last two were used for 2-ketogluconic acid. The organic acids were located by spraying, using 0.2% bromophenol blue in 99% ethanol and 2-ketogluconic acid using 2% *o*-phenylenediamine dihydrochloride in 80% ethanol.

Analysis. The amount of succinic acid was determined manometrically using succinic dehydrogenase from the muscle of pig's heart according to Singer and Kearney (69). 2-Ketogluconic acid was determined by a spectrophotometric measurement of the complex formed between the acid and *o*-phenylenediamine according to the method of DeMoss (68). Protein was determined according to the method of Lowry *et al.* (63).

Preparation of cell extract. The cells in the culture broth (1000 ml) were harvested by centrifugation and were washed with 0.01 M potassium phosphate buffer, pH 7.0. The washed cells were suspended

in appropriate volumes of the above buffer and treated with a 19 kHz Kaijo-Denki ultrasonic oscillator for 10 min. The cells and debris were removed by centrifugation at 12,000 x *g* for 30 min. The resultant supernatant was used as the cell extract.

Measurement of succinate dehydrogenase activity. The succinic dehydrogenase in the organism was determined by a manometric method (70). The complete reaction system in a Warburg flask contained 300 μmoles of sodium succinate, 2 μmoles of methylene blue, 50 μmoles of potassium cyanide, 200 μmoles of potassium phosphate buffer (pH 7.0), enzyme and water to 2.3 ml, a filter paper wick laden with 0.2 ml of 40% KOH was placed in the center well. The activity was expressed as Q_{O_2} which was defined as the μliter of oxygen absorbed per mg protein during 1 hr.

Measurement of respiratory activity. Q_{O_2} values for glucose oxidation were determined by the manometrical techniques. Each flask contained 200 μmoles of glucose, 200 μmoles of potassium phosphate buffer (pH 7.0), cells or cell extract and water to 2.3 ml, and 0.2 ml of 40% KOH in the center well.

RESULTS

Isolation of an organic acid-forming psychrophile

Bacteria which were able to grow at 5° to 10°C were isolated from samples of soil, or from river or lake water. Among the 1000 isolates, one bacterium, strain B-71, showed a marked accumulation of an organic acid after cultivation at 10°C. This organism was used in the following experiments.

Taxonomic studies of the isolated bacterium, strain B-71

The results of taxonomic studies are summarized in Table 1. The strain was a Gram-negative, Kovacs' oxidase positive, asporogenous rod with a pollar flagellum which produced diffusible yellow pigment and fluorescent pigment and was oxidative in Hugh and Leifson's medium. On the basis of its properties, the organism was placed in the genus *Pseudomonas*: most of the characteristics of the organism are similar to those of *Ps. fluorescens* which are described in Bergey's Manual of Determinative Bacteriology, 7th edition,

TABLE 1. BACTERIOLOGICAL CHARACTERISTICS OF STRAIN B-71

1. Morphological	
Rod, average 0.5 to 1.2 μ, without endospore. Motile with a polar flagellum. Gram negative.	
2. Physiological	
Gelatin liquefaction	Positive
Action on milk	Alkaline
Indole formation	Negative
Nitrate reduction	Positive
H_2S formation	Positive
Catalase	Positive
Urease	Positive
Hugh and Leifson's medium	Oxidative
Kovacs' oxidase	Positive
Diffusible pigment	
King's medium A	Pale yellow
King's medium B	Fluorescent yellow
Production of acid from glucose, sucrose starch and dextrin.	

although some differences were seen in the carbohydrate assimilations. The organism was tentatively assigned the name *Pseudomonas* sp. B-71.

Growth temperature of the organism

Figure 1 shows the growth curves of the organism at various temperatures. The organism was able to grow over the temperature range from 0° to 35°C. The optimum temperature was found to be

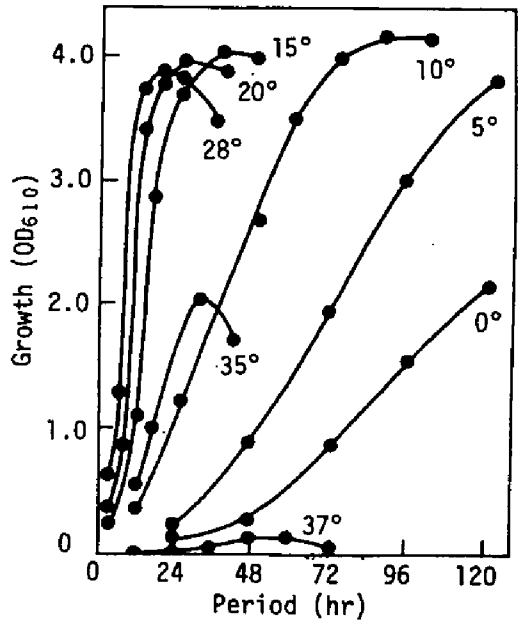

Fig. 1. Effect of Temperature on Growth of *Pseudomonas* sp. B-71.

The composition of the medium as follows: peptone, 1.0 g; yeast extract, 0.5 g; glucose, 0.5 g; NaCl, 0.5 g, in 100 ml of tap water, pH 7.0. Cultivation was carried out on a reciprocal shaker (130 rpm).

28°C. Therefore, the organism was classified as a facultative psychrophilic bacterium, according to the description of Stokes (12).

Determination of organic acid accumulation in culture broth at 10° and 28°C

The cultivation was carried out in a 2 liters flask containing 500 ml of medium at 10° and 28°C for 8 and 5 days, respectively. The culture broth was acidified to pH 2.0 with concentrated hydrochloric acid; cells of the broth were then removed by centrifugation. An aliquot of the broth was employed for the identification of organic acid by paper chromatography as described in Materials and Methods. The succinic acid spot on the paper chromatogram was detected clearly in the broth cultured at 10°C, while in the broth cultured at 28°C, a 2-ketogluconic acid spot, which was colored green with *o*-phenylenediamine, was found to have an identical *Rf* value with the authentic acid, but succinic acid could not be detected.

The organic acid in the broth cultured at 10°C was isolated by the usual ether-extraction methods. The melting points of the isolated acid and authentic succinic acid were 183° to 185°C and 184° to 185°C, respectively. The infrared spectrum of the acid agreed well with that of the authentic acid.

The broth cultured at 28°C was concentrated *in vacuo* at 50°C to syrup, followed by the addition of an appropriate volume of water and then to the solution was added a slight excess of oxalic acid. After filtering out the precipitate of calcium oxalate, excess oxalic acid was extracted out with ether. The aqueous layer was neutralized with 2 N potassium hydroxide and it was concentrated *in vacuo*. When the concentrate was permitted to stand at a low temperature, plate crystals were formed. The crystals were recrystallized from diluted ethanol solution. The melting points of the crystalline potassium salt and authentic potassium 2-ketogluconate were 148.5° to 152° and 149° to 152°C, respectively. The infrared spectrum of the product agreed well with that of authentic potassium 2-ketogluconate.

Effect of temperature on organic acid accumulation by Pseudomonas *B-71*

From the above data, it was established that the kind of organic acid accumulated varied with the incubation temperatures. Thus, the influence of temperature on organic acid accumulation was investigated. Table II shows the variation of the organic acid accumulation with temperature. In the broth cultured at 20°C, both succinic acid and 2-ketogluconic acid were present. Below this temperature, succinic acid was predominantly detected in the cultured broth; At 25° to 35°C, the main product was found to be 2-ketogluconic acid. The maximum accumulation of succinic acid and 2-ketogluconic acid were observed at 10° and 28°C, respectively. Figure 2 shows the time course for succinic acid formation at 10°C. The acid was accumulated up to 16.2 mg/ml for 8 days at this temperature.

TABLE II. EFFECT OF TEMPERATURE ON ORGANIC ACID FORMATION

The composition of the medium as follows; glucose, 7.0 g; NH_4Cl, 0.4 g; K_2HPO_4, 0.2 g; KH_2PO_4, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; yeast extract, 0.2 g; $CaCO_3$, 2.0 g; in 100 ml of tap water, pH 7.0.

Cultured at (°C)	Cultured for (day)	2-Ketogluconic acid (mg/ml)	Succinic acid (mg/ml)
5	14	-	15.0
10	10	-	16.2
15	10	-	8.4
20	8	7.0	6.0
28	7	25.0	-
30	5	24.2	-
35	5	12.0	-

-: Nondetectable.

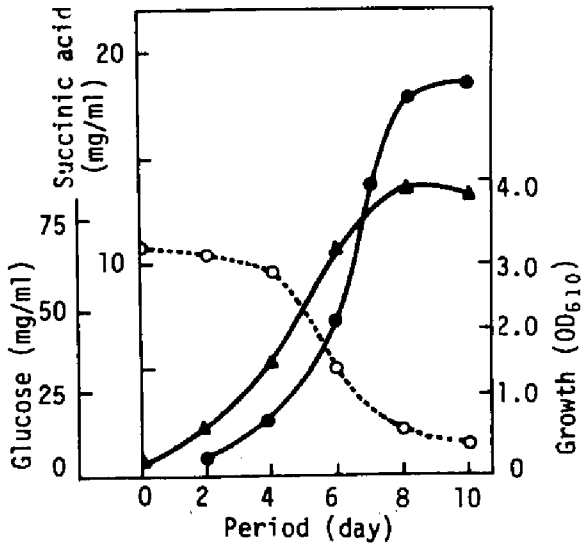

Fig. 2. Time Course for Succinic Acid Formation at 10°C.

Cultural conditions were described in Materials and Methods. (○), glucose; (▲), growth; (●), succinic acid.

Effect of aeration on organic acid formation

It is well known that succinic acid accumulation is influenced by aeration (71,72). The solubility of oxygen in water varies with temperature. The cultivation was carried out at several levels of aeration with a changing volume of medium in a 500 ml flask. The results are shown in Table III. In proportion to the increase

TABLE III. EFFECT OF AERATION ON ORGANIC ACID FORMATION AT 10° AND 28°C

The medium was the same as described in Table II. Cultivation was carried out on a reciprocal shaker at 130 rpm.

Volume in flask* (ml)	10°C		28°C	
	Succinic acid (mg/ml)	2-Keto-gluconic acid	Succinic acid	2-Keto-gluconic acid (mg/ml)
10	18.3	-	-	20.0
30	17.6	-	-	23.4
50	16.5	-	-	23.0
70	8.0	-	-	15.0
100	-	-	-	11.2
200	-	-	-	8.0

-: Nondetectable.
*Volume of medium in 500 ml flask.

in aeration, an increase was observed in the amount of succinic acid and 2-keto-gluconic acid at 10° and 28°C, respectively. However, alteration in the main product by changing the aeration was not observed.

Succinic acid formation by the intact cells

Succinic acid formation from various organic acids and glucose was investigated. The intact cell reaction was carried out under fully aerated conditions. The results are presented in Table IV. In the reactions at 10°C, succinic acid was formed from pyruvate, citrate, and α-ketoglutarate, but was scarcely formed at all from fumarate and malate. In the reaction at 28°C, succinic acid was only slightly formed from citrate and α-ketoglutarate, but was scarcely formed at all from fumarate and malate. In the reaction at 28°C, succinic acid was only slightly formed from citrate and α-ketoglutarate. No accumulation of this acid from malate or fumarate was observed under the conditions employed.

Effect of temperature on succinate dehydrogenase activity

TABLE IV. SUCCINIC ACID PRODUCTION FROM VARIOUS ORGANIC ACID BY INTACT CELLS

The reaction mixture in a test tube (25 x 200 mm) consisted of each organic acid or glucose, 100 μmoles; sodium phosphate buffer (pH 7.0), 300 μmoles; cells, 100 mg, water to a total volume of 10 ml. The cells used in the reaction were obtained by culturing at each corresponding temperature. The reaction was carried out on a reciprocal shaker (130 rpm) at 10 and 28°C for 5 and 2 hr, respectively.

Substrate	Succinic acid formed	
	at 10°C (μmoles/ml)	at 28°C (μmoles/ml)
Glucose	4.0	-
2-Ketogluconic acid	-	-
Pyruvate	8.3	-
Citrate	10.2	0.7
α-Ketoglutarate	21.0	1.2
Fumarate	0.8	-
Malate	0.9	-

-: Nondetectable.

From the above results, it was expected that the activity of succinate dehydrogenase would be reduced at low temperatures. Therefore, the activity was determined at 10° and 28°C. The results are presented in Table V. The specific activity (Q_{O_2}) of the enzyme in cells grown at 10°C was considerably lower than that in cells grown at 28°C, at both reaction temperatures.

TABLE V. EFFECT OF GROWTH TEMPERATURE ON SUCCINATE DEHYDROGENASE ACTIVITY

The reaction was carried out as described in Materials and Methods.

Cells grown at	for	Q_{O_2} Reacted at 10°C	Q_{O_2} Reacted at 28°C
10°C	3 days	0.03	0.11
10	5	0.04	0.10
28	3	5.5	12.5
28	5	6.0	13.5

The results presented thus infer that at low temperature the formation of the enzyme is depressed.

Glucose oxidation by intact cells and cell extract

In order to determine whether the nature of the cell itself was altered by the incubation temperature, the temperature dependence of the glucose oxidation of the whole cell and of its extract were investigated. Intact cell suspensions were obtained by culturing the organism at 10°

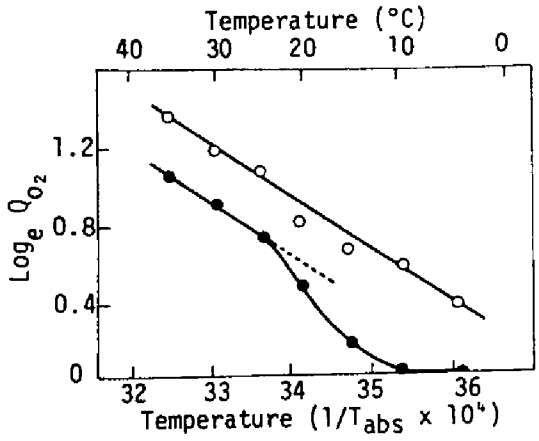

Fig. 3. Comparison of the Effect of Temperature on the Activity of Glucose Oxidation by Intact Cells Grown at 10° and 35°C.

The detailes of the reaction were described in Materials and Methods.
(○), cells grown at 10°C; (●), cells grown at 35°C.

or 35°C. The rate of glucose oxidation by these intact cells was measured at 5° to 35°C. Arrhenius plots of the rate of glucose oxidation by the intact cells are shown in Fig. 3. The graph obtained for the cells grown at 10°C is linear over the temperature range from 35° to 5°C, whereas the plots of the cells grown at 35°C deviated from linearity at temperatures below 25°C. When cells were broken, any differences in the temperatures dependence of glucose oxidation between the cells cultured at 10° and 25°C disappeared. From these data, it might be thought that an alteration of the biochemical organization of the organism was caused by changing the culture temperature, in addition to the change in the enzyme activity or in its formation.

DISCUSSION

It has been reported that 2-ketogluconic acid was produced by certain species of *Pseudomonas* (73, 74). The psychrophilic *Pseudomonas* sp. B-71 was found to be able to produce a considerable amount of 2-ketogluconic acid when cultured at optimum or superoptimum temperatures. Whereas, at the suboptimum temperatures, below 20°C, no accumulation of this acid was observed, and succinic acid was predominantly produced. Lewis *et al.* (75) and Koepsell (76) showed that one strain of *Ps. fluorescens* which oxidized glucose to 2-ketogluconic acid, on further oxidation produced pyruvic acid or α-ketoglutaric acid as major products. In the case of *Pseudomonas* sp. B-71, however, no accumulation of pyruvic acid and α-ketoglutaric acid or succinic acid from 2-ketogluconic acid was detected in the intact cell reactions at 28°C. From these data, this conversion of fermentative products by culture temperature might be said to be the result of some alteration in the metabolic systems due to temperature. As one of the alterations a considerable reduction of succinate dehydrogenase activity at lower temperatures was observed. The phenomenon was explained on the basis of the depression of the enzyme formation at low temperature. Okada *et al.* (71) studied the conversion from glutamic acid to succinic acid production by *Brevibacterium flavum* and suggested that the specific activity of succinate dehydrogenase in the succinic acid producing cells was considerably reduced, compared with that in glutamic acid producing cells. Thus, the reduction of the enzyme activity of *Pseudomonas* sp. B-71 in low temperature cultivation seems to be one of the factors influencing the succinic acid formation at this temperature.

The rate of glucose oxidation of cells grown at 30°C was depressed at suboptimum temperatures for growth, compared with that of cells grown at 10°C, but this difference disappeared in the reaction with cell extracts. It has so far been reported that temperature coefficient differences between the whole cells of mesophiles and psychrophiles have been found for catabolic processes, such as glucose oxidation and so on (24 ~ 28). Ingraham and Bailey (24) suggested that these phenomena might be due to differences in some aspects of the biochemical organization of the organism, such as the biomembrane. Baxter and Gibbons (25) suggested that the main factors determining the temperature characteristics of the mechanism for transporting solutes into the cells. Further, some informations with respect to alterations in the fatty acid composition of microbes by culture temperature have been accumulated (30 ~ 33). It might be thought that the conversion of a fermentative product by culture temperature may be partly attributed to the alteration of the nature of whole cells. The details of this problem are a subject for future study.

SUMMARY

A psychrophilic bacterium which formed succinic acid at 10°C was isolated from the water of Lake Biwa. The organism was classified as belonging to the genus *Pseudomonas*. This bacterium was able to grow over the temperature range from 0° to 35°C, and its optimum temperature was found to be 28°C. When the organism grew at the optimum temperature, accumulations of both succinic acid and 2-ketogluconic acid was predominantly accumulated, while at suboptimum temperatures, only succinic acid was accumulated. Of factors influencing the conversion of product by the temperature, the activity of succinate dehydrogenase in the cells grown at low temperature was found to be reduced. On the other hand, information regarding the alteration of the biochemical nature of cells by culture temperatures were obtained.

CHAPTER III

FORMATION AND CHARACTERIZATION OF PROTEASES FROM A MARINE-PSYCHROPHILIC BACTERIUM

SECTION 1

PROTEASE FORMATION BY A MARINE-PSYCHROPHILIC BACTERIUM, *PSEUDOMONAS* SP. No. 548[d)]

INTRODUCTION

Numerous psychrophilic or marine bacteria have been known to produce proteases which are found in culture fluid (45, 77 - 82). Among these enzymes, alkaline protease from an obligate psychrophilic bacterium (80) and aminopeptidase from a marine bacterium, *Aeromonas proteolytica* (82) have been hitherto isolated in ultracentrifugally and electrophoretically homogeneous forms. The physico-chemical and enzymatic properties of the purified proteases have been reported. However, most of the other reports of protease from psychrophilic bacteria are concerned primarily with the detection of enzymatic activities or with the general properties of partially purified enzymes. Thus, detailed information on the characteristics of proteases of psychrophilic or marine microorganisms is limilted.

During the course of studies on the physiological characteristics of psychrophilic bacteria isolated from marine or terrestrial samples, it was shown that proteolytic activities were found in the culture fluids of numerous organisms. Among these organisms, a marine psychrophilic bacterium, strain No. 548, showed the highest proteolytic activity. The properties of the protease will be described in this chapter, the present section being concerned with some specific cultural conditions, such as the effects of the salts in sea water or culture temperature on this strain.

MATERIALS AND METHODS

Organism. A marine psychrophilic bacterium strain No. 548, which was isolated from sea water collected in Sagami Bay, Japan, was principally used throughout this work. The procedure of microbial isolation from marine materials is described in Chapter IV.

Materials. The Hammersten quality casein and ferric phosphate soluble were products of E. Merck AG, Darmstadt. The DEAE-cellulose was a gift from Green Cross Corporation, Osaka, Japan. All other chemicals used were commercial products.

Media

a) Isolation media. ZoBell's medium, composed of 0.5 g peptone, 0.1 g yeast extract, 0.01 g ferric phosphate soluble, and 1.5 g agar in 100 ml of artificial sea water, pH 7.4, was used for the isolation of marine bacteria. Nutrient agar,

which was composed of 0.1 g peptone, 0.5 g meat extract, 0.5 g NaCl, and 2.0 g agar, in 100 ml of tap water, pH 7.0, was used for the isolation of terrestrial bacteria.

b) Media for protease formation. ZoBell's medium except agar, was used for the screening of marine bacteria for protease formation; a medium composed of 0.1 g peptone, 0.3 g yeast extract, 0.5 g glucose, and 0.5 g NaCl, in 100 ml of tap water, pH 7.0, was used for screening the terrestrial bacteria.

A liquid medium, composed of 2.0 g bean-meal, 1.0 g meat extract, and 0.5 g yeast extract, in 100 ml of artificial sea water, pH 7.0, was used for protease formation by strain No. 548. When growth was being measured, bean-meal extract was used in place of the meal in the medium. The extract was prepared by extracting 2 g bean-meal with 100 ml sea water at 120°C for 20 min.

The medium used for the culture of marine bacteria was prepared with artificial sea water of the following composition: NaCl, 23.5 g; KCl, 0.66 g; $CaCl_2 \cdot 2H_2O$, 1.1 g; Na_2SO_4, 3.91 g; KBr, 0.01 g; $SrCl_2$, 0.024 g; $NaHCO_3$, 0.19 g: $MgCl_2 \cdot 6H_2O$, 4.98 g; H_3BO_3, 0.03 g and 100 ml of deionized water (83).

Cultivation. Fifty milliliters of the liquid medium was placed in a 500 ml shake-flask and inoculated with 2 ml of a cell suspension in sterile sea water, freshly prepared from a slant culture. Incubation was carried out on a reciprocal shaker (130 rpm) at the desired temperature.

A Jar fermenter scale of cultivation was carried out under the following conditions: equipment, 30 liters Waldhof type jar fermenter; medium volume, 13 liters; revolution, 250 rpm; aeration, 1 v/v/m (in 0.6 atm.); and inoculum size, 1 liter.

Estimation of proteolytic activity.

To 1 ml of 2% casein solution (pH 7.5) was added 1 ml of enzyme solution which was appropriately diluted with 0.05 M Tris-HCl buffer containing 0.01 M calcium acetate, pH 7.5. After 10 min of incubation at 37°C, the reaction was stopped by the addition of 2 ml precipitation-reagent B (84), followed by 20 min incubation at 37°C. The liberated tyrosine in the filtrate of the resultant mixture was estimated by Folin-Ciocalteau's reagent (85). The absorbancy at 660 mμ was read using a Hitachi 101 spectrophotometer. In the case of screening for protease formation, the optical density of the filtrate was directly measured at 280 mμ. An unit of proteolytic activity was defined as the enzyme quantity which liberated 1 μg of tyrosine per ml of reaction mixture per minute under standard conditions.

RESULTS

Screening for protease-producing psychrophiles

About 670 cultures isolated from terrestrial materials and 348 cultures from marine environments were used in the experiment. The organisms were cultured in 5 ml of each medium in a test tube, with shaking, at 5°C. After 7 days cultivation, proteolytic activities in the culture fluids were estimated by the digestion of casein at 37°C for 30 min. A brief description of the distribution of the strains which produce protease during cultivation at 5°C is given in Table I.

TABLE I. DISTRIBUTION OF PROTEASE-FORMING BACTERIA AT 5°C

Cultivation was carried out at 5°C for 7 days. Protease activity was estimated by the method described in Materials and Methods.

Protease activity in broth (OD at 280 mμ)	Terrestrial organism (670 strains)	Marine organism (340 strains)
0 - 50	93.7%	87.6%
50 - 150	0.4	1.7
150 - 250	4.1	11.1
250 <	1.6	3.5

Higher proteolytic activities were found in the culture fluid of marine bacteria than in that of terrestrial bacteria. Significant proteolytic activity was observed during cultivation of one particular marine bacterium, strain No. 548. The strain was used in the following experiment.

Taxonomic studies of the isolated bacterium, strain No. 548

The taxonomic studies were conducted for the most part according to Shewan's method (86) which is applicable only to Gram-negative bacteria from marine environments. The results are summarized in Table II and Fig. 1. The strain was Gram-negative, Kovacs' oxidase (87)

TABLE II. BACTERIOLOGICAL CHARACTERISTICS OF STRAIN 548

1. Morphological: Rod, average 0.7 by 1.5 μ. Without endospore. Motile. Polar flagellum. Gram-negative.	
2. Physiological:	
Gelatin liquefaction	Positive
Action on milk	Acid coagulation
Indole formation	Positive
Nitrate reduction	Negative
H_2S formation	Positive
Catalase	Positive
Urease	Positive
Hugh and Leifson's medium	Oxidative
Kovacs' oxidase	Positive
Difusible pigment	
King's medium A	Thin reddish brown
King's medium B	Non-fluorescent yellow
Production of acid from glucose, sucrose, starch, dextrin.	

positive, an asporogenous rod with a polar flagellum, which produced diffusible pigments and was oxidative in Hugh and Leifson's medium (88). On the basis of the above characteristics, the organism was placed in the genus *Pseudomonas*, and then tentatively assigned the name *Pseudomonas* sp. No. 548. The bacterium does not appeared to be identical to any described in Bergey's Manual of Determinative Bacteriology (89).

Requirement of salt in sea water for growth and protease formation

Marine bacteria have been defined as bacteria from the sea which on initial isolation require for growth a medium containing sea water (90). *Pseudomonas* sp. No. 548 did require sea water for growth when freshly isolated. Even after 2 years of storage, the organism failed to grow in a medium with a greatly reduced salt content.

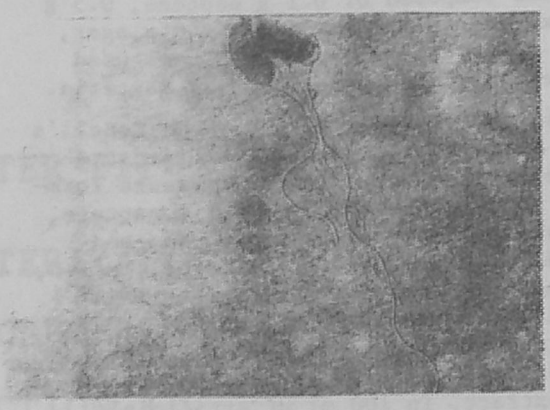

Fig. 1. Electron Micrograph of Strain 548.

The effect of varying levels of sea water on growth and protease formation was examined. The results are shown in Table III. The organism was able to grow in media containing over 25% sea water. Proteolytic activity was detected in the culture fluids when a medium containing over 50% sea water was used. Both the growth and the enzyme activity were at a maximum in a medium containing sea water at full strength. The ratio of protease activity to growth increased directly with an increase in the percentage of sea water in the medium. After being cultured in a medium of reduced salt content (50 to 75%) for 4 days, the growth reached the same extent as in the culture with sea water at full strength, but the ratio of activity to growth did not increase.

Effect of temperature on growth and protease formation

Figure 2 shows time course for protease formation and growth curves at various

TABLE III. EFFECT OF SEA WATER CONCENTRATIONS ON GROWTH AND PROTEASE FORMATION

The medium was the same as described in Fig. 2. Cultures were carried out at 10°C.

Sea water concentration (%)	Growth (OD at 610 mμ) after		Protease activity (OD at 660 mμ) after		Activity/Growth after	
	2 days	4 days	2 days	4 days	2 days	2 days
0	0.00	0.00	0.00	0.00	-	-
5	0.00	0.00	0.00	0.00	-	-
10	0.00	0.00	0.00	0.00	-	-
25	0.72	1.20	0.00	0.03	0.00	0.03
50	1.89	2.40	1.66	2.10	0.88	0.88
75	2.20	2.39	3.39	4.30	1.78	1.80
100	2.43	2.62	5.43	5.85	2.22	2.24

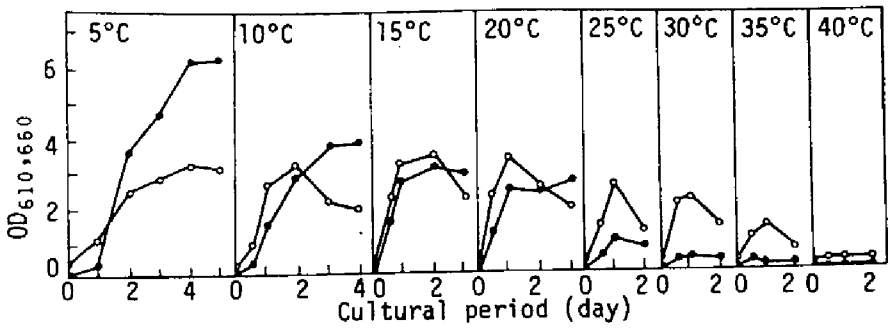

Fig. 2. Effect of Temperature on Growth and Protease Formation.

The composition of the medium was as follows: bean-meal extract, 0.2 g (as bean-meal); meat extract, 1.0 g; and yeast extract, 0.5 g, in 100 ml of artificial sea water, pH 7.0. ○ represents growth and ● represents protease activity.

temperatures. The optimum temperature for both the rate and extent of growth was found to be 20°C. At 5°C, the maximum extent of growth was observed after 3 to 4 days cultivation. No growth occurred above 40°C. Formation of enzyme was greatest at 5°C and decreased with increasing temperature until the smallest quantity of enzyme was found in broth cultured at 35°C. Thus, the optimum temperature for protease formation by the organism was a great deal lower than that for growth.

Figure 3 shows the relationships between proteolytic activity and growth at various temperatures; approximately linear relationships exist between the protease activity and extent of growth. The slope of the linear portion of the curves becomes more steep as the temperature decreases.

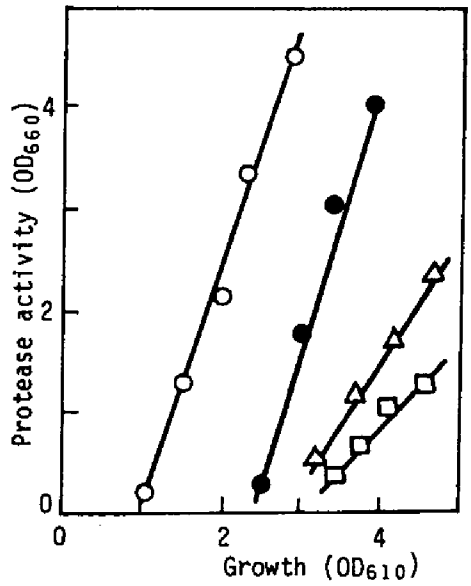

Fig. 3. The Relationship between Protease Formation and Growth at Various Temperatures.

The medium was the same as described in Fig. 2. Cultivation was carried out at 5°C (○), 10°C (●), 20°C (△), and 25°C (□).

Protease formation in resting cell suspension

Resting cell suspensions were obtained by growing the organism at 10° or at 28°C. Cells were harvested by centrifugation and washed three times with sea water.

TABLE IV. PROTEASE FORMATION IN RESTING CELL SUSPENSION

The cells were obtained by cultivation at 10°C for 2 days and at 28°C for 1 day, respectively. Incubation mixture: Glucose, 150 mg; $(NH_4)_2SO_4$, 30 mg; cells, 40 mg; and sea water, 5.0 ml (pH 7.0).

Temperature		Protease activity (OD at 660 mμ)			
Culture	Reaction	Reaction period (hr)			
		0	3	6	12
10°C	10°C	0.14	1.28	2.20	3.05
10	28	0.14	0.26	0.00	0.00
28	10	0.27	0.40	0.38	0.32
28	28	0.27	0.35	0.00	0.00

Using these cell suspensions, the protease formations were carried out at 10° and 28°C, respectively. Results are shown in Table IV. When the protease formation was carried out at 10°C using cells grown at 10°C, a remarkable activity was detected in the resultant fluid.

Effect of aeration on protease formation

The solubility coefficient for O_2 in water is 0.026 at 30°C and 0.038 at 10°C,

TABLE V. EFFECT OF AERATION ON PROTEASE FORMATION

Medium: 2.0 g of bean-meal, 1.0 g of meat extract, and 0.5 g of yeast extract in 100 ml of sea water, pH. 7.0.

Medium volume* (ml)	Protease activity (OD at 660 mμ) 10°C after (day) 2	5	25°C after (day) 1	2
20	2.94	7.20	2.15	1.55
50	2.93	6.78	1.10	0.52
100	3.39	5.28	0.08	0.80
200	2.97	5.18	0.81	0.65

*Medium volume in 500 ml flask.

or 46% greater. Therfore, the availability of O_2 to microorganisms is influenced by the cultural temperature. To determine the effect of aeration and temperature, cultivation was carried out at several levels of aeration with changing volumes of medium in a 500 ml-flask. The results are shown in Table V. In proportion to increasing aeration, an increase was observed in the amount of enzyme formation at 25°C as well as at 10°C. However, the aeration seems not to be the only important factor in the lower yields of protease at higher temperatures.

Column chromatography of the protease on DEAE-cellulose

The enzyme solution to be applied was prepared as follows. After centrifugation of the broth, the supernatant was mixed with four volumes of cold acetone and kept 1 hr in the cold. The resulting precipitate was suspended in 5 ml of 0.05 M Tris-HCl buffer containing 0.01 M calcium acetate, pH 7.5, and dialyzed over-night against the same buffer. The dialyzed solution was applied to a column of DEAE-cellulose (1.8 X 22 cm), equilibrated with the buffer. An elution by the buffer was began, and a further elution was carried out by a stepwise increase in the NaCl concentration.

The proteases of the organism were fractionated by column chromatography on DEAE-cellulose. Examples of the results are shown in Table VI. The proteases of the culture fluid were divided into two active fractions. The protease fraction initially eluted by 0.1 M NaCl was designated as Fraction I; the fraction eluted by 0.3 M NaCl, as Fraction II. The two proteases were found in the broths cultured at 10° to 25°C; the activity ratios of Fraction I to Fraction II varied with the culture temperature. The individual ratios were 3:1, 6.5:1 and 12:1 in the broths cultured at 10°, 20° and 25°C, respectively. Increasing temperature tends to decrease the accumulation of Fraction II.

TABLE VI. FORMATION OF PROTEASE FRACTIONS I AND II AT VARIOUS TEMPERATURES

Cultivation was carried out by using a mini-jar apparatus (medium volume, 1000 ml). The method of fraction by DEAE-cellulose chromatography is described in the text.

Temperature	Protease activity (total unit/liter) Fraction I	Fraction II
5°C	810,000	270,000
20	140,000	22,000
25	58,000	4,460

Effect of components of medium on protease formation

An investigation into the components of the medium which favor the formation of protease was made. Results are shown in Table VII. The medium (E) containing bean-meal was suitable for enzyme formation. Furthermore, the optimum concentration of bean-meal was observed to be 2.0 %. Compared with the case of medium

TABLE VII. EFFECT OF MEDIA ON PROTEASE FORMATION

Cultivation was carried out at 10°C for 6 days.

Medium composition (in 100 ml sea water)	Protease activity (OD at 660 mμ)
A Glucose, 5 g; $(NH_4)_2SO_4$, 0.8 g; yeast extract, 0.2 g.	2.43
B Starch, 5 g; Na-glutamate, 0.8 g; yeast extract, 0.2 g.	4.44
C Peptone, 5 g; yeast extract, 0.4 g.	5.53
D Peptone, 1 g; meat extract, 0.5 g; yeast extract, 0.3 g.	5.10
E Bean-meal, 2.0 g; meat extract, 0.5 g; yeast extract 0.3 g.	15.2
F Bean-meal, 1.0 g; meat extract, 1.0 g; yeast extract, 0.5 g.	9.5
G Bean-meal, 3.0 g; meat extract, 1.0 g; yeast extract, 0.5 g.	10.8
H Bean-meal, 4.0 g; meat extract, 1.0 g; yeast extract, 0.5 g.	10.1
I Bean-meal, 2.0 g; meat extract, 0.2 g.	11.0

(E), the addition of yeast extract only resulted in a decrement of protease by 15%. However, medium I, of which the components were more simplified, was used in further experiments, in the interest of the ease of purification of the enzyme.

Time-course of proteases formation at various temperatures on a jar-fermenter scale of cultivation

A cultivation was carried out with a 30 liters jar-fermenter at 5°, 10° and 28°C. Results are shown in Fig. 4. Maximum yields of protease were obtained by cultivation for 5 and 3 days, at 5° and

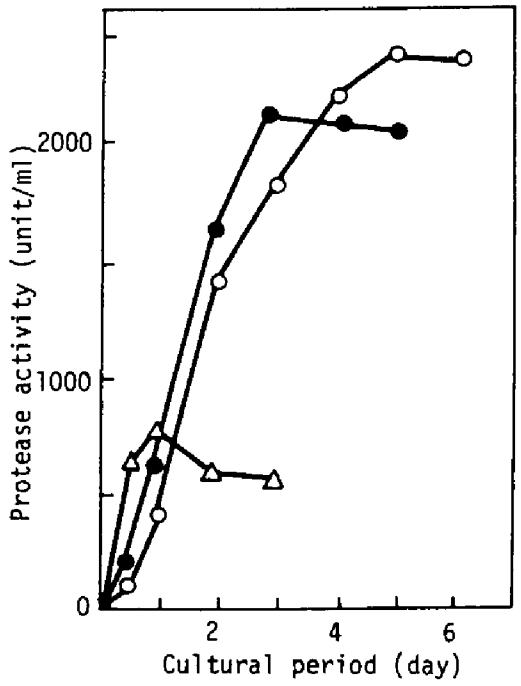

Fig. 4. Protease Formation during a Jar Fermenter Scale Cultivation.

The medium was as follows; 2.0 g of beanmeal and 0.2 g of yeast extract in 100 ml of sea water, pH 7.0. Cultural conditions are described in the text. Cultivation was carried out at 5°C (○), 10°C (●), and 28°C (△).

10°C, respectively. Under these conditions, the protease activity found in broth cultured at 10°C was almost the same as that in broth at 5°C. In the broth cultured at 28°C, little activity was found, as in the case of a flask-culture.

DISCUSSION

In view of the origins of the organism, the physiological properties of marine bacteria might be expected to be more extremely affected by various inorganic ions in comparison with terrestrial bacteria. Drapeau *et al.* (91) observed that a marine pseudomonad required sodium ion, at relatively high levels, for the transport of various organic substances. On the other hand, it is known that some terrestrial organisms require cations for extracellular enzyme production. The accumulation of extracellular alkaline phosphatase of *Micrococcus sodonensis* is the result of a selective permeation process and is totally dependent upon the presence of divalent cations (92). Morihara (93, 94) reported that calcium ion was required from a precursor substance in some steps of the enzyme synthesizing reaction of *Pseudomonas myxogenes*. In the case of *Pseudomonas* sp. No. 548, the ratios of proteolytic activity to growth increased with a higher content of sea water in the media. It is assumed that a relatively high concentration of the salts was required for the actual synthesis or release from the cell (or both) of protease.

The optimum temperature of protease formation by strain 548 was much lower than that of growth. Peterson and Gunderson (45) reported that the extracellular protease elaboration by *Pseudomonas fluorescens* was shown to be inversely proportional to the culture temperature. This phenomena has been explained on the basis of the thermolability of the enzyme. As shown in next section, the proteases of *Pseudomonas* sp. No. 548 were stable at near 30°C in a buffer containing 0.01 M calcium ion; the concentration of calcium ion was at the same level as in sea water.

Sinclair and Stokes (50) reported that the higher cell yields at the lower temperature were due simply to the increased solubility and, therefore, availability of O_2. The protease activity in the culture fluids of strain No. 548 increased with an increase in aeration at 25°C. However the increments by aeration were not so great as those obtained by the lowering of the culture temperature. This factor has some influence on the accumulation of the protease at each temperature, but seems not to be the only important factor influencing the higher yields of protease at lower temperatures.

Based on the results of the protease formation in resting cell suspension, it would appear that growth at a low temper-

ature was necessary for protease accumulation by the organism. It is assumed that the variation brought about by changing the culture temperature reflects a substantial difference in the organization of the cell and/or metabolic processes at each temperature.

SUMMARY

Proteolytic activity was found in the culture fluids of numerous psychrophilic bacteria isolated from terrestrial or marine samples. Among these organisms, a marine psychrophilic bacterium *Pseudomonas* sp. No. 548, showed the highest proteolytic activity. This organism required salts of sea water for both growth and protease formation. The optimum temperature for the growth of this organism was 20°C. The formation of protease was the greatest at 5°C and decreased with increasing temperature. The extracellular protease system was fractionated into at least two components having proteolytic activities by chromatography with DEAE-cellulose. Increasing culture temperature tended to increase the activity ratio of Fraction I to Fraction II. Some cultural conditions for protease formation were investigated.

SECTION 2

PURIFICATION AND PROPERTIES OF PROTEASES FROM *PSEUDOMONAS* SP. No. 548[e)]

INTRODUCTION

Production of extracellular proteases by an obligate psychrophilic bacterium, and purification and characterization of the enzymes have been reported by McDonald *et al.* (78, 80). Prescott *et al.* showed that a marine bacterium, *Aeromonas proteolytica*, produced an aminopeptidase (82, 95). The aminopeptidase has been purified and characterized. Crystalline preparations of alkaline protease and elastase have been reported to be obtained from *Pseudomonas aeruginosa* by Morihara *et al.* (96, 97) and these enzymes have been well characterized. In a preceding section, it was shown that a marine-psychrophile, *Pseudomonas* sp. No. 548, accumulated protease in broth cultured at a low temperature.

The present section describes the steps in the purification of the proteases and the general properties of the purified enzymes.

MATERIALS AND METHODS

Organism. A marine psychrophile, *Pseudomonas* sp. No. 548, was used in this work.

Materials. Diisopropylfluorophosphate was purchased from Sigma Chemical Company, U.S.A. The colodion bag was a product of the Sartorius Membrane Filter Company, W. Germany.

Estimation of protease activity. Protease activity in the culture fluid or purified enzyme preparation was estimated by the digestion of casein at 37°C. The method was the same as described in previous section.

Protein determination. The protein was determined spectrophotometrically by measuring its absorbance at 280 mμ.

Acrylamide gel electrophoresis. The electrophoresis was carried out according to Davis (98). After electrophoresis, the gel was stained with coomassie blue (99).

RESULTS

Purification of proteases

Pseudomonas sp. No. 548 was inoculated into a 2-liter jar fermenter containing 1 liter of the medium described under the preceding section. The subculture was carried out at 7°C for 48 hr under aeration. This subculture was inoculated into a 30-liter jar fermenter containing 14 liters of the medium; cultivation was carried out at 7°C for 60 hr under aeration (1 v/v/m, in 0.6 atm.). The cells and residue of bean-meal were removed from the culture broth by a continuous-flow centrifuge. The resulting clear supernatant was used as the starting material.

Step 1. Tannin precipitation. The procedure of this step was carried out according to Bergkvist (100). The bulk of the protein was precipitated by slowly adding a 10% solution of tannin to the final concentration of 50 g of tannin per 1000 ml of the supernatant. After settling for 3 hr, the resulting precipitate was isolated by filtration. In order to remove tannin, the precipitate was washed by centrifugation three times with 80%

cold acetone. The final precipitate was dissolved in 1000 ml of 0.01 M calcium acetate solution. The enzyme solution was dialyzed for 24 hr against two changs of 20 liters of 0.01 M calcium acetate solution.

Step 2. Acetone fractionation. Cold acetone was added to the dialyzate to 30% (v/v). After standing for 20 min, the precipitate was removed by filtration through a layer of Hyflo Super-Cel on a Buchner funnel. The acetone concentration of the filtrate was increased to 80% (v/v). After standing for 20 min, the precipitate was collected by filtration. The enzyme was extracted with 0.05 M Tris-HCl buffer containing 0.01 M calcium acetate (pH 7, 7.5) from the resulting cake. Hyflo Super-Cel was removed by filtration through a filter paper. The filtrate was dialyzed for 24 hr against the same buffer.

Step 3. First DEAE-cellulose column chromatography. The dialyzate obtained in the preceding step was applied to a DEAE-cellulose column (6 x 60 cm) which had been equilibrated with 0.05 M Tris-HCl - 0.01 M calcium acetate buffer (pH 7.5). An elution by the buffer was began, followed by a stepwise elution involving a gradual increase in the NaCl concentration from 0.1 to 0.4 M. The enzyme solution applied was divided into two fractions with protease activity. The first protease fraction was eluted initially by 0.1 M NaCl (Fraction I) and the second fraction was eluted by 0.3 M NaCl (Fraction II). Four volumes of cold acetone was added to each fraction showing activity, and then the resulting precipitate was collected by filtration and dissolved in the buffer.

Step 4. Second DEAE-cellulose column chromatography. The dialyzed enzyme preparation of Fraction I was rechromatographed with a linear gradient on a column of DEAE-cellulose (3 x 42 cm). The buffer was allowed to flow at a rate of 30 ml/hr and 10 ml fractions were collected. The reservoir contained 2 liters of 0.05 M Tris-HCl - 0.01 M calcium acetate buffer which was 0.2 M in NaCl, while the mixing chamber contained 2 liters of the same buffer without NaCl. A typical chromatogram is shown in Fig. 1. Two active peaks appeared, in which the front and rear peaks were designated as Ia and Ib, respectively. Similarly, the dialyzed enzyme solution of Fraction II was rechromatographed. The reservoir contained 2 liters of 0.05 M Tris-HCl - 0.01 M

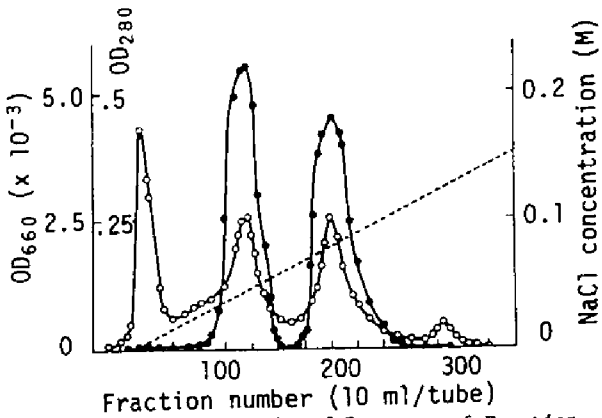

Fig. 1. Rechromatography of Protease of Fraction I on DEAE-Cellulose.

The column (3 x 42) was loaded with 400 mg of enzyme. The flow rate was 30 ml/hr. The reservoir contained 2 liters of 0.05 M Tris-HCl - 0.01 M calcium acetate buffer (pH 7.5) which was 0.2 M in NaCl; the mixing chamber contained 2 liters of the same buffer without NaCl. ● represents protease activity in absorbance at 660 mμ; ○ the absorbance at 280 mμ of the eluate; ----, the NaCl concentration of the eluate.

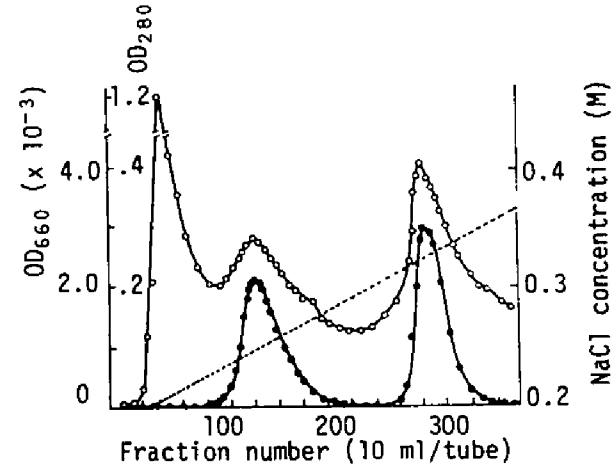

Fig. 2. Rechromatography of Protease of Fraction II on DEAE-Cellulose.

The reservoir contained 2 liters of 0.05 M Tris-HCl - 0.01 M calcium acetate buffer with 0.4 M NaCl (pH 7.5); the mixing chamber contained 2 liters of the same buffer with 0.2 M NaCl. The other conditions were the same as described in Fig. 1. ● represents protease activity in absorbance at 660 mμ. ○, absorbance at 280 mμ of the eluate; -----, the NaCl concentration of the eluate.

calcium acetate buffer with 0.4 M NaCl, while the mixing chamber contained 2 liters of the same buffer with 0.2 M NaCl. A typical chromatogram is shown in Fig. 2. The front and rear peaks with the activity were designated as IIa and IIb, respectively. The active fractions of Ia, Ib, IIa

and IIb were individually collected by precipitation with acetone, and then the resulting precipitates were dissolved in 0.05 M Tris-HCl - 0.01 M calcium acetate buffer.

Step 5. Sephadex G-100 filtration. The four enzyme preparations obtained in the preceding step were individually subjected to Sephadex G-100 filtration. The sephadex was packed into a column (2 x 100 cm) and equilibrated with the 0.05 M Tris-HCl - 0.01 M calcium acetate buffer. The enzyme solution was then placed on the column and the buffer was allowed to flow at a rate of 10 ml/hr and 3 ml fractions were collected. The enzyme preparations of Ia and Ib were obtained as symmetric protein peaks and the proteolytic activities were associated with these protein peaks.

Step 6. Sephadex G-75 filtration. Enzyme preparations of IIa and IIb obtained in the preceding step were further purified using a Sephadex G-75 column. Proteases of IIa and IIb were individually obtained as symmetric protein peaks and the proteolytic activities were associated with these protein peaks.

Step 7. Crystallization. The active fractions which were obtained in the preceding steps were collected and then concentrated by using a collodion bag. Cold acetone was cautiously added to the concentrated enzyme solution until a faint turbidity appeared, and then the suspension was stored overnight in a refrigerator. The protease of Ia was crystallized. Figure 2 is a photograph of the crystalline protease of Ia.

Homogeneity

The crystalline enzyme preparation (Ia) gave a single band on acrylamide gel electrophoresis carried out at pH 9.0. The enzyme preparation sedimented as a single peak in the ultracentrifuge in 0.05 M Tris-HCl - 0.01 M calcium acetate buffer (pH 7.5), at 20°C.

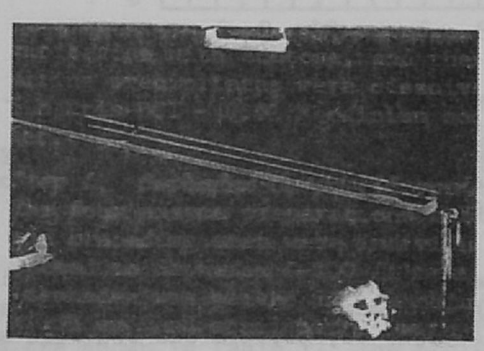

Fig. 3. Photomicrograph of Crystalline Protease Ia.

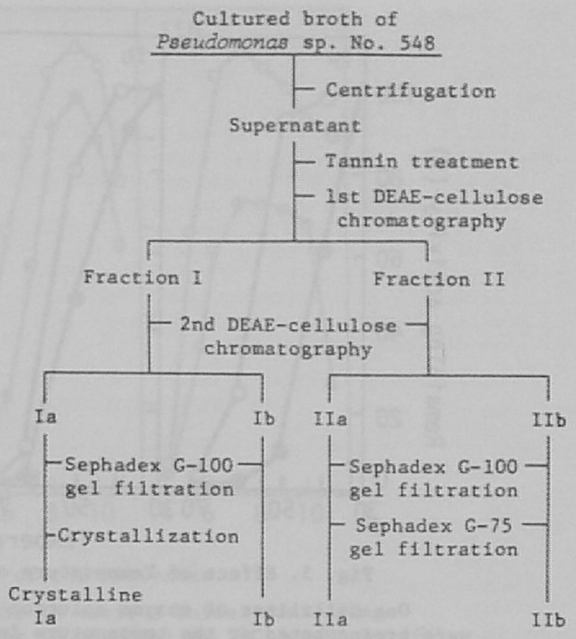

Fig. 4. Purification Procedure for Extracellular protease of *Pseudomonas* sp. No. 548.

The purified preparations of the proteases of Ib, IIa and IIb gave two or three bands of electrophoresis. The protease activity was detected only in individual main bands. The purity of each protease preparation was checked by electrophoresis. The purities of the proteases of Ib, IIa, and IIb were 90, 85 and 80%, respectively.

The enzyme preparations obtained by the above procedures (see Fig. 4) were used as purified enzymes.

Enzyme activity of the purified proteases

1) Stability to temperature. Figure 5 shows the effect of temperature on the stability of the proteases. Relative values are shown, with the maximum activity for enzyme in a buffer solution with and without calcium ion assigned a value of 100%. All proteases formed by the organism were thermolabile. Among the enzymes, IIa was somewhat more stable than the others. By the addition of 0.01 M calcium acetate, the enzymes Ia, Ib and IIb become more stable to temperature, whereas IIa was little affected.

2) Temperature optimum. The effect of temperature on the protease activities of the four enzymes was investigated

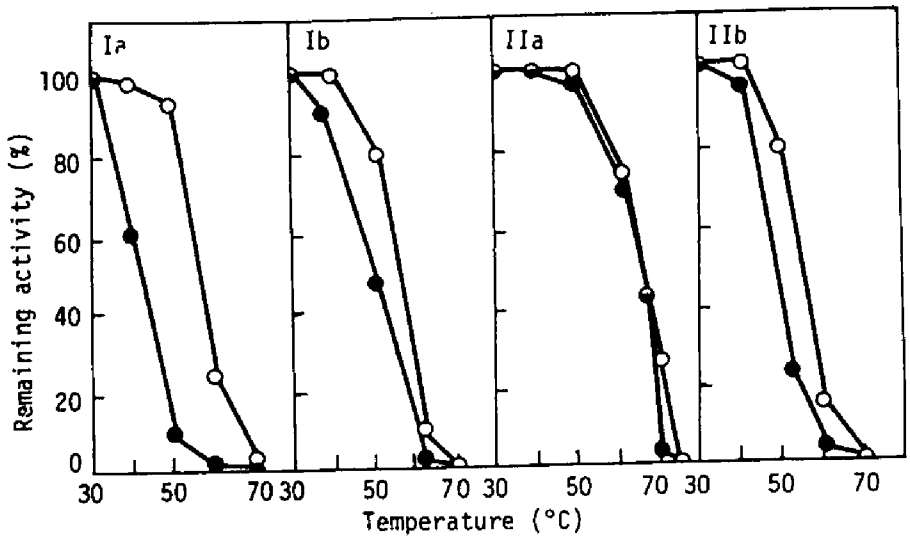

Fig. 5. Effect of Temperature on Stability of the Four Enzymes.

One milliliter of enzyme solution (0.005%) in 0.05 M Tris-HCl buffer, pH 7.5, were preincubated at the temperature indicated. After 10 min, each enzyme solution was rapidly cooled. The remaining activity was determined. ● represents the results without calcium ion and ○ represents the results in the presence of 0.01 M calcium acetate.

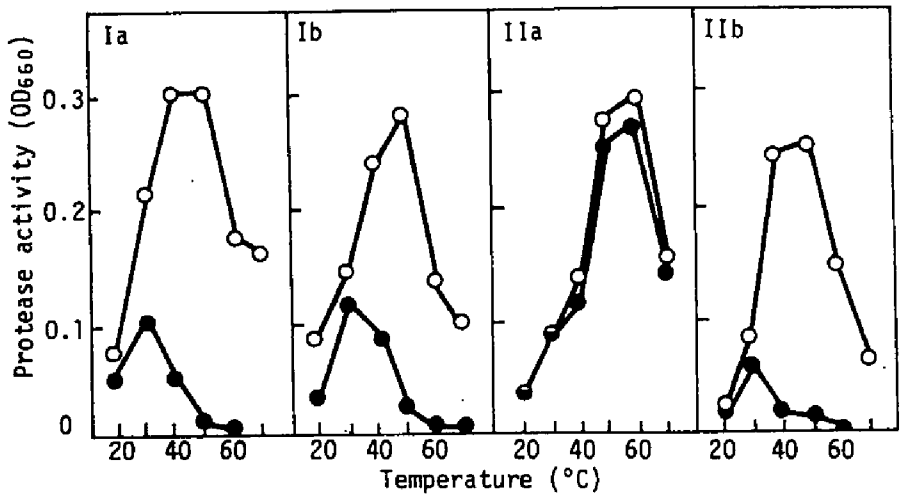

Fig. 6. Effect of Temperature on Protease Activity.

The reaction was carried out at various temperatures for 10 min. ● represents the results without calcium ion and ○ represents the results in the presence of 0.01 M calcium acetate.

(Fig. 6). The optimum temperature of proteases of Ia, Ib and IIb was at 30°C and that of IIb was at 50°C. In the presence of 0.01 M calcium acetate, the optimum of the former enzymes was shifted to 40°C, accompanied by an increase in activity but that of the latter enzyme was not shifted.

3) *Stability to pH.* The enzymes were incubated at a given pH for 10 min at 50°C or for 20 hr at 5°C, and then tested for protease activity at pH 7.5. As shown in Fig. 7, the enzymes of Ia, Ib and IIb were stable over the pH range 6.0 to 10.0, whereas IIa was stable from pH 8.0 to 10.0.

4) *pH optimum.* The variations of enzyme activity with pH are shown in Fig. 8. The optimum activity of the proteases of fractions Ia, Ib, IIa and IIb were at pH 9.0 to 10.0, pH 7.5 to 8.0, pH 7.0 to 7.5, and pH 7.5 to 8.0, respectively.

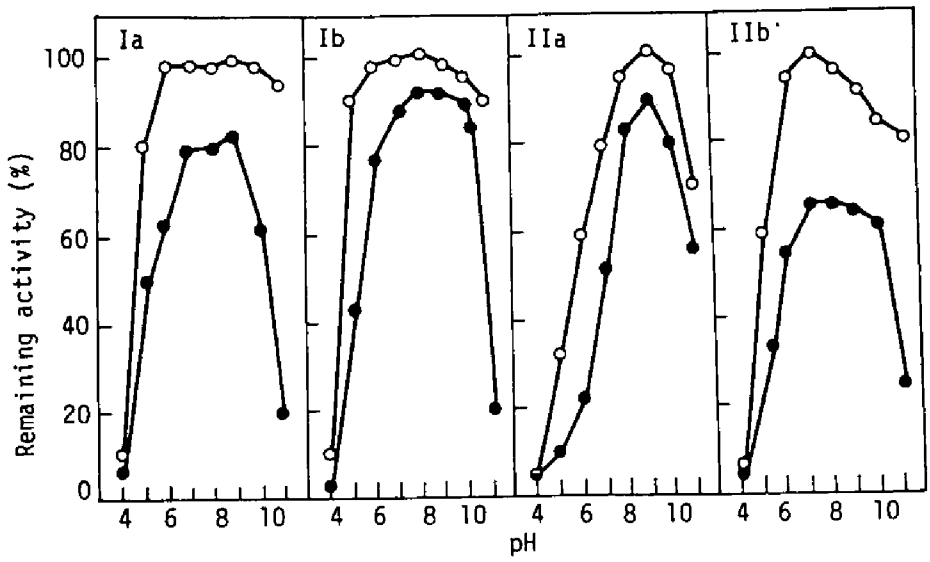

Fig. 7. Effect of pH on the Stability of the Proteases.

One milliliter of the enzyme solutions of 0.05% in various buffer containing 0.01 M calcium acetate were incubated at 50°C for 10 min (●) or 5°C for 24 hr (○). After incubation, the pH of the enzyme solutions was adjusted to 7.5 and then final volumes were made up to 10 ml. The remaining activities were determined. The buffer solutions used were acetate buffer (pH 5.0), Tris-HCl buffer (pH 7.5 to 8.0), Palitzsh borate buffer (pH 8.0 to 9.0), and borate-NaOH buffer (pH 10 to 11). Concentration of the buffers used was 0.01 M and the buffer contained 0.01 M calcium acetate.

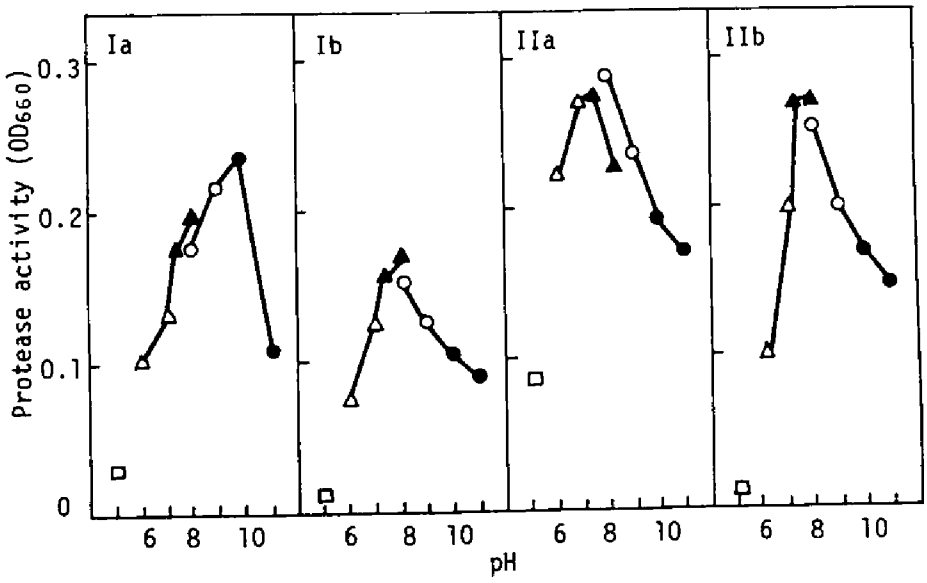

Fig. 8. Effect of pH on Proteolytic Activity.

The reaction mixture contained 1 ml of 0.5% casein at various pH values and 1 ml of the enzymes in various buffer solutions (0.005%). After 10 min, 1 ml of 5% trichloroacetic acid was added to stop the reaction. The other procedures were same as the standard methods. The buffers used were the same as those described in Fig. 7.

5) *Effect of various inhibitors.* The results are shown in Table I. The proteolytic activities of all four enzymes were not inactivated by sulfhydryl reagents such as *p*-CMB or monoiodo-acetate. On the other hand, all the enzymes were

TABLE I. EFFECT OF INHIBITOR ON PROTEASE ACTIVITY

One milliliter of 0.05 M Tris-HCl-0.01 M calcium acetate buffer (pH 7.5) containing 0.005% of enzyme and each inhibitor were kept at 37°C for 1 hr. In the case of metal-complexing agents, 0.05 M Tris-HCl buffer (pH 7.5) without calcium ion was used. In the case of DFP, 0.1 ml of 10 mM DFP solution containing 10% of isopropanol and 0.05 M Tris-HCl buffer (pH 7.5) was added to 0.9 ml of the enzyme solutions. To the treated enzyme solutions, 1 ml of 2% casein was added and remaining activities were determined.

Inhibitor	mM*	Remaining activity (%) Ia	Ib	IIa	IIb
None	-	100	100	100	100
Na·thioglycerate	1	116	118	103	129
KCN	1	88	109	95	90
$KMnO_4$	1	0	0	0	0
p-CMB	1	92	98	100	104
Monoiodoacetate	1	117	89	100	104
EDTA	1	0	0	0	0
o-Phenanthrolin	1	109	105	30	96
α,α'-Dipyridyl	1	98	118	87	96
Citrate	100	0	0	60	0
Oxalate	100	0	0	66	0
DFP**	1	9	8	101	0

*Final concentration
**Trypsin was completely inactivated at a concentration of 1 mM.

completely inactivated by 1 mM EDTA. The proteases, except for the protease of IIa, however, were not inhibited by *o*-phenanthrolin. DFP 1 mM completely inactivated the proteases of Ia, Ib and IIb but not IIa.

TABLE II. EFFECT OF METAL ION ON PROTEASE ACTIVITY

One milliliter of 0.05 M Tris-HCl buffer (pH 7.5) containing 0.005% of enzyme and each salt (1.0 mM) were preincubated at 37°C for 1 hr. To the treated enzyme solutions, 1 ml of 2% casein was added and the remaining protease activities were determined.

Salt	Remaining activity (%) Ia	Ib	IIa	IIb
None	100	100	100	100
Ba·acetate	62	86	91	69
$NiSO_4$	45	49	4	31
$CdCl_2$	94	52	42	89
$MnCl_2$	102	105	73	91
Zn·acetate	86	28	68	63
$CoCl_2$	97	91	76	83
$MgCl_2$	104	98	104	100
$CaCl_2$	121	105	101	104
$CuSO_4$	85	90	38	77
$HgCl_2$	57	91	8	76
$FeSO_4$	10	25	54	39
$AgNO_3$	100	20	15	94
$SrCl_2$	100	103	104	98
Pb·acetate	9	17	10	13

6) Effect of metal ions. As shown in Table II, the four enzymes behaved in a somewhat different manner with respect to metal ions.

7) Effect of calcium ion. From the results of temperature stability experiments, calcium ion appeared to markedly stabilize the enzymes. The enzymes were incubated at 30°C with or without calcium ion and then the remaining activities

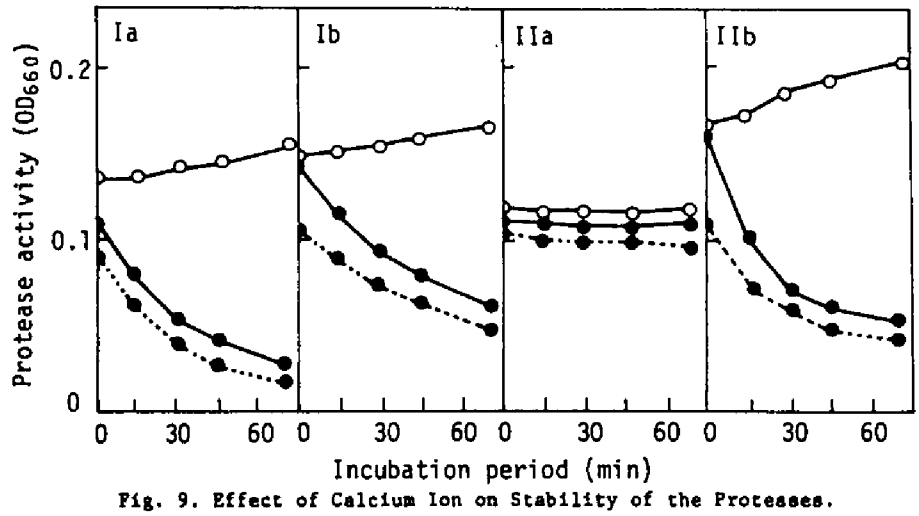

Fig. 9. Effect of Calcium Ion on Stability of the Proteases.

The enzymes were dissolved in 0.05 M Tris-HCl buffer (pH 7.5) containing 0.01 M calcium acetate (○—○) or in the buffer without calcium ion (●—●, ●--●). The enzyme solutions were incubated at 30°C for various periods. Remeining activities were determined. When the remaining activity was estimated, 0.1 ml of calcium acetate solution (0.1 M) was added (●—●) or was not added (●--●).

were estimated. The results are shown in Fig. 9. In the presense of calcium ion, the proteases of Ia, Ib and IIb were stable at 30°C for 75 min and the activities increased rather slightly. When the three enzymes were incubated without calcium ion, the enzyme activities were not completely restored by the addition of 0.01 M calcium acetate at the time of the measurement of the activity. Calcium ion had little effect on the stability of IIa.

DISCUSSION

Protease which was found in the culture fluid of *Pseudomonas* sp. No. 548 was fractionated into four components with protease activity by a two step column chromatographies with DEAE-cellulose. The respective protease preparations obtained by the chromatography were further purified by gel filtration on Sephadex G-100 and/or G-75. The protease of Ia was obtained in a crystalline form and the protease preparation was shown to be homogeneous by electrophoresis. The purities of proteases Ib, IIa and IIb were 80 to 90% and the contaminating proteins did not shown protease activity.

The enzyme of Ia was an alkaline protease of which the optimum pH was at 10.0 and the others were neutral or semialkaline. All the enzymes were somewhat unstable in acidic solution. The stable pH-range of IIa was more narrow than that of the others.

The optimum temperature of Ia, Ib and IIb was 30°C in the absence of calcium ion. However, the temperature was shifted to 40°C by the addition of 0.01 M calcium acetate. As shown in Fig. 9, the proteases of Ia, Ib and IIb were unstable in the absence of calcium ion. The concentration of calcium ion used in the experiments is about the same as that of sea water. Therefore, in view of the origin of the organism, it would seem that the normal state of the enzyme was obtained by the addition of calcium ion. The optimum temperature of IIa was at 50°C and the temperature did not shifted with the addition of calcium ion.

All the protease were inactivated by 1 mM EDTA. On the other hand, the enzymes of Ia, Ib and IIb were also completely inactivated by 1 mM DFP. According to the classification by Hartley (101), the enzymes of Ia, Ib and IIb may be classified as metal protease and concurrently calssified as serine protease. However, it is not clear that a metal and serine residue participate simultaneously in the catalitic action of the protease. While the enzyme of IIa was classified as metal protease. It has been shown that the proteases of obligate psychrophilic bacterium were inactivated by both EDTA and DFP (80). As reported by Morihara (96) and Ogino *et al.* (102), proteases of pseudomonads have been found to be inhibited by EDTA but not by DFP. Similarly, a protease from a marine bacterium was also inactivated by EDTA (82).

The proteases Ia, Ib, and IIb were quite different from IIa with respect to inhibition by DFP and optimum temperature. The optimum pH of Ia was different from the others. Ib and IIb differed from one another in their inhibitions by various metal ions. Thus, the four proteases from *Pseudomonas* sp. No. 548 have different properties each other.

SUMMARY

Protease which was found in the culture fluid of *Pseudomonas* sp. No. 548 was fractionated into four components with protease activity by a two step chromatography using DEAE-cellulose. Each protease was further purified by gel filtration on Sephadex G-100 and/or G-75. The protease of Ia was obtained crystalline form and was shown to be homogeneous by analysis with electrophoresis, while the other three enzymes were also highly purified. The enzymatic properties of the proteases were investigated. All of the four enzymes were inactivated by EDTA. Protease Ia, Ib and IIb were inactivated by DFP. The optimum activity of protease Ia was shown to be at pH 10.0, and that of the other enzymes were at pH 7.0 to 8.0. The proteases of Ia, Ib

and IIb were stabilized by calcium ion. The effect of temperature, pH, and metal ions on the activity of the enzyme were also investigated.

SECTION 3

SUBSTRATE SPECIFICITIES OF PROTEASES FROM *PSEUDOMONAS* SP. No. 548[f)]

INTRODUCTION

In general, proteases have been classified by their origin, behavior against inhibitors, or pH optimum. Recently, Morihara *et al.* (103 - 105) showed that microbial proteases were able to be classified also by their substrate specificities. The present study was undertaken to investigate the relation between the behavior against inhibitors and the specificities against oxidized insulin B-chain and various synthetic substrates on the two proteases from *Pseudomonas* sp. No. 548. Furthermore the proteases were classified from the standpoint of their specificity.

MATERIALS AND METHODS

Enzymes. Proteases from *Pseudomonas* sp. No. 548 were fractionated into four components, Ia, Ib, IIa and IIb, by a two step chromatographies on DEAE-cellulose, according to the methods described previously. Among the four enzymes, the proteases Ia and IIa were further purified by gel filtrations using Sephadex G-100 and G-150, respectively. The purified protease preparations were found to be of a homogeneous form by a cellulose acetate-membrane electrophoresis. The purified preparation of Ia was most active at pH 10.0 and was inhibited by both DFP and EDTA. The purified IIa was most active at 7.5 to 8.0 and was inactivated by EDTA but not by DFP. The two purified protease preparations were used in this study.

Substrates. Oxidized insulin B-chain and various synthetic peptides used were pure in analyses by paper chromatography and paper electrophoresis. All other chemicals were obtained from commercial sources and were used without further purification.

Inactivation by various reagents. One milliliter of 0.5 M Tris-HCl - 0.1 M calcium acetate buffer (pH 7.5) containing 0.005% of enzyme and each inhibitor was kept at 37°C for 1 hr. In the case of EDTA, 0.5 M Tris-HCl buffer (pH 7.5) without calcium ion was used. In the case of DFP, 0.1 ml of 10 or 5 mM DFP solution containing 10% of isopropanol and 0.05 M Tris-HCl buffer (pH 7.5) was added to 0.5 ml of the enzyme solution. To the treated enzyme solution, 1 ml of 2% casein was added and remaining activities were determined as described previously.

Determination of the point of cleavage in oxidized insulin B-chain. Fifty milligrams of oxidized insulin B-chain were dissolved in 5 ml of 0.01 M calcium acetate, the pH of which was adjusted to about 8.0 with 0.1 N ammonia water, and 5 mg of the enzyme was mixed with the solution. The reaction was carried out at 37°C. The reaction was stopped by placing the tube in a boiling water bath for 10 min, and the mixture was evaporated to dryness under reduced pressure.

Separation of peptides in the digest was performed by paper chromatography and/or paper electrophoresis. The dried digest was dissolved in a minimal quantity of water, and spotted on several sheets of Toyo Roshi No. 50 filter-paper (40 x 40cm). In the case of protease Ia, the filter papers were subjected to two dimensional paper chromatography according to Tsuru *et al.* (106). The solvent systems used were *n*-butanol, acetic acid, and water

(4:1:1, v/v/v) for the first run and *n*-butanol, pyridine, and water (1:1:1, v/v/v) for the second run. In the case of protease IIa, the spotted filter papers were subjected to paper electrophoresis (2 kv, 40 min), using a solvent containing pyridine, acetic acid, and water (10:0.4:90, v/v/v, pH 6.5). In order to prepare the standard peptide map, one of the developed filter-papers was treated with a ninhydrin reagent (0.2% in acetone and ethanol, 6:4.v/v), heated at 50°C for 30 min, and then, to detect the peptides containing histidine or tyrosine, treated with Pauly reagent (107). The other papers were used for the elution of peptide. The papers were dipped in a 0.02% ninhydrin solution made with an acetone and ethanol mixture (6:4.v/v), and then allowed to stand for 20 hr in the dark at 20°C. Each colored spot was cut out from the papers and the paper strips which were regarded as containing the identical peptide, after a comparison with the standard peptide map, were combined in a test tube and washed with acetone to remove excess ninhydrin. The peptides were eluted from the strips with 5 ml water, and then the eluates were concentrated to dryness under reduced pressure. The purities of individual peptides were checked by paper chromatography, using a solvent containing *n*-butanol, acetic acid, pyridine and water (30:6:20:24, v/v/v/v) (108). The pure peptides were hydrolyzed with 6 N hydrochloric acid for 24 hr at 110°C in sealed and evacuated tubes. The hydrolyzates were subjected to amino acid analysis with a Hitachi auto-analyzer, type KLA-5. The amino-terminal residues of some of the peptides were identified by the usual dinitrophenyl (DNP) method (109), if necessary, to characterize them.

Enzyme assays. The rate of hydrolysis of the various synthetic peptides was determined according to Morihara *et al.* (103) and Morihara and Tsuzuki (104). The reaction mixture consisted of 50 μmoles of Tris-HCl buffer, pH 7.5, 10 μmoles of calcium acetate, 2.0 μmoles of substrate (except where specified), a suitable amount of enzyme, and water, to a total volume of 1.0 ml. The reaction was carried out at 37°C. At various intervals, 0.1 ml of the reaction mixture was withdrawn and put into a test tube in which had been placed 1 ml of 0.5 M citrate buffer, pH 5.0, containing 0.01 M EDTA. The extent of hydrolysis was determined by the ninhydrin method, according to Yemm and Cocking (110). The hydrolyzates formed during the reaction were analyzed by paper- or thin layer-chromatography.

The esterase activity was determined with the aid of a Radiometer titrimetric set (PM26/TT1C/ABU12/SBR2C) equipped with a pH meter, a syringe drive, a recorder, and a thermostatically controlled reaction vessel. The reaction mixture (2.5 ml), containing 0.2 to 10 mM substrate, 0.1 M calcium chloride, and 4% ethanol, was adjusted to pH 8.0 by the addition of a minute volume of 0.5 N NaOH. As the titrant, 0.001 M sodium hydroxide was used and the reaction was performed at 30°C.

RESULTS

Effect of inhibitors on the proteolytic activity of the protease Ia and IIa

The data on the effect of inhibitor on the activity are shown in Table I. The protease Ia was completely inhibited by DFP and EDTA and even with dialysis but not with STI, TLCK and *p*-CMB. The enzyme

TABLE I. EFFECT OF VARIOUS INHIBITORS ON THE ACTIVITY OF PROTEASES Ia AND IIa

The procedures were described in Materials and Methods.

Inhibitor	mM*	Remaining activity (%) Ia	IIa
DFP	1.0	5	97
	0.5	20	100
STI	0.1	98	98
TLCK	1.0	92	98
EDTA	1.0	0	0
	0.1	12	25
(Dialysis**)		20	100
p-CMB	1.0	100	100

*Final concentration.
**The enzyme was dialyzed against 5 liter volume of 0.05 M Tris-HCl buffer (pH 7.5) without calcium ion at 5°C for 24 hr.

inactivated with EDTA or dialysis was not reactivated by a dialysis against this buffer containing 0.01 M calcium acetate at 5°C for 24 hr. While, the protease IIa was inhibited only by EDTA. When calcium acetate was added in a final concentration of 2 mM after the EDTA treatment, the enzyme was completely reactivated.

Determination of the points of split in the oxidized insulin B-chain

Digestion of oxidized insulin B-chain

TABLE II. AMINO ACID COMPOSITION OF PEPTIDES OBTAINED FROM DIGESTS OF OXIDIZED INSULIN B-CHAIN BY PROTEASE Ia

Peptide No.	N-Terminal	Pauly reaction	Amino acid composition (molar ratio)	Possible sequence in the B-chain
1	Tyr	Tyr	Tyr, Leu, Val, Cys, Gly, Glu, Agr 1.1 0.9 1.0 0.8 1.3 1.0 0.9	16-22
2		His	His, Leu, Cys, Gly, Ser, (Tyr) 1.8 1.0 1.0 0.9 1.0 0.2	5-10
3	Phe		Phe, Val, Asp, Glu, (Leu) 0.7 1.0 0.9 1.0 0.3	1-4
4			Phe	6
5			Gly, Phe, (Arg) 1.0 1.0 0.2	23-23
6			Thr, Pro, Lys, Ala 1.2 0.9 1.1 1.0	27-30
7			Ala	14 or 30
8			Ala, Leu 1.0 1.0	14-15
9	Leu		Leu, Val, Cys, Gly, Glu, Arg 1.0 1.0 1.2 0.9 0.9 1.1	17-22
10		Tyr	Tyr	16 or 26
11	Leu		Leu, Val, Glu 1.0 1.0 0.8	11-13
12	Agr		Arg, Gly, Phe 1.2 1.0 2.1	22-25

TABLE III. AMINO ACID COMPOSITION OF PEPTIDES OBTAINED FROM DIGESTS OF OXIDIZED INSULIN B-CHAIN BY PROTEASE IIa

Peptide No.	N-Terminal	Pauly reaction	Amino acid composition (molar ratio)	Possible sequence in the B-chain
1		His	Cys, Gly, Ser, His 0.9 1.0 1.1 0.9	7-10
2	Val	His	Val, Asp, Glu, His, Leu 1.0 0.8 0.9 1.1 1.0	2-6
3		His	Phe, Val, Asp, Glu, His, Leu 0.9 1.0 0.9 1.1 0.7 1.0	1-6
4			Gly, Glu, Arg 2.0 1.0 0.8	20-23
5	Val	Tyr	Val, Glu, Ala, Leu, Tyr, Cys, Gly, Arg 1.9 2.1 0.9 2.0 1.1 0.8 1.9 0.8	12-23
6		Tyr	Tyr	16 or 26
7			Phe	1, 24 or 25
8			Leu	6, 11, 15 or 17
9		Tyr	Ala, Leu, Tyr, (Glu) 1.0 1.0 0.9 0.2	14-16
10		Tyr	Tyr, Thr, Pro, Lys, Ala 1.1 1.2 0.8 0.9 1.0	26-30
11			Thr, Pro, Lys, Ala 0.9 1.1 1.2 1.0	27-30
12	Val	His	Val, Asp, Glu, His 1.0 0.9 1.0 1.1	2-5
13	Leu		Leu, Val, Glu, Ala (Glu, Tyr) 1.0 1.0 1.0 0.9 0.2 0.3	11-14
14		His	Leu, Cys, Gly, Ser, His 1.0 0.8 0.7 0.8 0.9	6-10

by each of the alkaline- and neutral-protease preparations obtained from the marine-psychrophilic *Pseudomonas* sp. No. 548 was carried out by the method described in Materials and Methods. The peptides digested by the proteases were separated by paper chromatography and/or paper electrophoresis, and the amino acid composition of each peptide was analyzed. The molar ratios of constituent amino acids are indicated in Table II and III. Comparing the amino acid compositions of the peptides obtained with the known structure of the insulin B-chain, the points of cleavage in the B-chain by these protease were determined. From the results, it could be deduced that major cleavages had occurred at the peptide bonds of Gln-His (4-5), His-Leu (10-11), Glu-Ala (13-14). Leu-Tyr (15-16), Tyr-Leu (16-17), Leu-Val (17-18), Glu-Arg (21-22), Arg-Gly (22-23), Phe-Phe (24-25), Phe-Tyr (25-26), and Tyr-Thr (26-27) in the alkaline protease Ia, and at those of Phe-Val (1-2), His-Leu (5-6), His-Leu (10-11), Leu-Val (11-12), Ala-Leu (14-15), Tyr-Leu (16-17), Gly-Phe (23-24), Phe-Phe (24-25), and Phe-Tyr (25-26) in the neutral protease IIa.

Hydrolysis of various synthetic substrates by the proteases

On the basis of the above results, a detailed study was undertaken to elucidate the substrate specificity of the two proteases.

1) The alkalin protease Ia. An experiment on the specificities of this alkaline protease was made, using, for the most part, Z-Gly-X-NH_2 (X = various amino acids residues) as substrates. The results shown in Table IV indicated that the amide bonds containing the carboxyl group of L-phenylalanine, L-tyrosine, or L-leusine were strongly hydrolyzed by the enzyme, and the amide bonds of L-alanine and L-valine were also hydrolyzed. The rate of hydrolysis of Ac-Gly-Leu-NH_2 by the enzyme was considerably smaller than that of Z-Gly-Leu-NH_2. The enzyme was unable to hydrolyze the amide bond of D-leucine.

The esterase actvities of the enzyme were determined using Bz-Arg-OEt, Ac-Tyr-OEt, and Ac-Phe-OEt as substrates. All the substrates tested were hydrolyzed by the enzyme. The *Km*s of Bz-Arg-OEt, Ac-Tyr-OEt, and Ac-Phe-OEt for the enzyme were 26.3, 50, and 66.7 mM, respectively.

2) The neutal protease IIa. The synthetic peptides, Z-Gly-X-NH_2, were subjected to hydrolysis by the neutral protease IIa, in a manner similar to the above. The results are shown in Table V. The enzyme had some hydrolyzing activity against Z-Gly-Ileu-NH_2, A-Gly-Phe-NH_2, and Z-Gly-Leu-NH_2, and the cleavage points in the substrates were the peptide bonds containing the amino groups of L-isoleucine, L-phenylalanine, and L-leucine, respectively.

In order to determine the effect of the neighboring residues around the hydrolyzing peptide bond, some peptides containing a leucine residue were also tested as substrates. The rate of hydrolyses of Ac-Gly-Leu-NH_2 and Z-Ala-Leu-NH_2 were con-

TABLE IV. HYDROLYSIS OF SYNTHETIC SUBSTRATES BY THE PROTEASE Ia

All reactions were performed at 37°C for 2 and 23 hr. The conditions of the reaction are described in Materials and Methods.

Substrate	%Hydrolysis 2 hr	23 hr
Z-Gly-Ileu-NH_2	0	0
Z-Gly-Pro-NH_2	0	0
Z-Gly-Ser-NH_2	0	0
Z-Gly-Val†-NH_2	0	30
Z-Gly-Ala†-NH_2	0	60
Z-Gly-Phe†-NH_2*	30	>95
Z-Gly-Tyr†-NH_2*	34	>95
Z-Gly-Try†-NH_2*	20	>95
Z-Gly-Leu†-NH_2	15	>95
Z-Ala-Leu†-NH_2	50	>95
Z-Gly-D-Leu-NH_2	0	0
Ac-Gly-Leu†-NH_2	0	25

† shows the bond split by the enzyme.
*The reaction mixture contained 4% ethanol.

TABLE V. HYDROLYSIS OF SYNTHETIC SUBSTRATES BY THE PROTEASE IIa

All reactions were performed at 37°C for 1 and 20 hr. The conditions of the reaction are described in Materials and Methods

Substrate	%Hydrolysis 2 hr	23 hr
Z-Gly-Val-NH_2	0	0
Z-Gly-Tyr-NH_2*	0	0
Z-Gly-Ser-NH_2	0	0
Z-Gly-Pro-NH_2	0	0
Z-Gly-Ala-NH_2	0	5
Z-Gly†-Ileu-NH_2	5	40
Z-Gly†-Phe-NH_2*	15	57
Z-Gly†-Leu-NH_2	5	85
Z-Gly-D-Leu-NH_2	0	0
Z-Gly-Leu	0	0
Z-Ala†-Leu-NH_2	3	22
Ac-Gly†-Leu-NH_2	0	29

† shows the bond split by the enzyme.
*The reaction mixture contained 4% ethanol.

siderably reduced, compared with that of Z-Gly-Leu-NH_2; no hydrolytic activity against Z-Gly-D-Leu-NH_2 was observed.

The *Km* values of the neutral protease-catalyzed hydrolysis of Z-Gly-Leu-NH_2 and Ac-Gly-Phe-NH_2 were 2.2 and 1.3 mM, respectively.

DISCUSSTION

The alkaline protease Ia is inactivated by DFP and EDTA but not by STI and TLCK. From its behavior against these inhibitors, except for EDTA, the protease seems to be analogous to those enzymes which have been classified as subtilisin-like alkaline proteases by Morihara (105). The specificity of the alkaline protease Ia toward the oxidized insulin B-chain is shown in Fig. 1, in which those of a microbial alkaline-serine protease, and α-chymotrypsin are presented for comparison. The protease Ia is able to hydrolyze the all peptide bonds which are cleavage points of α-chymotrypsin. However, the specificity of the protease Ia is somewhat broader than those of the other microbial alkaline proteases cited in this figure. It is uncertain whether the differences of specificities against the insulin B-chain are fundamental ones or are contributed to differences of experimental conditions. The protease Ia is able to cleave amide or ester bonds of Z-Gly-X-NH_2, Ac-X-OEt, *etc.*, where X is L-tyrosine, L-phenylalanine, or L-leusine. The specificities of the enzyme against synthetic substrates agree fairly well with the alkaline-serine proteases shown by Morihara and Tsuzuki (104), in the following respects; a) the enzyme exhibits specificities toward amide bonds containing the carboxyl groups of aliphatic amino acids, such as L-leucine, L-alanine and L-valine as well as aromatic ones, b) the enzyme posesses esterase activity against Bz-Arg-OEt which is a specific substrate for trypsin, c) the *Km* values for the enzyme of Ac-Tyr-OEt and Ac-Phe-OEt were considerably higher than those of α-chymotrypsin (104).

The alkaline protease Ia is inactivated by both DFP and EDTA. Therefore, it has been unclear whether the enzyme should be classified as a serine protease or as a metalic one. Morihara (104) decided that microbial proteases should be classified by their specificities, independent of their origin. On the basis of the specificity, the protease Ia might be classified as a serine protease. If this is so, it might be thought that a metal in the protein molecule, which is chelated with EDTA, participates in the maintenance of the conformation of the enzyme protein. Although a number of enzymes called serine protease have so far been isolated from various microbes, few information on the

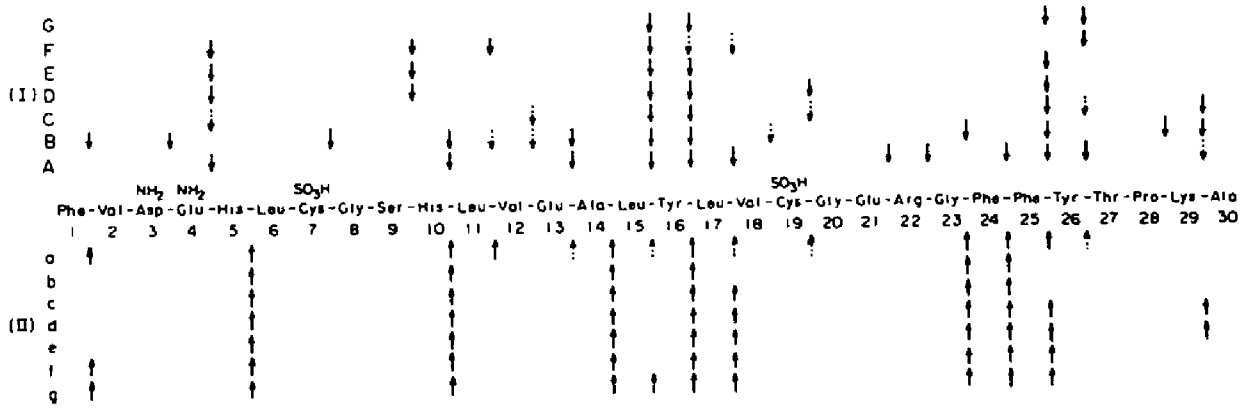

Fig. 1. Cleavage Points of Oxidized Insulin B-Chain by Alkaline-Serine Proteases (I) and by Neutral Proteases (II).

(I) A: Protease Ia from *Pseudomonas* sp. No. 548, B: Serine protease from a mold (111), C: from *Aspergillus oryzae* (104), D: from *Streptomyces fradiae* (104), E: from *Bacillus subtilis* (104), F: from *Bacillus subtilis* (112), G: α-Chymotrypsin (113).

(II) a: Protease IIa from *Pseudomonas* sp. No. 548, b: neutral protease from *Bacillus subtilis* (114), c: from *Bacillus subtilis* (103), d: from *Bacillus thermoproteolyticus* (115), e: from *Pseudomonas aeruginosa* (116), f: from *Streptomyces griseus* (103), g: from *Aspergillus oryzae* (103).

serine protease from Gram-negative bacterium was given. The protease Ia might be thought to be unique in the following respects; this Gram-negative bacterium produces a serine protease, the protease is inhibited by both DFP and EDTA, and the protease is irreversibly inactivated by a dialysis against the buffer without calcium ion.

The protease IIa from the organism acts optimally at a neutral pH range and is inhibited by EDTA but not by DFP. These properties agree closely with those of the enzymes classified as neutral proteases (103, 105). In Fig. 1, the cleavage points in the oxidized insulin B-chain by the various neutral proteases so far reported and by the neutral protease IIa are summarized. Morihara *et al.* (103) have shown that eight kinds of neutral proteases from various microorganisms exhibit remarkably similar specificities against the insulin molecule. From the figure, it is clear that the cleavage points by protease IIa are also exactly similar to those of the other neutral proteases. The protease IIa was active against synthetic substrates such as Z-Gly-X-NH_2, where X was L-leucine, L-phenylalanine, or L-isoleucine residues, and the split points were the peptide bonds to which these amino acid residues contribute the amino group. From the susceptibilities of some peptides involving the amino group of leucine to the enzyme, the structure of peptides which are able to be hydrolyzed by the enzyme has to satisfy the following requirements: a) the leucine residue is of the L-configuration, and b) the carboxyl group of the leucine residue is blocked. Furthermore, the rate of hydrolysis of Ac-Gly-Leu-NH_2 was considerably reduced, in comparison with that of Z-Gly-Leu-NH_2, and that of Z-Ala-Leu-NH_2 was slower than that of Z-Gly-Leu-NH_2. All of these phenomena, except for the last case, were in fair agreement with the susceptibilities of peptides to five kinds of microbial neutral proteases which were reported by Morihara *et al.* (103).

SUMMARY

The specificities of the alkaline and neutral proteases from the marine-psychrophilic *Pseudomonas* sp. No. 548 were investigated using oxidized insulin B-chain and various synthetic peptides as substrates. The alkaline protease was able to cleave the peptide or amide bonds containing a carboxyl group of amino acid residues, such as L-alanine, L-glutamic acid, L-arginine, L-leucine, L-phenylalanine, L-tryptophan, L-tyrosine, and L-valine. On the basis of the specificities, the enzyme might be classified as a serine protease, comparing the specificities of the proteases so far reported, although the enzyme is inactivated with EDTA as well as DFP.

The neutral protease hydrolyzed the peptide bonds containing an amino group of hydrophobic amino acid residues, such as L-isoleucine, L-leucine, and L-phenylalanine. These specificities agree closely with those of the neutral proteases which have been reported.

CHAPTER IV

UTILIZATION OF NON-CARBOHYDRATE SUBSTRATES BY YEASTS AT LOW TEMPERATURES

SECTION 1

UTILIZATION OF *n*-PARAFFIN BY A MARINE YEAST, *CANDIDA* SP. MM313[g)]

INTRODUCTION

It is well known that numerous microorganisms isolated from marine samples are able to utilize hydrocarbons. ZoBell *et al.* (117) studies on the oxidation of hydrocarbon by marine sediments and reported that hydrocarbon-oxidizing bacteria were found in every sample of the sediments. Recently, principal types of hydrocarbon-oxidizing microorganisms isolated from marine sediments were summarized to include *Nocardia, Actinomyces, Pseudomonas, Micromonospora and Mycobacterium* (118).

The author has isolated a number of microorganisms from sea, a typical environment of low temperature. Among the organisms, a yeast isolated from marine sediment was found to be able to utilize *n*-paraffins at low temperature. The cultural conditions for cell production from *n*-paraffins and the effect of temperature on assimilation of *n*-paraffins by the isolate are described in this section.

MATERIALS AND METHODS

Microorganism. The organism mainly used in this experiments was a yeast, strain MM313, isolated from marine sediment of Sagami Bay.

Chemicals. The mixture of *n*-paraffins used in the experiments was a gift from Mr. Minami, Maruzen Petroleum Industries Co. Ltd. The composition of the mixture was as follows; 0.4% *n*-C_{13}, 68% *n*-C_{14}, 29.9% *n*-C_{15}, 0.8% *n*-C_{16} and 1.3% aromatic hydrocarbons. Pure *n*-paraffins were obtained commercially.

Medium. The *n*-paraffin medium composed of the *n*-paraffin mixture, 3.8 g (5% v/v); $(NH_4)_2SO_4$, 0.4 g; K_2HPO_4, 0.2 g; KH_2PO_4, 0.1 g; $FeCl_3 \cdot 6H_2O$, 0.01 g; Tween 20, 0.005 g; yeast extract, 0.2 g; in 100 ml of artificial sea water, pH 6.0. The composition of the artificial sea water was described in Chapter III.

Isolation of marine microorganisms. The sea water and sediment samples were collected at the stations of Sagami and Suruga Bay, 34°50.1'N-138°38.4'E, 35°00.4'N-138°39.1', 34°59.7'N-139°19.5'E, 35°09.7'N-139°16.5'E and 35°06.8'E-139°24.7'E, during the cruise in October, 1968, by the research vessel "Tanseimaru" of the Ocean Research Institute, University of Tokyo. ORIT type of microbiological sampler (119) was used for collection of the sea water samples from the vertical depths. The

sediment samples were collected by Gravity Corer. Microorganisms in the sea water samples were collected on the sterilized Milipore Filter by filtration. The filter was placed on the plate of ZoBell's 2216E agar. The sediment sample suspended in the sterilized sea water was smeared on the same agar. The plate was incubated at room temperature or below 10°C. After one or two weeks, the microbial colonies grown on the plate were transferred to the agar slant of the same composition. Concurrently, the enrichment culture in *n*-paraffin medium was carried out. Small amount of the sea water or sediment sample was added into the *n*-paraffin medium and incubated under shaking for 10 days. After two times subcultures, the cultured broth was plated on the *n*-paraffin agar, and then transferred to *n*-paraffin agar slant. All of the incubations were carried out at 10°C.

Cultivation of microorganism. One loopful cells grown on the *n*-paraffin agar slant was inoculated into 4 ml of culture medium in a test tube. In the case of flask culture, the test-tube culture was used as seed culture. The cultures were carried out under reciprocal shaking at 120 rpm.

Determination of cell yield. The cells in the cultured broth were collected by centrifugation at 5000 x *g* for 10 min and washed twice with the artificial sea water. The cell concentration of final suspenstion was measured by optical density at 610 mμ. The cells treated with sea water were washed with deionized water and dried at 105° to 110°C for 6 hr. Then the dry cell weight was measured.

Analysis of n-paraffin. Analysis of *n*-paraffins was carried out by a gas chromatography under the following conditions: The apparatus, Shimazu Model GC-3AH; column dimention, 200 x 0.3 cm; solid support, celite 545 (80 to 100 mesh); stationary phase, 5% SE-30; temperature, 90° to 250°C; carrier gas, helium. The extraction of *n*-paraffins from cultured broth was performed according to the method of Tanaka and Fukui (120).

RESULTS AND DISCUSSION

Isolation of n-paraffin-utilizing marine microorganisms

Among 650 pure cultures isolated from marine materials, 20 strains of bacteria and 4 strains of yeasts were able to grow at 10°C on the medium containing *n*-paraffins as carbon source. Table I shows the cell yield from *n*-paraffins by the isolates. A yeast, MM313, which was isolated from marine sediment, showed the highest cell yield. This yeast was used in the following experiments.

TABLE I. UTILIZATION OF *n*-PARAFFINS BY MARINE MICROORGANISMS

Medium used was the same as described in Materials and Methods. Cultivation was carried out under shaking at 12°C.

Strain	Origin	Cell produced
Bacteria		
M3	water	3.0 mg/ml
M25	water	7.2
M81	water	5.1
M100	water	4.7
M171	water	2.0
M172	water	3.1
MM95	sediment	3.2
MM303	sediment	2.5
MM502	sediment	6.8
MM611	sediment	2.0
Yeast		
M97	water	4.6
M345	water	5.0
MM215	sediment	11.0
MM313	sediment	20.7

Taxonomic studies on the yeast, strain MM313

Diagnostic tests of the strain MM313 were carried out according to the methods of Lodder and Kreger-van Rij (121) and of Iizuka and Goto (122).

Descriptions of the strains are as follows:

Growth in malt extract; After 3 days at 15°C, cells were oval to cylindrical 2 - 4 x 1 - 2 μ, single or in pair (Fig. 1). After 3 days, a ring and sediment were formed.

Growth on malt agar; After 5 days at 15°C, the streak culture was white to cream-colored, flat, smooth, butyrous and entire at the margin.

Slide culture; Pseudomycelium was formed (Fig. 2).

Sporulation; Not observed.

Fermentation; Glucose and sucrose were fermented slightly.

Assimilation of carbon compounds

Glucose	+	D-Xylose	+
Galactose	+	L-Arabinose	-
L-Sorbose	-	D-Arabinose	-
Maltose	+	D-Ribose	-

Sucrose	+	Ethanol	+
Cellobiose	-	Glycerol	+
Trehalose	-	D-Mannitol	+
Lactose	-	D-Sorbitol	+
Raffinose	+	Methyl-α-glucoside	+
Inulin	-	Salicin	-

Assimilation of potassium nitrate; Negative.

Splitting of arbutin; Positive.

Production of starch like compound; Negative.

Growth in vitamin-free medium; No increase.

Reaction of litmus milk; No reaction.

Origin of the strain studied; The strain was isolated from marine sediment sample at depth of 1120 m at Sagami Bay, 35°06.8' N-139°24.7'E.

Fig. 1. Photomicrograph of Cells of Strain MM313.

Cells were grown in malt extract for 3 days at 15°C.

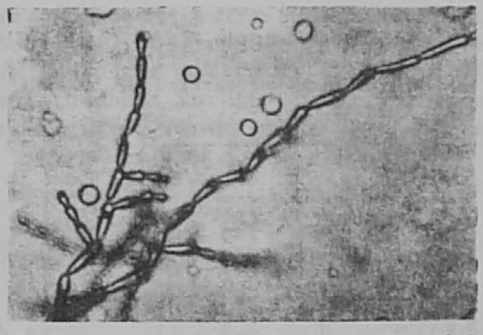

Fig. 2. Photomicrograph of Pseudomycelia of Strain MM313.

Cells were cultured on slide glass for 5 days at 15°C. Potato-glucose agar medium was used.

From these results, the strain was identified as a *Candida* sp. MM313 referring to the description of Lodder (123). Although most of morphological and physiological characteristics of this yeast were similar to those of *Candida solani*, some differences were found in the carbon assimilation patterns. The isolate could assimilate galactose, raffinose, mannitol and *n*-paraffins but not sorbose, cellobiose, trehalose and salicin, whereas the strain of *C. solani* obtained from Institute for Fermentation, Osaka (IFO 0162) assimilated sorbose, cellobiose, trehalose, and salicin but not galactose, raffinose, mannitol and *n*-paraffins.

Effect of carbon source on the growth

The cultivations of the strain MM313 were carried out at 12°C using the media containing glucose, acetate and *n*-paraffins as carbon source, respectively. The results are shown in Fig. 3. The highest growth

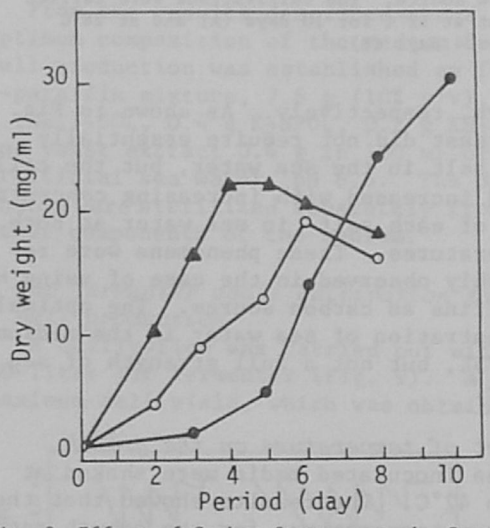

Fig. 3. Effect of Carbon Sources on the Growth.

Medium contained 0.4 g of $(NH_4)_2SO_4$, 0.2 g of K_2HPO_4, 0.1 g of KH_2PO_4, 0.01 g of $FeCl_3 \cdot 6H_2O$, 0.005 g of Tween 20, 0.2 g of yeast extract and carbon sources in 100 ml of artificial sea water (pH 6.0). As carbon sources, 5.0% glucose (▲), 3.8% *n*-paraffins (●) and 4.0 % ammonium acetate (○) were used. The cultivations were carried out under shaking at 12°C.

rate was recognized in the cultivation with the glucose medium, followed by the acetate medium. The maximum cell yields from glucose, acetate and *n*-paraffins were about 45, 50 and 84% to the initial weight of carbon sources, respectively.

Effect of sea water concentration on the growth

The requirement of inorganic salt by the yeast was tested. Glucose or *n*-paraffins were used as carbon source. The yeasts were inoculated in media prepared with the artificial sea water which was successively diluted with deionized water, and incubated at 12° and 28°C for 10 and

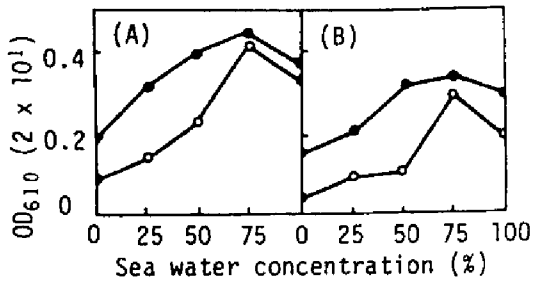

Fig. 4. Effect of Sea Water Concentration on the Growth.

Medium used was the same as described in Fig. 3. Diluent, the artificial sea water, was diluted with deionized water. Glucose (●) and *n*-paraffins (○) were used as carbon source. The cultivations were carried out at 12°C for 10 days (A) and at 28°C for 4 days (B).

4 days, respectively. As shown in Fig. 4, the yeast did not require essentially each salt in the sea water, but the cell yield increased with increasing concentration of each salt in sea water at both temperatures. These phenomena were remarkably observed in the case of using *n*-paraffins as carbon source. The optimal concentration of sea water in the medium was 75%, but not a full strength of sea water.

Effect of temperature on the growth

The inoculated media were shaken at 5° to 42°C. The results showed that the optimum temperatures for the growth rate and for the growth level existed around 28° and 10°C, respectively, in the *n*-paraffin medium (Fig. 5). The organism grew

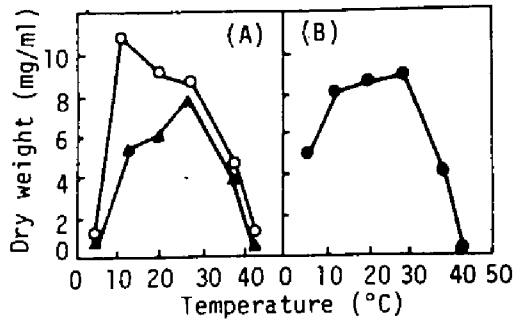

Fig. 5. Effect of Temperature on the Growth.

(A): *n*-Paraffins were used as carbon source. (▲), growth after 5 days; (○), maximum cell yield after appropriate period. (B): Glucose was used as carbon source, (●), growth after 3 days.

well at 5°C in the glucose medium (Fig. 5-B), but the growth was not observed at the temperature in the *n*-paraffin medium (Fig. 5-A). This is because the *n*-paraffin mixture used was solidified at the

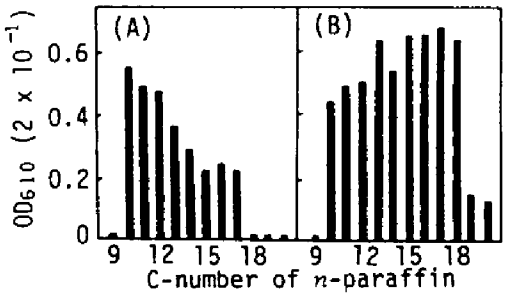

Fig. 6. Utilization of Pure *n*-Paraffins.

Each *n*-paraffin of 5% (v/v) was added to the medium mentioned in Fig. 3. (A): Cultivation was carried out at 12°C for 7 days. (B): Cultivation was carried out at 28°C for 3 days.

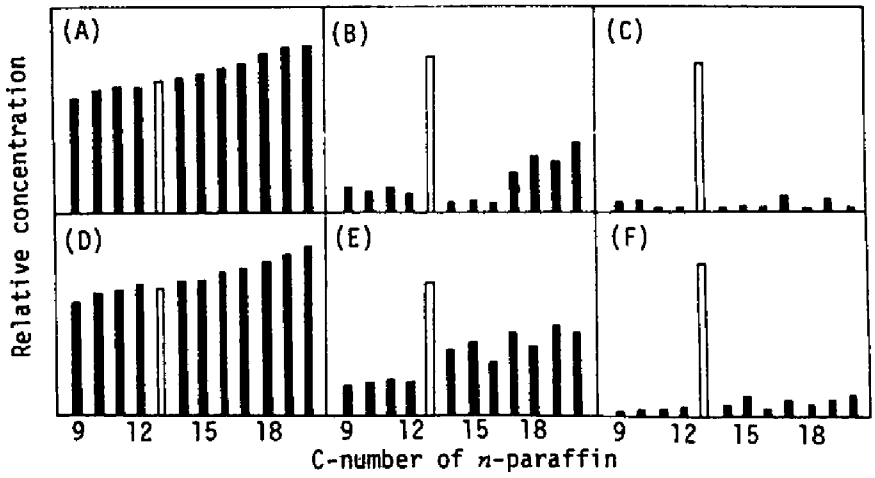

Fig. 7. Relative Utilizability of *n*-Paraffins.

The method of the experiment was described in the text. The analysis of residual *n*-paraffins was performed after 0 (A), 2 (B) and 3 (C) days cultivation at 28°C and after 0 (D), 7 (E) and 10 (F) days at 12°C.

temperature.

Relation between kind of n-paraffin and its utilization by the yeast

The specificity of *n*-paraffin as carbon source for the growth was investigated using each pure *n*-paraffin, *n*-C_9 to *n*-C_{20}. The cultivations were carried out under shaking at 12°C for 7 days and at 28°C for 3 days. As shown in Fig. 6, the organism was able to assimilate *n*-paraffins of *n*-C_{10} to *n*-C_{17} and especially *n*-C_{10} to *n*-C_{12} at 12°C with good cell production. At 28°C, *n*-paraffins of *n*-C_{10} to *n*-C_{18} were assimilated.

The relative utilizability of *n*-paraffins was investigated. The mixture of equivalent volume of pure *n*-paraffins, *n*-C_9 to *n*-C_{20}, except *n*-C_{13}, was used as carbon source. Tridecane was chosen as a control of extraction of *n*-paraffins from cultured broth with *n*-hexan. Thirty milliliters of the medium in 300 ml-flask was shaken at 12° or 28°C. Tridecane was added to the cultured broth after the separation of cells. The residual *n*-paraffins were estimated by gas chromatographic techniques. As shown in Fig. 7, all of *n*-paraffins, *n*-C_9 to *n*-C_{20}, were utilized at both temperature.

C/N ratio

Effect of C/N ratio (*n*-paraffin concentration per $(NH_4)_2SO_4$ concentration) on the growth rate and the cell yield were investigated. *n*-Paraffin mixture was added to the medium in the concentration of 3.8 and 7.6%, respectively. The results showed that the medium of C/N ratio of 19 was the best for the initial growth rate (Fig. 8). For cell yield, the ratio of 7.6 was the most suitable under the experimental conditions.

A marked drop of pH of the culture medium occurred in presence of $(NH_4)_2SO_4$ as nitrogen source. When the cultivation was carried out under the control of pH with ammonia water, the acceleration of the growth rate in the medium of higer C/N ratio and the increase of the cell yield in the medium of lower C/N ratio were observed.

Moreover, effects of phosphate concentration and growth factor on the growth of the organism were investigated. As the results, addition of 0.4% K_2HPO_4 and 0.2% KH_2PO_4, and 0.4% yeast extract were suitable for the growth.

From the results described above, the optimum composition of the medium for cell production was established as follows: *n*-paraffin mixture, 7.6 g (10% v/v), 0.4 g $(NH_4)_2SO_4$, 0.4 g K_2HPO_4, 0.2 g KH_2PO_4, and yeast extract 0.4 g in 100 ml of artificial sea water, pH 6.5. The phosphates were sterilized separately from other components of the medium.

A jar fermenter scale cultivation for cell production

A cultivation was carried out with 30-liter jar fermenter (Fig. 9). A maximum cell yield, which was obtained

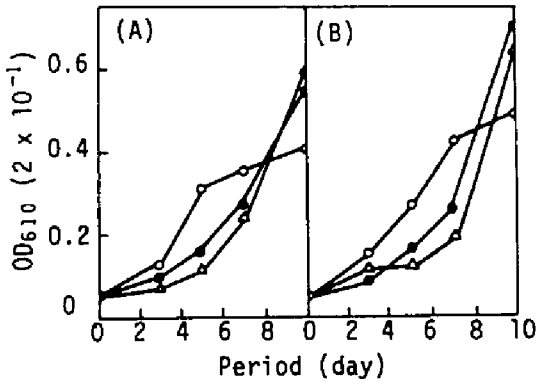

Fig. 8. Effect of C/N Ratio on the Growth.
Basal medium used was the same as mentioned in Materials and Methods. Concentrations of *n*-paraffins added were 3.8% (A) and 7.6% (B). (○) C/N ratio, 19; (●) C/N ratio, 15.2; (△) C/N ratio, 7.6.

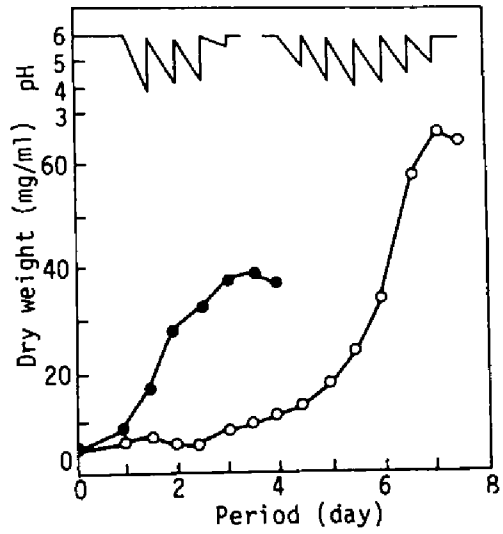

Fig. 9. Time Course of Cell Production by Strain MM313 at 13° and 28°C.
The medium was the same as Table IV. The conditions of cultivation were as follows: Equipment, 30-liter Waldhof type jar fermenter; medium volume, 13 liters; revolution, 550 rpm; aeration, 2.0 v/v/m (in 0.6 atm). The pH of medium was controlled in the range of 5.0 to 6.0 with 14% ammonia water. Cultivations were carried out at 13°C (○) and 28°C (●).

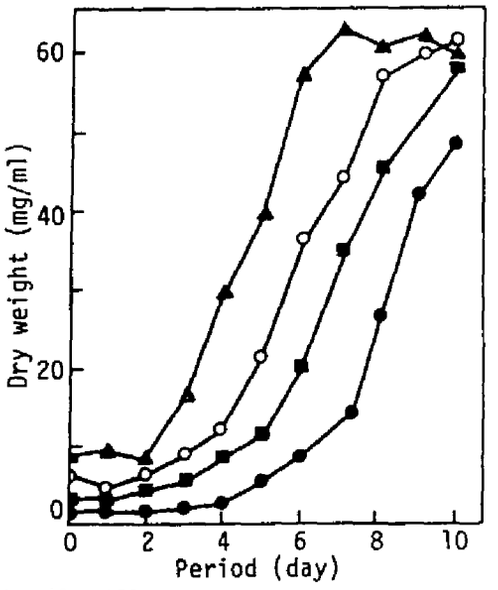

Fig. 10. Effect of Inoculum Size on the Cell Production of the Strain MM313.

Cultivation was carried out under the same conditions as that shown in Fig. 10, except for cultivation temperature (15°C) and inoculum size. Inoculum size; (▲), 10%; (○), 7%; (■), 5%; (●), 2%.

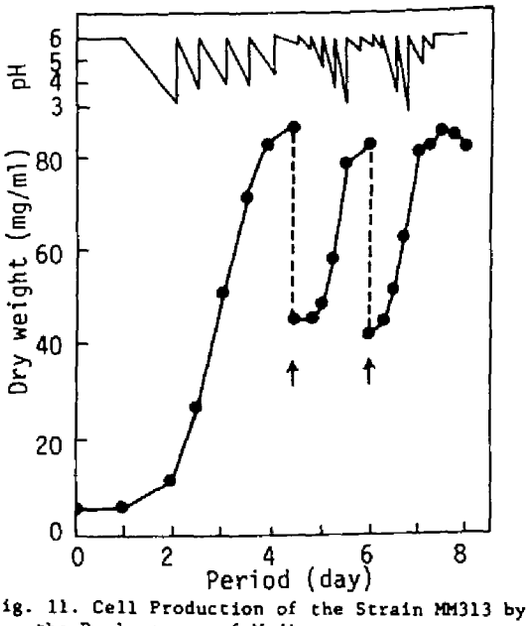

Fig. 11. Cell Production of the Strain MM313 by the Replacement of Medium.

Concentration of *n*-paraffins added was 10% (w/v). After 4.5 and 6 days ↑, a half volume of cultures was replaced by the fresh medium. Cultural conditions were the same as described in Fig. 10.

after 3 days cultivation at 28°C, was 40 g of dry cells per liter from 76 g of *n*-paraffins. While, the cell yield by 7 days cultivation at 13°C reached about 85% to initial weight of *n*-paraffins. Though the cell yield at 13°C was 1.6 times higher than at 28°C, a long period was required for the cell production.

The effect of the size of inoculum on the growth rate was investigated. The seed cultures of logarithmic phase were inoculated into the medium at various concentrations. At concentration of inoculum of 10%, the growth rate was the most rapid without decrease of the value of maximum cell yield (Fig. 10).

Effect of the replacement of medium on the cell production was examined with the cultivation at 15°C (Fig. 11). The maximum cell yield was obtained after 4.5 days, and then a half volume of culture was replaced with the fresh medium successively. After the first replacement, it took 1.5 days to obtaine the maximum cell yield, and took one day after the second replacement. The results showed the effectiveness of the half replacement of medium on the rapid cell production at low temperatures.

When the cell production from *n*-paraffin is conducted at low temperatures, the following disadvantages for the practical use were considered; time is necessary for an organism to reach the maximum level of growth and energy is necessary for cooling of a fermentor in the course of cultivation. However, using *Candida* sp. MM313, a fairly high ratio (85%) of cell yield to *n*-paraffin was obtained at 15°C, even if the medium containing 10% *n*-paraffin was used. Further, the succesive replacement of medium in the course of cultivation was effective on the reduction in period of cultivation at low temperatures.

SUMARRY

A marine yeast, strain MM313 was isolated from a marine sediment sample at depth of 1120 m. The organism was identified as a *Candida* sp. MM313.

The yeast was able to utilize *n*-paraffin, *n*-C_{10} to *n*-C_{20}. Regardless of its origin, the organism grew in a medium prepared with fresh water. However, the cell yield increased with increasing concentration of each salt in sea water in the medium and reached a maximum value at the concentration of 75%. The cultivation temperature for the maximum rate of growth and that for the maximum level of growth were 28° and 10°C respectively. Several cultural conditions were investigated. The cell yields to *n*-paraffins were about 85% at 15°C after 4 days and 56% at 28°C after 3 days under the optimum conditions obtained.

SECTION 2

UTILIZATION OF METHANOL BY YEASTS[h)]

INTRODUCTION

Recently, interest in the microbial utilization of methanol has increased. As to fermentation substrate, methanol is superior to other carbon sources, including normal alkane, methane and so on, in following respects; a) methanol is available as highly pure substrates, b) methanol is completely miscible with water, c) cells utilizing methanol have lower oxygen demand on the basis of constant productivity and d) heat evolution of methanol culture, which is directly proportion to the oxigen demand, is lower than that of the other substrate cultures.

In 1906, Söhgen first reported the isolation of a methane-oxidizing bacterium (124). Bassalik observed growth of *Bacillus extorquens* on methanol in 1914 (125); yet until after the work of Dworkin and Foster (1956) (126) little effort was devoted toward the elucidation of the mechanism of aerobic C_1 metabolism. Recenty, as the interest in methanol as fermentation substrate increases, informations describing microbial growth on methanol increases. Many methanol assimilating bacteria belonging to different genera have been well known for bacteria, for example *Pseudomonas* sp. PRL-W4 (127), *Pseudomonas methanica* (126, 128, 129) *Methanomonas methano-oxidans* (130, 131), *Pseudomonas* AM1 (132), *Pseudomonas* M27 (133), *Vibrio extorquens* (134), some species of *Methylomonas* (135), *Bacillus cereus* (136), *Arthrobacter rufescens* (136), *Hyphomicrobium* sp. WC, B522 and so on. On the other hand, no methanol-assimilating yeast had been described until only recently. In 1969, Ogata *et al.* first reported the assimilation of an yeast, *Kloeckera* sp. No. 2201 (138). Asthana *et al.* reported the assimilation of methanol by another yeast presumed to be a strain of *Torulopsis glabrate* (139). Sahm and Wagner studies the growth of a *Candida boidinii* on methanol (140). Fujii and Tonomura (141) reported the assimilation of methanol by some strains of *Candida*, *Saccharomyces* and *Torulopsis*. Oki *et al.* isolated twenty strains of methanol assimilating yeasts from rotten plants, and indicated to include two new species; *Candida methanolica* and *Torulopsis methanolovescens* (142). Hazeu *et al.* found to be able to utilize methanol by 15 strains of yeasts belonging to genera, *Hansenula*, *Phichia*, *Torulopsis* and *Candida*, among the type culture of the Centraal Bureau voor Schimmelculture (CBS) (143).

The availability of quantitative data describing microbial growth on methanol has been increased cellular yields are found to vary from 0.19 to 0.45 gram dry cell weight per gram of methanol with most cultures having yield of about 0.4. Specific growth rates or mass doubling times vary tremendously among the various isolates depending on the growth environment and the methanol concentration. Methanol utilizers are capable of doubling their mass in as little as 2 hr.

It is of interest to examine to biochemical problems which are posed by energy transduction and biosynthesis of cell constituents from the reduced one carbon compound in the organism. The pathway of aerobic methanol oxidation has been shown to proceed in the manner first proposed by Dworkin and Foster (126),

$(CH_4 \rightarrow)\ CH_3OH \rightarrow HCHO \rightarrow HCOOH \rightarrow CO_2$

The enzyme catalizing each step is confirmed in cell extracts or purified enzymes. (126 - 129, 156).

As to the biosynthesis of cellular constituents during growth on methanol, it is now clear that at least two distict mechanisms exist. One of these, the serine pathway (145 - 148), seems to occure in facultative methylotrophs (135, 144)

which are able to grow on various carbon compounds as well as reduced C_1 compounds. The other one, the allulose pathway (149 - 148), has so far been described only in the obligate methylotrophs (135, 144). These two pathways have been examined in detail by Quayle and his co-workers. The serine pathway involves a hydroxymethylation of formaldehyde with glycine to form serine. The serine formed is metabolized to oxaloacetate *via* hydroxypyruvate, glycerate, 3-phosphoglycerate and phosphoenol pyruvate. In this pathway, glycine is formed by the transamination between glyoxalate and serine. However, it has not been clear whether glyoxalate is synthesized by a splitting of an unknown C_4 compound or by a condensation of carbon dioxide and the C_1 compound.

C_1 units are incorported into the allulose phathway by condensation of three formaldehyde with three ribose 5-phosphate molecules to form the six-carbon, allulose 6-phosphate. The allulose 6-phosphate is epimerized to fructose 6-phosphate and then phosphorylated to fructose 1,6-diphosphate. The fructose 1,6-diphosphate is splitted to glyceraldehyde 3-phosphate and dihydroxyacetone 3-phosphate. The dihydroxyaceton 3-phosphate can then enter the glycolytic pathway, and the glyceraldehyde 3-phosphate molecules to regenerate three ribose 5-phosphate *via* the reaction being similar to the Calvin cycle.

So far most studies on biochemical aspects of assimilation of reduced C_1-compound such as methanol have been carried out using the bacterial strains. Recently a few information of metabolism of methanol utilizing yeasts have been obtained. A methanol-oxidizing enzyme of the supposed *Kloeckera* sp. No. 2201 was isolated and purified by Tani *et al.* (152, 153). The enzyme is a kind of alcohol oxidase containing FAD as prosthetic group. The properties are different from those of methanol-oxidizing enzyme isolated from methanol-utilizing bacteria. Thereafter, Fujii and Tonomura also recognized the similar enzyme in *Candida* sp. (141). Further, with the yeast, Fujii and Tonomura (154) showed that carbon of methanol was rapidly incorporated initially into hexose phosphates.

In this section, cultural conditions for methanol utilization of yeasts are described. The cultural conditions for *Kloeckera* sp. No. 2201 have been already documented (138, 155). But it is thought that the cell yield is lower and the growth rate is slower, comparing with other methanol utilizing yeasts. In order to obtain the better conditions for culturing on methanol of *Kloeckera* sp. No. 2201, following experiments are performed.

MATERIALS AND METHODS

Microorganism. *Kloeckera* sp. No. 2201 was mainly used in the experiment. Partly, four strains of yeast, *Torulopsis pinus* IFO 0741, *Hansenula capsulata* IFO 0974, *Pichia pinus* IFO 1342, *Pichia trehalophile* IFO 1282, which were found to be able to assimilate methanol by Hazeu *et al.* (143) and two yeasts, *Candida methanolica* and *Torulopsis methanolovescens*, which were isolated by Oki and Kouno (142) were also used.

Medium. The basal medium consisted of methanol 2 g, NH_4Cl 0.4 g, K_2HPO_4 0.1 g, KH_2PO_4 0.1 g, $MgSO_4 \cdot 7H_2O$ 0.05 g and growth substance in 100 ml of tap water, pH 6.0. Methanol was added to the medium which had been separately sterilized.

Cultivation. One loopful cells grown on the methanol agar slant was inoculated into 5 ml of the medium in a test tube (1.6 x 16 cm). The cultivation was carried out at 28°C on a reciprocal shaker (130 rpm). In the case of 300 ml-flask culture, 1 ml of the tube culture was used as seed. The cultivation was carried out at a desired temperature on a rotary shaker (240 rpm).

Determination of cell yield. The cells in the cultured broth were collected by centrifugation at 5000 x *g* for 10 min and washed twice with deionized water. An optical density at 610 mμ of the final suspension was measured.

RESULTS AND DISCUSSION

Effect of several growth factors on the growth of Kloeckera *sp. No. 2201*

It has been shown that *Kloeckera* sp. supplementally required thiamine for growing on methanol (155). Conventional medium of the organism contained a vitamin mixture as growth factor. The effect of several growth factor on the growth of the yeast was examined. The yeast was cultured on the medium containing vitamin mixture, yeast extract or corn steep

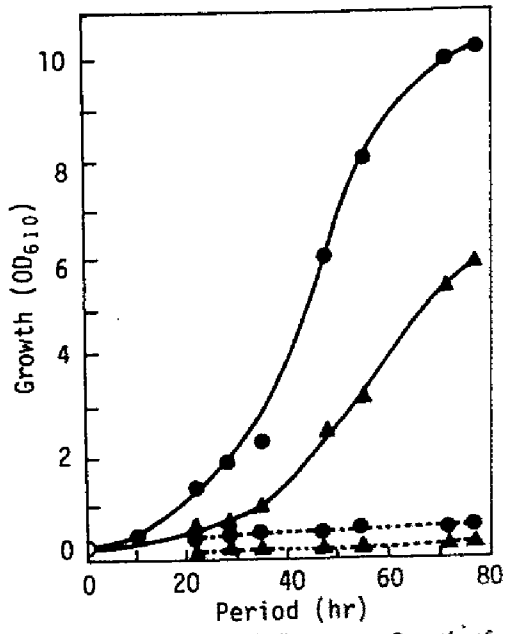

Fig. 1. Effect of Growth Factor on Growth of *Kloeckera* sp. No. 2201.

The basal medium contained 2.0 g methanol, 0.4 g NH_4Cl , 0.1 g K_2HPO_4, 0.1 g KH_2PO_4, and 0.05 g $MgSO_4 \cdot 7H_2O$ in 100 ml tap water, pH 6.5. CSL was added in the concentration of 0.2%. The concentration of vitamin mixture was described in Materials and Methods. (●—●) CSL added, (●--●) CSL added and methanol less, (▲—▲) vitamin mixture added, (▲--▲) vitamin mixture added and methanol less.

liquor (C.S.L). Among the growth factors, C.S.L was the most effective for both growth rate and cell yield. As shown in Fig. 1, the cell yield on the medium containing 0.2% C.S.L was about 2.7 times that on the medium containing vitamin mixture. In these cases, the growth on each methanol-less medium was negrigible. In the following experiments, the medium containing 0.2% C.S.L was used.

Effect of methanol concentration on the growth

As shown in Fig. 2, the optimal concentration of methanol in the batch-wise culture was found to be 1 to 2%. In the higher concentrations, e.g. greater than 5% methanol, the growth was extremely inhibited.

Effect of pH on the growth

The effect of initial pH of medium on the growth was examined. As shown in Fig. 3, the optimum pH for the growth was found to be 5.0 to 6.5. During the growth on methanol, the drop of pH of the medium was observed. Thus, to obtaine a

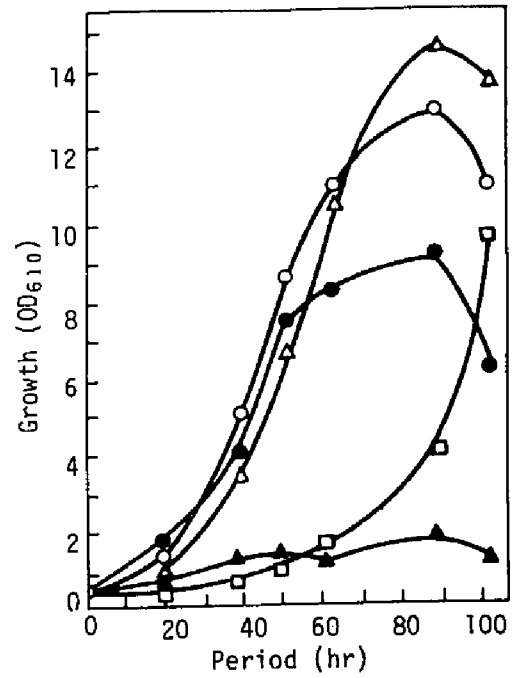

Fig. 2. Effect of Methanol Concentration on Growth of *Kloeckera* sp. No. 2201.

The composition of medium was the same as described in Fig. 1, except for methanol concentration. Methanol: (▲) 0%, (●) 1%, (○) 2%, (△) 3%, and (□) 5%.

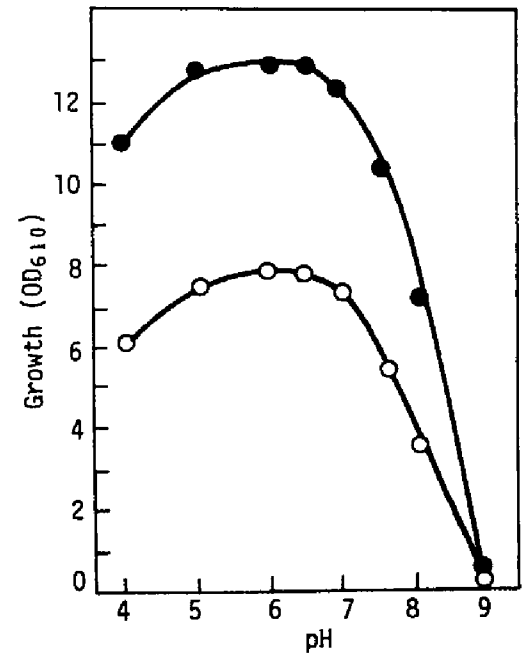

Fig. 3. Effect of pH on the Growth of *Kloeckera* sp. No. 2201.

The medium contained 2.0 g methanol, 0.4 g NH_4Cl, 0.1 g K_2HPO_4, 0.1 g KH_2PO_4, 0.05 g $MgSO_4 \cdot 7H_2O$, and 0.2 g CSL in 100 ml of tap water at various pH indicated. 71 hr cultivation (●), 44 hr cultivation (○).

better growth yield, the pH should be controlled.

Relationship between temperature and growth of Kloeckera *sp. No. 2201 and other yeasts*

Figure 4 shows the growth curves of *Kloeckera* sp. No. 2201 at various temperatures. The organism was able to grow up to 30°C. The optimum temperature was found to be 25°C. The cell yield reduced at 30°C, compared with 20°C. It is appeared that the yeast has relatively low

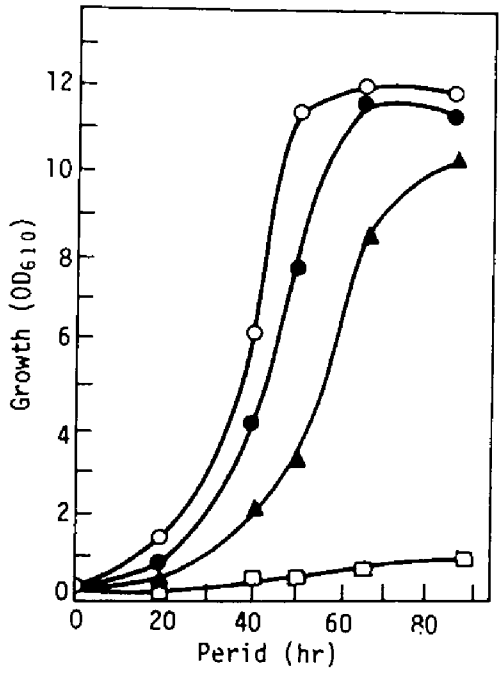

Fig. 4. Effect of Temperature on the Growth of *Kloeckera* sp. No. 2201.

The composition of the medium was the same as described in Fig. 3. (pH 6.5). Temperature: (●) 20°C, (○) 25°C, (▲) 30°C, and (□) 35°C.

growth temperatures. This temperature relationship coincides with the fact that the activities of alcohol oxidase and formaldehyde dehydrogenase from the yeast are optimum at the temperature below 30°C as described latter.

The relationships between temperature and growth of several methanol-utilizing yeasts were investigated. The results are presented in Table I. The optimum temperature for growth of most of the yeasts were found to be 25° to 30°C. However, *Torulopsis pinus* IFO 0741 was anable to grow at 28°C and its optimum temperature was found to be 20°C. Although

TABLE I. GROWTH TEMPERATURE OF METHANOL UTILIZING YEASTS

Medium used was the same as described in Materials and Methods. Cultivation was carried out under shaking at various temperatures.

Strain	Growth temperature	
	Optimum	Maximum
Kloeckera sp.	25°C	30°c
Candida methanolica	25-30	35
Torulopsis pinus	20	25
Torulopsis methanolovescens	25-30	35
Hansenula capsulata	25	28
Pichia pinus	28	30
Pichia trehalophile	30	35

no data regarding the growth at low temperatures was obtained, on the basis of optimum and maximum growth temperature, the yeast may be classified as a psychrophile.

A jar fermenter scale cultivation for cell production

A cultivation was carried out with 30-liter Waldhof-type fermenter in the following conditions; temperature, 25°C; revolution, 300 rpm; aeration, 2.0 v/v/m and medium volume, 15 liters. Under the best conditions obtained preceding ex-

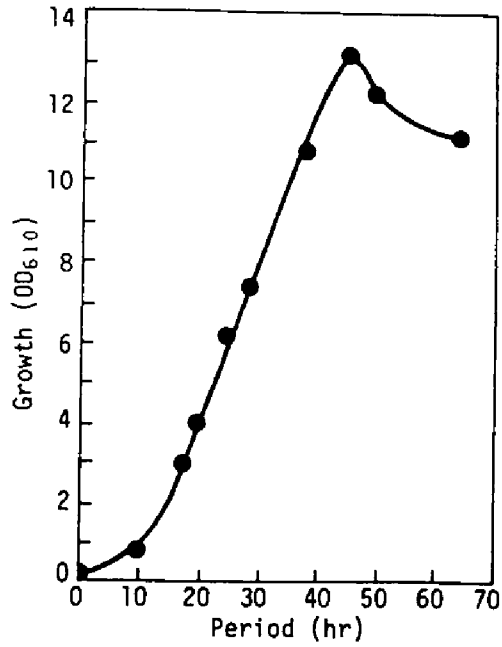

Fig. 5. Jar-fermenter Scale Cultivation for Cell Production.

The compostion of the medium was the same as described in Fig. 3. Cultural conditions were as follows: temperature, 25°C; revolution, 300 rpm; aeration, 1 v/v/m (0.6 atm).

periments, growth rate and cell yield were calculated to be μ_{max}, 0.13 hr^{-1}, and 0.40 g dry cell weight per g methanol, respectively. These values obtained are higher than values described previously (138, 155).

Among the factors investigated, the addition of C.S.L and the lowing temperature may be thought to be the most effective to rise both growth rate and cell yield.

SUMMARY

Some cultural conditions for cell production of a yeast, *Kloeckera* sp. No. 2201, from methanol were investigated. As for growth factor, C.S.L was the most effective for both growth rate and cell yield. The cell yield on the medium containing 0.2% C.S.L was about 2.7 times that on the medium containing a vitamin mixture. The optimum concentration of methanol was found to be 1 to 2% and at the higher concentration, the growth was depressed. The optimum pH was 5.0 to 6.5. The best cell yield from methanol was attained at 25°C. Under the best conditions obtained, the growth rate and the cell yield were calculated to be μ_{max} = 0.13 hr^{-} and 0.40 g dry cell weight per g methanol, respectively. The relationships between temperature and growth of the six types of methanol-utilizing yeast were tested. Among the yeasts, *Torulopsis pinus* IFO 0741 grew optimally at 20°C.

CHAPTER V

OXIDATION OF C_1 COMPOUNDS BY METHANOL-UTILIZING YEASTS

SECTION 1

PURIFICATION AND PROPERTIES OF FORMALDEHYDE DEHYDROGENASE IN A METHANOL-UTILIZING YEAST, *KLOECKERA* SP. No. 2201[i)]

INTRODUCTION

It has been suggested that the methanol oxidation by methanol utilizing bacteria, such as pseudomonads, proceeds as follows (126 - 129).

$CH_3OH \longrightarrow HCHO \longrightarrow HCOOH \longrightarrow CO_2$

Some informations dealing with the properties of enzymes which catalyze each step of the methanol oxidizing system has been accumulated (126 - 129, 156).

Tani *et al.* (152, 153) have reported on the properties of a methanol oxidizing enzyme from the methanol utilizing yeast, *Kloeckera* sp. No. 2201. The enzyme was isolated in crystalline form from the cell-extract, and was characterized as an alcohol oxidase containing FAD as a prosthetic group, in which it differed from other methanol-oxidizing enzymes in methanol-utilizing bacteria.

In the present section the author wishes to clarify a subsequent reaction in the methanol-oxidation system of *Kloeckera* sp. No. 2201. A formaldehyde oxidizing enzyme was found in the cell-extract of this yeast. This section describes the formation, purification, and characterization of the enzyme.

MATERIALS AND METHODS

Organism. *Kloeckera* sp. No. 2201, which was able to grow on methanol as a sole source of carbon, was used throughout this study (138).

Materials. DEAE-cellulose was a gift from Green Cross Co., Osaka. Hydroxylapatite was purchased from Seikagaku Kogyo Co., Ltd., Tokyo. Selecta-electrophoresis-membrane (cellulose acetate membrane) was purchased from Carl Schlicher and Schule, Dassel. Formaldehyde solution (37%) of guaranteed reagent grade was obtained from Nakarai Chemicals, Ltd., Kyoto, and this solution was standardized by the method of Yoe and Reid (157). All other chemicals were obtained from commercial sources and were used without further purification.

Medium and cultivation. The medium for culture consisted of 3 g methanol, 0.4 g NH_4Cl, 0.1 g KH_2PO_4, 0.1 g K_2HPO_4, 0.05 g $MgSO_4 \cdot 7H_2O$ and 0.2 g yeast extract in 100 ml of tap water, pH 6.0. Methanol was added to the medium after the sterilization of the other components of the medium. The medium for subculture contained 1% of glucose instead of methanol as the carbon

source.

Preparation of cell-extract. The cells in the cultured broth were harvested by continuous flow centrifugation and were washed with 0.01 M potassium phosphate buffer, pH 7.5. The washed cells were suspended in appropriate volumes of the above buffer containing 0.001 M dithiothreitol (DTT) and treated with a 19 kHz Kaijo Denki ultrasonic oscillator for 5 hr. The cells and debris were removed by centrifugation at 12,000 x *g* for 30 min. The resultant supernatant was used as the cell-extract. The above procedures were carried out below 5°C.

Measurement of formaldehyde dehydrogenase activity. The complete reaction system in a 3.5 ml silica cell (1 cm light path) consisted of 1.2 μmoles of formaldehyde, 0.67 μmole of NAD, 0.67 μmole of reduced glutathione (GSH), 6.6 μmoles of potassium phosphate buffer (pH 7.5), enzyme, and water to a total volume of 3.0 ml. The blank cell lacked formaldehyde. The reaction was started by the addition of formaldehyde, and the increase in the extinction at 340 mμ was followed in a Shimadzu double beam spectrophotometer Type UV-200.

Specific activity of the enzyme was expressed as units per mg of protein, where one unit is defined as the increase of 0.10 in the optical density at 340 mμ during the initial 1 min incubation.

Analytical method. Formaldehyde was determined by the spectrophotometric measurement of the complex formed between formaldehyde and chromotropic acid according to the method of Frisell and Mackenzie (158). Formic acid was determined using a colorimetric method according to Grant (159), which was based on the reduction to formaldehyde by means of magnesium, with subsequent measurement of the formaldehyde by the method described above. Protein was determined by measurement of the absorbancy at 280 mμ.

For the investigation of the purity of the enzyme preparation, electrophoresis on cellulose acetate membrane was carried out at 0.5 mA/cm (560 v) on cooling plates for 1 hr, according to the procedure of Kohn (160). Amido black was used for the staining of protein.

RESULTS

Formation of formaldehyde dehydrogenase

Kloeckera sp. No. 2201 was grown on a medium containing 3.0% methanol or 2.0% glucose as carbon sources. Figure 1 shows the time courses of the formation of formaldehyde dehydrogenating enzyme in

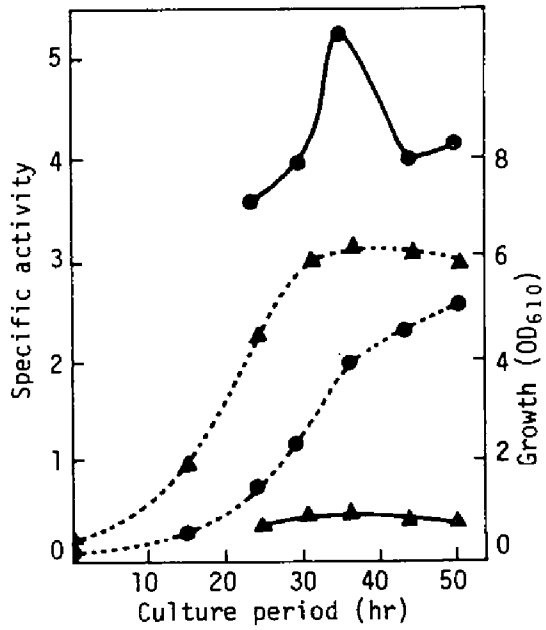

Fig. 1. Specific Activity of Formaldehyde Dehydrogenating Enzyme in Methanol- or Glucose-Grown Cells.

Cultivations were carried out at 28°C with shaking. The enzyme activity was measured under the standard assay conditions. (▲–▲); growth on glucose, (●–●); growth on methanol, (▲–▲); specific activity in glucose-grown cells, (●–●); specific activity in methanol-grown cells.

cells which were grown on each of the media. The specific activity of the enzyme from methanol-grown cells was higher than that from glucose-grown cells by about 10 times.

Purification of the enzyme

In 0.01 M potassium phosphate buffer, pH 7.5, the enzyme activity was lost almost completely within a week at 0° to 5°C. But, by the addition of DTT (final concentration: 0.001 M) to the buffer, the enzyme activity could be maintained at these temperatures without measurable loss for at least 2 weeks. All operations were carried out at 0° to 5°C throughout the purification procedures.

Step 1. Preparation of the cell-extract. A cell-paste was obtained from 30 liters of cultured broth, and the cell-extract was prepared in the same manner as described in Materials and Methods.

Step 2. Ammonium sulfate fractionation. Solid ammonium sulfate was added to 670 ml of the cell-extract to 0.40 saturation. After standing overnight, the precipitate was removed by centrifugation at 12,000 x *g* for 30 min and discarded. The ammonium sulfate concentration of the supernatant was increased to 0.70 saturation. After standing overnight, the precipitate was collected by centrifugation and dissolved in 0.01 M potassium phosphate buffer, pH 7.5, containing 0.001 M DTT. The enzyme solution was dialyzed for 18 hr against four changes of 5 liter volumes of the same buffer.

Step 3. 1st DEAE-cellulose column chromatogrphy. The dialyzed enzyme solution (120 ml) was chromatographed on a DEAE-cellulose column. An elution was carried out with stepwise increases in the buffer concentration. The elution pattern of the chromatographed enzyme is shown in Fig. 2. The enzyme was eluted with 0.1 M

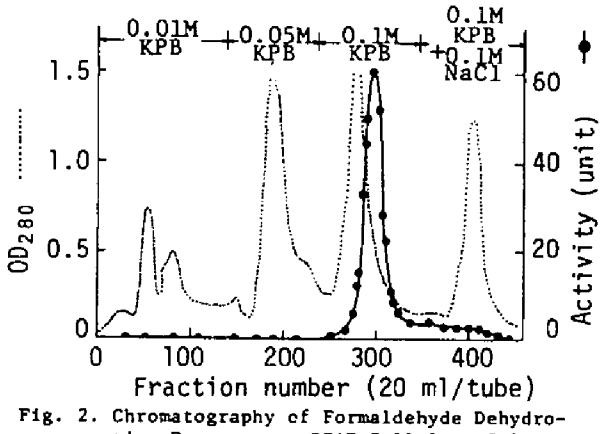

Fig. 2. Chromatography of Formaldehyde Dehydrogenating Enzyme on a DEAE-Cellulose Column.

The dialyzed enzyme solution (120 ml) was applied to a DEAE-cellulose column (5 x 40 cm) which had been equilibrated with 0.01 M potassium phosphate buffer, pH 7.5, containing 0.001 M DTT, and then washed with the same buffer, followed by a stepwise elution involving an increase in the buffer concentration. The buffers were allowed to flow at a rate of 1 ml/min and 20 ml fractions were collected.

potassium phosphate buffer, pH 7.5, containing 0.001 M DTT. The active fractions were combined to give 750 ml, which was concentrated by the addition of solid ammonium sulfate to 0.70 saturation. The precipitate obtained by centrifugation at 12,000 x *g* for 30 min was dissolved in a small volume of the buffer (0.01 M), and dialyzed for 18 hr against four changes of 2 liters volumes of the same buffer.

Step 4. 2nd DEAE-cellulose column chromatography. The dialyzed enzyme solution was rechromatographed on a column of DEAE-cellulose. The enzyme solution was placed on the column (3 x 45 cm) and eluted by application of a 4 liter-linear salt gradient (from 0 to 0.1 M NaCl in 0.05 M potassium phosphate buffer, pH 7.5, containing 0.001 M DTT; fractions, 10 ml; flow rate, 0.5 ml/min). The active fractions were combined, and subsequently concentrated in the same manner as described in the above step. The concentrated enzyme solution was dialyzed for 18 hr against four changes of 2 liters volumes of the 0.01 M buffer.

Step 6. Hydroxylapatie column chromatography. The dialyzed enzyme solution was placed on a column of hydroxylapatite (2.5 x 21 cm) which had been equilibrated with 0.01 M potassium phosphate buffer, pH 7.5, containing 0.001 M DTT. The successive elutions were carried out with 0.01, 0.02, 0.05, and 0.1 M potassium phosphate buffer. The active fractions were found in the eluate with the 0.1 M potassium phosphate buffer. The enzyme solution collected (300 ml) was concentrated by ultrafiltration, using a collodion bag.

Step 7. Sephadex G-200 gel filtration The enzyme solution obtained in the preceding step was subjected to Sephadex G-200 gel filtration. Figure 3 shows a typical elution pattern of the enzyme. These active fractions were combined to give 60 ml, and concentrated by ultrafiltration.

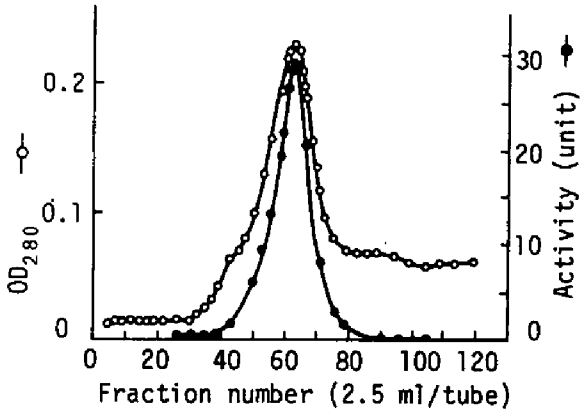

Fig. 3. Gel Filtration of the Enzyme on Sephadex G-200.

The Sephadex G-200 was packed into a column (2 x 100 cm) and equilibrated with 0.01 M potassium phosphate buffer, pH 7.5, containing 0.001 M DTT. The enzyme solution was placed on the column and then the buffer was allowed to flow at a rate of 10 ml per hr and 3 ml fractions were collected.

TABLE I. SUMMARY OF THE PURIFICATION OF THE FORMALDEHYDE DEHYDROGENATING ENZYME

Treatment	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Purification
Crude extract	22780	134000	6	1
Ammonium sulfate	5520	66240	12	2
1st DEAE-cellulose	1000	115200	115	19
2nd DEAE-cellulose	513	68400	133	23
Hydroxylapatite	57.3	29400	516	88
Sephadex G-200	19.5	12200	629	107

The enzyme was precipitated by the addition of solid ammonium sulfate to 0.70 saturation and stored in a refrigerator at 5° to 7°C.

A summary of the purification of the enzyme is presented in Table I. In the results, the enzyme preparation which was chromatographed on DEAE-cellulose had a specific activity of 115; recovery of the activity was about 175% of that of the preceding step. This excess yield of activity was probably due to the removal of NADH oxidase activity.

Homogeneity

As shown in Fig. 4, the analysis of the purified enzyme preparation by ultracentrifugation showed a nearly single and symmetrical Schlieren peak. An electrophoretic analysis was carried out under the conditions described in Materials and Methods. From the results, it appeared that the enzyme preparation was accompanied by a small amount of protein which did not exhibit enzyme activity. The purity of the enzyme preparation was assayed by comparing the extention of color-staining with amido black: the purity obtained was about 90%.

Properties of the purified enzyme

1) Absorption spectrum. The absorption spectrum of the purified formaldehyde dehydrogenating enzyme was measured with a Shimadzu double beam spectrophotometer Type UV-200. Figure 5 shows that the enzyme, in 0.01 M potassium phosphate buffer, pH 7.5, containing 0.001 M DTT, exhibited only one absorption maximum, near 280 mμ, over the range of wave lengths investigated.

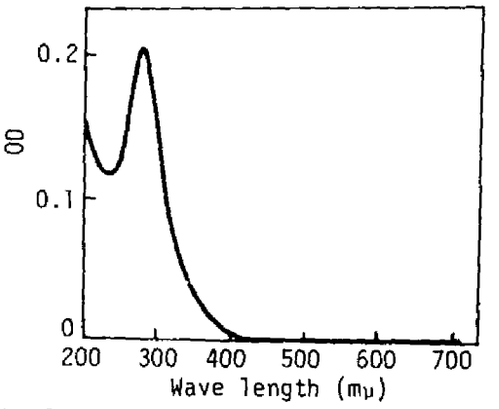

Fig. 5. Absorption Spectrum of the Purified Enzyme.

Purified enzyme was used in 0.01 M potassium phosphate buffer, pH 7.5, containing 0.001 M DTT.

2) Substrate specificity. The reaction system for formaldehyde dehydrogenation consisted of 1.2 μmoles formaldehyde or other aldehydes, 0.67 μmole NAD, 0.67 μmole GSH, 6.6 μmoles potassium phosphate buffer, pH 7.5, enzyme, and water to a total volume

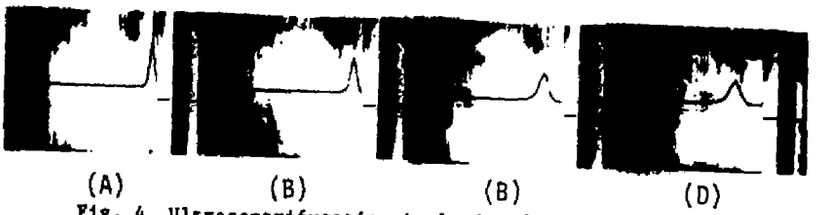

Fig. 4. Ultracentrifugation Analysis of the Purified Enzyme.

Photographs of Schlieren patterns were taken at 12((A), 18 (B), 42 (C), and 60 (D) min after reaching 54,000 rpm. Protein, 0.5% in 0.01 M potassium phosphate buffer, pH 7.5, containing 0.001 M DTT.

TABLE II. REACTION SYSTEM OF FORMALDEHYDE DEHYDROGENASE

The complete system consisted of 1.2 μmoles formaldehyde, 0.67 μmole NAD, 0.67 μmole GSH, 6.6 μmoles potassium phosphate buffer (pH 7.5), enzyme, and water to a total volume of 3.0 ml. The enzyme reaction was carried out under the standard assay conditions.

Reaction system	Activity (unit)
Complete system	4.0
minus formaldehyde	0.0
minus NAD	0.0
NADP*	0.0
minus GSH	0.0
minus enzyme	0.0

*A reaction system in which NAD was replaced by NADP was used.

of 3.0 ml. As shown in Table II, no reaction was observed when any constituent was omitted from the reaction system. The system was specific for NAD. No appreciable reduction of NADP occurred. The *Km* for NAD was 0.025 mM. The addition of GSH as a cofactor was necessary for the oxidation of formaldehyde by NAD; thiol compounds, such as cysteine 2-mercaptoethanol, DTT, or thioglycerol, were not able to replace GSH as a cofactor. When GSH was omitted from the reaction mixture described above, NAD-linked formaldehyde dehydrogenase could not to be detected even in the crude cell-extract.

The oxidation of various aldehydes by the dehydrogenase is shown in Table III. Methylglyoxal as well as formaldehyde was effective as substrate, but the enzyme was unreactive toward the other aldehydes tested. The *Km* of formaldehyde for the enzyme was found to 0.29 mM, while the *Km* for methylglyoxal was 2.8 mM.

TABLE III. SUBSTRATE SPECIFICITY OF THE ENZYME

The enzyme activity was measured under the standard assay conditions. A reaction mixture (3.0 ml) which contained 1.2 umoles of aldehyde was used.

Substrate	Activity (ΔE_{340} for 1 min)
Formaldehyde	0.09
Acetaldehyde	0.00
Propionaldehyde	0.00
Butylaldehyde	0.00
Isobutylaldehyde	0.00
Glutaraldehyde	0.00
Glyceraldehyde	0.00
Glycolaldehyde	0.00
Benzaldehyde	0.00
Glyoxal	0.00
Methylglyoxal	0.08

3) *Stoichiometry of formaldehyde dehydrogenation.* The stoichiometry of the reaction was established by the use of a reaction system containing 0.1 μmole of formaldehyde as substrate. The reaction was carried out at 30°C in a silica cell. When formaldehyde was completely consumed, the reaction was stopped by the addition of 0.2 ml of 4 N HCl. The concentration of NADH formed was determined by the measurement of the increment of the optical density at 340 mμ (extinction coefficient; $E_{340} = 6.22 \times 10^6$ cm^2/mole). An aliquot of the reaction mixture was taken up, and then formaldehyde and formic acid were determined. It was shown that one μmole each of formic acid and NADH were produced for each μmole of formaldehyde oxidized (Table IV). Thus, the reaction may be described by the following equation:

$$HCHO + NAD + H_2O \longrightarrow HCOOH + NADH + H^+$$

TABLE IV. STOICHIOMETRY OF FORMALDEHYDE DEHYDROGENATION BY THE ENZYME

The reaction was carried out at 30°C in a 3.5 ml silica cell (1 cm light path). The experimental methods are described in the text and the determination of the product was performed as described in Materials and Methods.

Formaldehyde decreased (μmole)	NAD formed (μmole)	Formic acid formed (μmole)
0.100	0.109	0.108

4) *Effect of temperature on the enzyme activity.* Figure 6 shows the variation of the enzyme activity with temperature. The maximum activity was observed at 30°C.

Figure 7 shows the effect of temperature on the stability of the enzyme. The enzyme was stable up to 20°C, but was unstable above 30°C. The activity was almost lost at 50°C under the standard assay conditions.

5) *Effect of pH on the enzyme activity.* The variations of enzyme activity with pH are shown in Fig. 8. The optimum pH of the enzyme was found to be at 8.0 in potassium phosphate buffer. The activity of the enzyme in Tris-HCl buffer was lower than that in the potassium phosphate buffer. It seemed that Tris-HCl buffer inhibited the activity of the enzyme.

Figure 9 shows the pH stability of the enzyme. The enzyme was incubated at

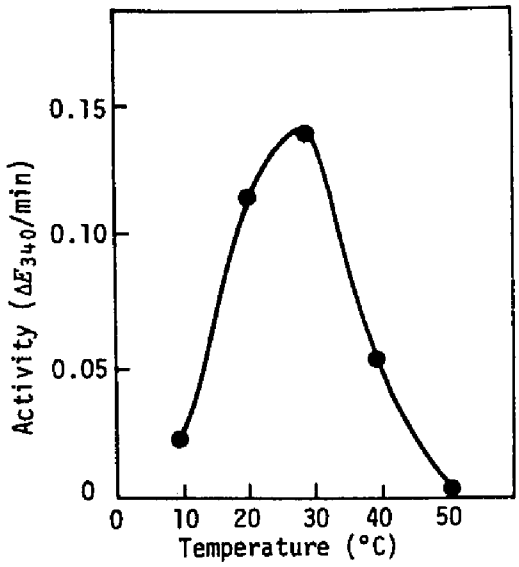

Fig. 6. Effect of Temperature on the Enzyme Activity.

The enzyme activity was measured under the standard assay conditions at various temperatures.

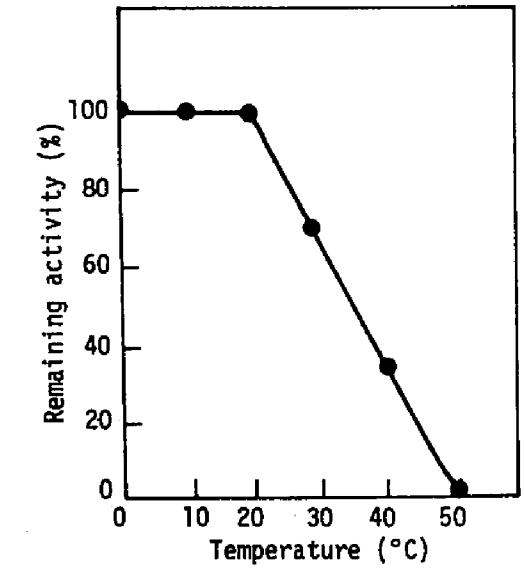

Fig. 7. Stability of the Enzyme against Temperature.

The enzyme, in 0.01 M potassium phosphate buffer, pH 7.5, containing 0.001 M DTT, was preincubated for 10 min at each temperature as indicated. The remaining activity was measured under the standard assay conditions.

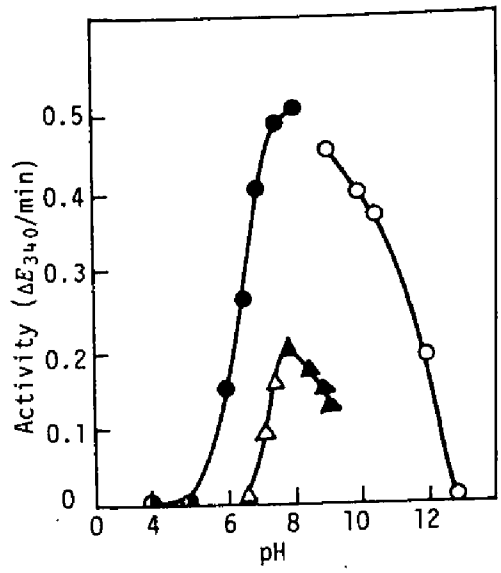

Fig. 8. Effect of pH on the Enzyme Activity.

The enzyme activity was measured under the standard assay conditions using the following buffers: potassium phosphate - citric acid buffer (◑), potassium phosphate buffer (●), boric acid NaOH buffer (○), Tris-maleate buffer (△), or Tris-HCl buffer (▲) at various pH values.

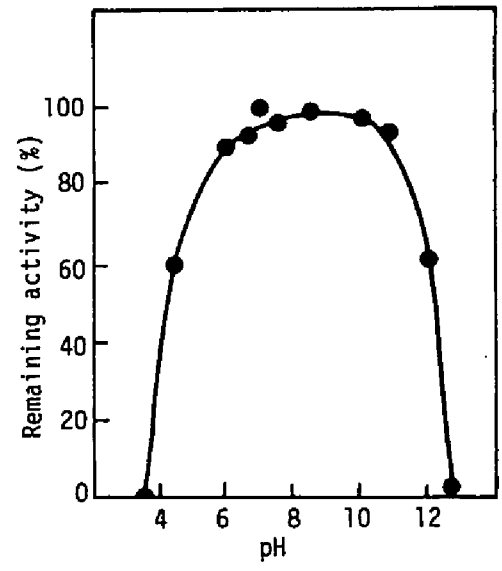

Fig. 9. Stability of the Enzyme against pH.

The enzyme, in each of the buffers described in Fig. 8, was preincubated at 20°C for 1 hr. The remaining activity was measured under the standard assay conditions.

a given pH for 1 hr at 20°C, and then assayed for the remaining activity under the standard assay conditions. The enzyme was stable over the pH range 6.0 to 11.0.

6) Effect of metal ion on the enzyme activity. As shown in Table V, the enzyme activity was completely inhibited by Cd^{2+}, Cu^{2+}, Hg^{2+}, and Ag^{+} at a concentration of 1 mM.

7) Effect of inhibitor on the enzyme activity. The results are shown in Table

TABLE V. EFFECT OF METAL ION ON THE ENZYME ACTIVITY

The enzyme was preincubated with each metal ion (1 mM) for 1 hr at 20°C, and then the remaining activity was measured under the standard assay conditions.

Salt	Inhibition (%)	Salt	Inhibition (%)
None	0	$CuSO_4$	100
$NiCl_2$	14	$HgCl_2$	100
$CdCl_2$	100	$AgNO_3$	100
$MnCl_2$	42	$SrCl_2$	0
$ZnCl_2$	73	Pb·acetate	0
$CoCl_2$	0	$(NH_4)_2Mo_7O_{24}$	33
Ba·acetate	14	$AlCl_3$	22
$CaCl_2$	0		

TABLE VI. EFFECT OF INHIBITOR ON THE ENZYME ACTIVITY

The enzyme was preincubated with each inhibitor for 1 hr at 20°C, and then the remaining activity was measured under the standard assay conditions.

Inhibitor	Concentration (mM)	Inhibition (%)
KCN	1	63
EDTA	1	0
	10	0
Oxalate	10	24
Citrate	10	0
o-Phenanthroline	1	59
α,α'-Dipyridyl	1	93
p-CMB	1	100
Iodoacetate	1	0
	10	100
DFP	1	0

VI. The enzyme activity was completely inhibited by sulfhydryl reagents, such as *p*-CMB and iodoacetate at concentrations of 1 mM and 10 mM, respectively. The activity was slightly inhibited by chelating reagents such as *o*-phenanthroline and α,α'-dipyridyl, but was not inhibited by EDTA even at higher concentration (10 mM).

DISCUSSION

These studies have shown the presence of an NAD-linked formaldehyde dehydrogenating enzyme in *Kloeckera* sp. No. 2201, which utilized methanol as a sole source of carbon. The enzyme was purified about 107-fold from the cell-extract of the yeast. Experiments with purified enzyme showed that the enzyme required NAD and GSH for activity. NAD was unable to be replaced by NADP. The GSH requirement of the enzyme could not be satisfied by other thiol-compounds. It may be thought that GSH participates directly in the dehydrogenation rather than action non-specifically to protect protein thiol-groups. The enzyme was reactive only towards formaldehyde and methylglyoxal among the aldehydes tested.

Most of the NAD- or NADP-linked aldehyde dehydrogenases that have been described cannot catalyze the dehydrogenation of formaldehyde (161), except for NAD- or NADP-dependent aldehyde dehydrogenase (aldehyde:NAD (P) oxidoreductase, EC 1.2.1.5) obtained from *Pseudomonas fluorescens* by Jakoby (162). On the other hand, Strittmatter and Ball (163) have identified a formaldehyde-specific, NAD-linked and GSH-dependent dehydrogenase (formaldehyde:NAD oxidoreductase EC 1.2.1.1) in beef liver. GSH was specifically required for the enzyme reaction. The role of GSH in the system has been postulated as follows: Formaldehyde non-enzymatically reacts with GSH to form S-hydroxymethylglutathione which is subsequently attacked by the enzyme (163). A similar type of formaldehyde dehydrogenating enzyme has been purified from baker's yeast (164), and from human and rat livers (165). Several enzymes which catalyze the oxidation of formaldehyde have been studied in the methanol-utilizing bacteria. Harrington and Kallio (128) have shown the presence of an NAD-linked, GSH-dependent formaldehyde dehydrogenating enzyme in the crude-extract of methanol-grown *Pseudomonas methanica*. Johnson and Quayle (129) demonstrated an aldehyde dehydrogenating enzyme of broader specificity in *Pseudomonas* AM1. This enzyme was different from other aldehyde dehydrogenases in its requirement of 2,6-dichlorophenol-indophenol or phenazine methosulfate for a primary electron acceptor.

From the facts of the GSH requirement and the narrow substrate specificity for the dehydrogenation, the enzyme from *Kloeckera* sp. No. 2201 was concluded to be a kind of formaldehyde dehydrogenase (formaldehyde:NAD oxidoreductase, EC 1.2.1.1). Several differences were noted between the NAD-linked and GSH-dependent formaldehyde dehydrogenase from *Kloeckera* sp. No. 2201 and baker's yeast reported by Rose and Racker (164). Glyoxal and methylglyoxal have been reported to be good substrates for the latter, but the enzyme from *Kloeckera* sp. No. 2201 was unreactive towards glyoxal. The *Km* of NAD

for the enzyme of *Kloeckera* sp. was 0.025 mM, while the *Km* for that of baker's yeast was reported to be 0.68 mM. The dehydrogenase from *Kloeckera* sp. No. 2201 has a pH optimum at 8.0, whereas it has been reported that the pH optimum of the enzyme from baker's yeast was broader, pH 6.0 to 8.5 (164). The *Km* of formaldehyde for the enzyme *Kloeckera* sp. No. 2201 is higher than that for the enzymes from mammalian livers (163, 164).

The enzyme of *Kloeckera* sp. No. 2201 was very unstable even in the presence of DTT. About 30% of the activity disappeared at 30°C within 10 min. This finding may be compatible with the fact that the optimum temperature for growth of the organism was 20° to 25°C (Chapter IV, section 2).

In the present investigation, the inducible formation of formaldehyde dehydrogenase of *Kloeckera* sp. No. 2201 in response to methanol and/or certain metabolites was observed. Namely, the specific activity of the enzyme in methanol-grown cells was about 10 times that in glucose-grown cells. It is suggested that the enzyme may play a significant role in the methanol metabolism of *Kloeckera* sp. No. 2201. In this yeast, methanol was oxidized by alcohol oxidase as reported by Tani *et al.* (152, 153) and the formaldehyde produced was further oxidized by NAD- and GSH-dependent formaldehyde dehydrogenase. The latter step may be coupled with an energy production system.

SUMMARY

An NAD-linked formaldehyde dehydrogenating enzyme was found in the cell extract of *Kloeckera* sp. No. 2201, which utilized methanol as a sole source of carbon. The enzyme was inducibly formed in methanol-grown cells. This fact suggests that the enzyme may play a significant role in the methanol metabolism of this yeast. The enzyme was purified from a cell extract by ammonium sulfate fractionation, column chromatographies on DEAE-cellulose and on hydroxylapatite, and Sephadex G-200 gel filtration. From an experiment with the purified enzyme, it was found that the enzyme specifically required reduced glutathione for activity, and was reactive toward methylglyoxal as well as formaldehyde. The enzyme catalyzed the following reaction:

$$\text{Formaldehyde} + \text{NAD} + H_2O \longrightarrow \text{formic acid} + \text{NADH} + H^+$$

The enzyme was concluded to be a kind of formaldehyde dehydrogenase (formaldehyde:NAD oxidoreductase, EC 1.2.1.1). Other properties of the enzyme were also investigated.

SECTION 2

PURIFICATION AND PROPERTIES OF FORMATE DEHYDROGENASE IN *KLOECKERA* SP. No. 2201[j)]

INTRODUCTION

It has been reported that in methanol utilizing-microorganisms, the last step of methanol oxidation was catalyzed by the NAD-linked formate dehydrogenase (127, 129, 141). In the earlier paper, Quayle showed the presence of the enzyme in a formate utilizer, *Pseudomonas oxalaticus* (167). However, most of the reports on the enzyme are concerned primarily with the detection of enzymatic activities or with the general properties of partially purified enzymes. Especially, the details of the properties of the enzyme from the methanol utilizing yeast have been limited.

In this section, the purification and characterization of formate dehydrogenase from *Kloeckera* sp. No. 2201 are described.

MATERIALS AND METHODS

Organism. A methanol-utilizing yeast, *Kloeckera* sp. No. 2201, was used throughout this study.

Materials. DEAE-cellulose was a gift from Green Cross Co. Osaka. Sodium ^{14}C-formate of which specific radioactivity was 40.8 mCi/mmole was purchased from Daiichi Pure Chemicals Co. Ltd., Tokyo. The Ultrathimble which was used for concentration of enzyme solution was a product of Carl Schleicher und Schull, Dassel. All other chemicals were obtained from commercial sources and were used without further purification.

Cultivation. The medium consisted of 2 g methanol, 0.4 g NH_4Cl, 0.1 g K_2HPO_4, 0.1 g KH_2PO_4, 0.05 g $MgSO_4 \cdot 7H_2O$ and 0.2 g yeast extract in 100 ml tap water, pH 6.0. The cultivation was carried out as previously described (Section 1).

Preparation of cell-extract. The cells in the cultured broth were harvested by continuous flow centrifugation and were twice washed with 0.01 M potassium phosphate buffer, pH 7.5. The washed cells were suspended in an appropriate volume of the above buffer and treated with a 19 kHz Kaijo Denki ultrasonic oscillator for 2 hr. The cells and debris were removed by centrifugation at 12000 x *g* for 15 min. The resultant supernatant was dialyzed against 0.01 M potassium phosphate buffer, pH 7.5. The above procedures were carried out below 5°C.

Measurement of formate dehydrogenase activity. The complete reaction system in a 3.5 ml silica cell (1 cm light path) consisted of 100 μmoles of sodium formate, 3.0 μmoles of NAD, 200 μmoles of potassium phosphate buffer (pH 7.5), enzyme, and water to a total volume of 3.0 ml. The blank cell lacked sodium formate. The reaction was started by the addition of formate, and the increase in the extinction at 340 mμ was followed in a Hitachi double beam spectrophotometer Type 124. Specific activity of the enzyme was expressed as unit per mg protein, where one unit was defined as the increase in optical density at 340 mμ during the initial 1 min incubation.

Protein determination. Protein was determined by measurement of the absorbancy at 280 mμ or by the methods according to Lowry *et al.* (63).

Determination of radioactive carbon dioxide produced during the enzyme reaction. The reaction was carried out in a Warburg flask with double arms. The main compart-

ment contained 200 μmoles of phosphate buffer, pH 7.5, 1.5 μmoles of NAD, 12.4 μmoles sodium ^{14}C-formate (80 nCi) and water to give a 2.0 ml. The side-arm 1 contained 0.2 ml of the enzyme solution and the side-arm 2 contained 0.2 ml of 2 N phosphoric acid. A filter paper wick laden with 0.2 ml of 40% NaOH was placed in center well in order to trap the CO_2 liberated. The reaction was initiated by tipping in the enzyme solution from the side-arm 1, and performed at 30°C for 30 min. The reaction was terminated by addition of 2 N phosphoric acid from the side arm 2, and then shaking was continued for 10 min. The radioactivity of filter-paper wick and of reaction mixture in main compartment were determined by a Beckman liquid scintillation counter. The redio-activity of the reaction mixture was established as radioactivity of formate.

RESULTS

Formation of formate dehydrogenase

The effect of carbon source of medium on the formate dehydrogenase activity was investigated. The cell extract was prepared from the cells which were grown on a medium containing 2% methanol or 2% glucose as carbon source. The specific activity in each cell extract was determined. As shown in Table I, the specific activity of the enzyme in the cell extract from methanol-grown cells was extremely higher than that from glucose-grown cells.

TABLE I. FORMATE DEHYDROGENASE ACTIVITY IN METHANOL- OR GLUCOSE-GROWN CELLS

Cultivations were carried out at 28°C with shaking. The enzyme activity was measured under the standard assay conditions.

Cell	Specific activity after	
	24 hr	48 hr
Methanol-grown	0.300	0.250
Glucose-grown	0.001	0.003

Purification of the enzyme

The enzyme activity was extremely lost by a treatment with ammonium sulfate. Therefore, the enzyme solution was concentrated with a filtration using a Ultra-thimble. All operations were carried out at 0° to 5°C (except where specified).

Step 1. Preparation of the cell extract. A cell paste was obtained from 10 liters of broth cultured on methanol medium, and the cell extract (120 ml) was prepared as described in Materials and Methods.

Step 2. Heat treatment. The cell extract was heated for 10 min in a water bath at 55°C. The extract was cooled in ice and centrifuged to remove denatured protein. The clean supernatant was dialyzed for 18 hr against 0.01 M potassium phosphate buffer.

Step 3. DEAE-cellulose column chromatography. The dialyzed enzyme solution (130 ml) was applied to a DEAE-cellulose column (3 X 40 cm) which had been equilibrated with 0.01 M potassium phsphate buffer (pH 7.5). An elution was carried out with stepwise increase in the concentration of potassium phosphate buffer (pH 7.5) as follows; 0.01, 0.05 and 0.1 M. The enzyme activity appeared in the eluate with the 0.05 M buffer. The active fractions were combined to give 350 ml, which was dialyzed for 18 hr against a 20 liter volume of 0.01 M potassium phosphate buffer.

Step 4. Hydroxylapatite column chromatography. The dialyzed enzyme solution was placed on a column of hydroxylapatite (3 X 15 cm) which had been equilibrated with 0.01 M potassium phosphate buffer, pH 7.5. The successive elutions were carried out with 0.01, 0.02, 0.05 and 0.1 M potassium phosphate buffer. The active fractions were found in the eluate with the 0.1 M buffer. The solution collected

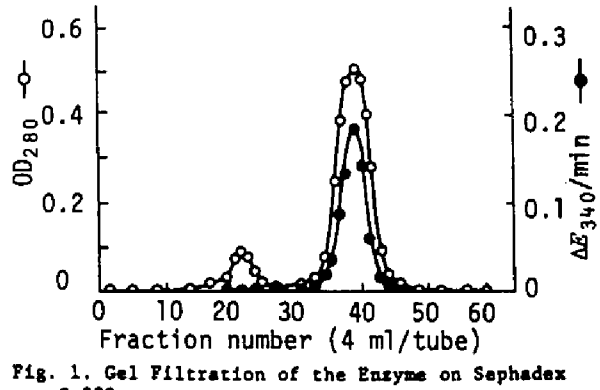

Fig. 1. Gel Filtration of the Enzyme on Sephadex G-200.

The Sephadex G-200 was packed into a column (2 x 100 cm) and equilibrated with 0.01 M potassium phosphate buffer, pH 7.5. The enzyme solution was placed on the column and then the buffer was allowed to flow at a rate of 10 ml per hr and 4 ml fractions were collected.

TABLE II. SUMMARY OF THE PURIFICATION OF THE FORMATE DEHYDROGENATING ENZYME

Treatment	Total Protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Purification
Crude extract	6240	160	0.025	1
Heat treatment	975	130	0.133	5.3
DEAE-cellulose	105	52	0.50	20
Hydroxylapatite	51	44	0.87	34.8
Sephadex G-200	40	35.6	0.89	35.5

(170 ml) was concentrated by filtration using a Ultra-thimble.

Step 5. Sephadex G-200 gel filtration. The enzyme solution obtained in the preceding step was subjected to Sephadex G-200 gel filtration. Figure 1 shows a typical elution pattern of the enzyme. These active fractions were combined to give 35 ml, and then concentrated.

A summary of the purification of the enzyme is presented in Table II.

Homogeneity

As shown in Fig. 2 the analysis of the purified enzyme preparation by ultracentrifugation showed a exactly single and symmetric Schlielen peak. Furthermore, an electrophoretic analysis was carried out using a cellulose acetate membrane under the conditions as described in the preceding section. The enzyme preparation gave a single band on the membrane.

Properties of the purified enzyme

1) Absorption spectrum. The enzyme, in 0.01 M potassium phosphate buffer (pH 7.5), exhibited only one absorption maximum, near 280 mμ, over the range from 200 to 700 mμ.

2) Stoichiometry of the formate dehydrogenase. The enzyme was incubated with ^{14}C-labelled formate and NAD in Warburg flask, and the consumed formate and carbon dioxide were determined as described in Materials and Methods. The results are presented in Table III. They show that the decrease of radioactivity of formate was nearly equivalent to the radioactivity of produced carbon dioxide. Thus, the reaction may be described by the following equation.

$$HCOOH + NAD \longrightarrow CO_2 + NADH + H^+$$

The reverse reaction, namely reduction of sodium bicarbonate by NAD, were unable to be detected spectrophotometrically. This demonstrated that the equilibrium of the reaction strongly favors the dehydrogen-

TABLE III. STOICHIOMETRY OF THE FORMATE DEHYDROGENASE REACTION

The reactions were carried out manometrically. The details of the reaction was described in Materials and Methods.

	Formate initial	Formate final	Formate consumed	CO_2 liberated
Radioactivity (c.p.m)	4321	2820	1501	1320
μmole			4.3*	3.9**

*Calculated from radioactivity consumed.
**Obtained from the reading of the manometer.

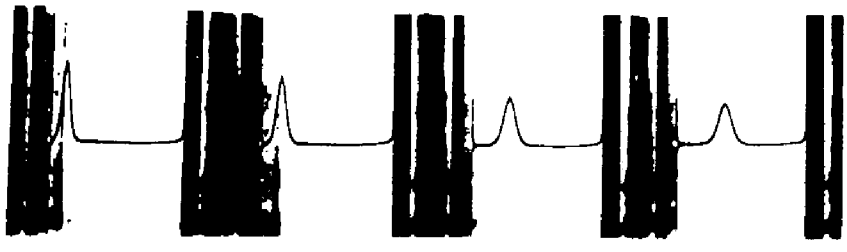

Fig. 2. Ultracentrifugation Analysis of the Purified Enzyme.

Photographs of Schlielen patterns were taken at 18 (A), 26 (B), 50 (C) and 66 (D) min after reaching 59780 rpm. Protein, 0.5 % in 0.01 M potassium phosphate buffer, pH 7.5.

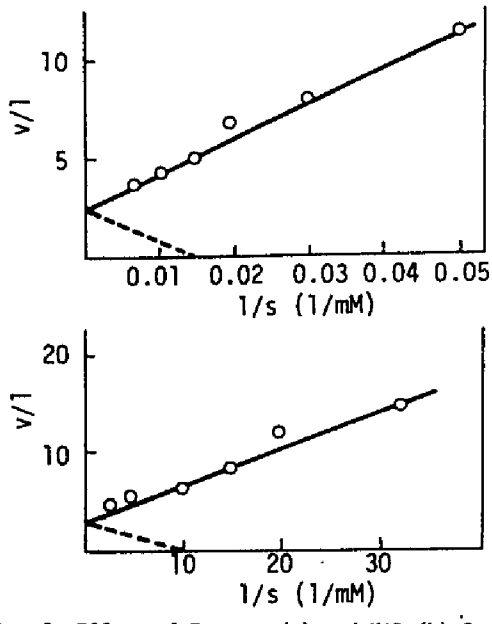

Fig. 3. Effect of Formate (a) and NAD (b) Concentration on the Reaction Rate.

The enzyme activity was measured under the standard assay conditions except for substrate concentration. Velocity (v) was expressed as ΔE_{340}/min.

ation of formate.

3) Substrate specificity. As far as tested, the enzyme was specific for formate. No activity was detected with acetate, propionate, pyruvate, malate, formadehyde, glyoxal or methylglyoxal under the conditions of assay. NADP was inactive as an electron acceptor for the reaction. The dehydrogenase activities with various concentrations of formate and NAD were investigated. The plots of Lineweaver and Burk were showed in Fig. 3. The Michaelis constants (*Km*) for formate and NAD were calculated to be 22 mM and 0.1 mM, respectively.

4) Effect of temperature on the enzyme activity. Figure 4-a shows the variation of the enzyme activity with temperature. The maximum activity was observed at 50°C. Figure 4-b shows the effect of temperature on the stability of the enzyme. The most of activity was remained at 50°C and the 60% activity was lost at 58°C under the standard assay conditions.

5) Effect of pH on the enzyme activity. The variation of enzyme activity with pH are shown in Fig. 5-a. The optimum pH of the enzyme was found to be 8.0. The enzyme was stable at neutral pH range under the standard assay conditions (Fig. 5-b).

6) Effect of various reagents on the enzyme activity. The enzyme was preincubated with individual reagents at a 1 mM concentration (except where specified) at 30°C for 30 min, and then the remaining activity was determined under the standard assay conditions. The enzyme activity was completely inhibited with potassium cyanide, p-CMB, or sodium azide

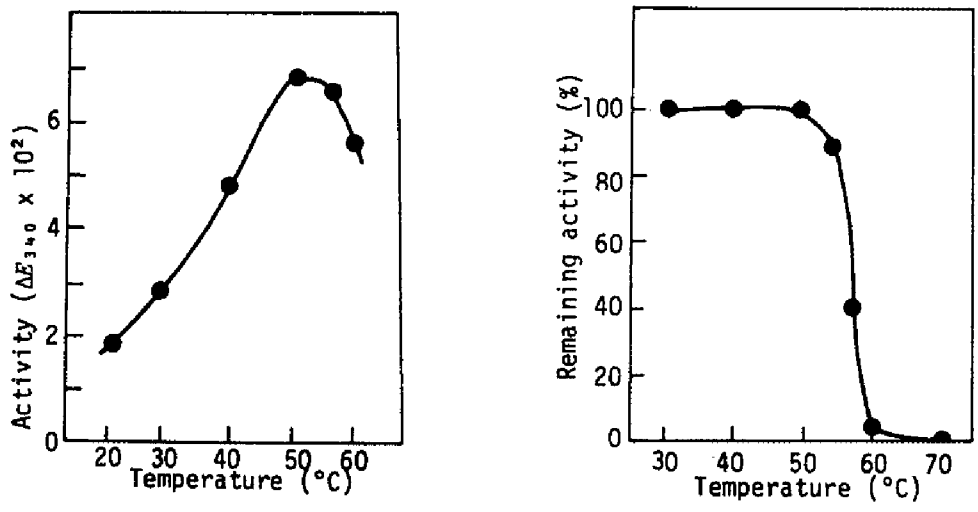

Fig. 4. Effect of Temperature on Activity (a) and Stability (b) of the Enzyme.

(a): The enzyme activity was measured under the standard assay conditions at various temperatures.

(b): The enzyme, in 0.01 M potassium phosphate buffer (pH 7.5), was preincubated for 10 min at each temperature as indicated. The remaining activity was measured under the standard assay conditions.

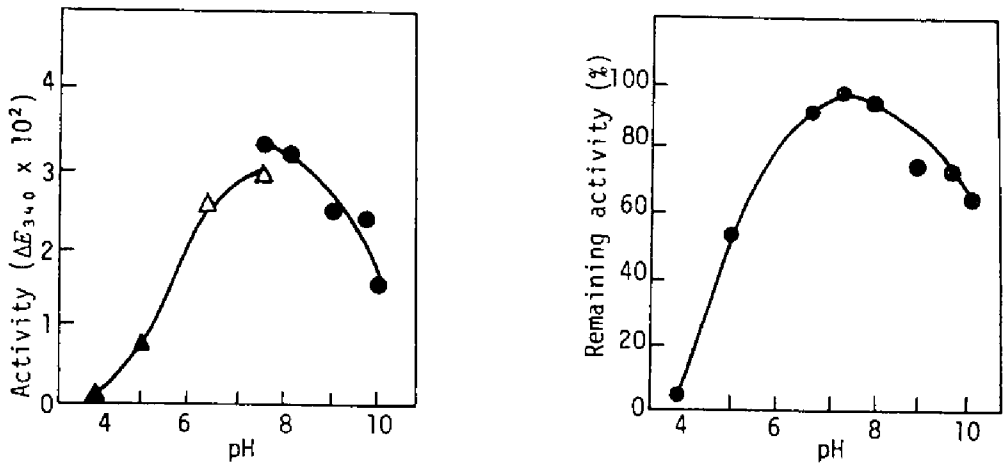

Fig. 5. Effect of pH on Activity (a) and Stability (b) of the enzyme.

(a): The enzyme activity was measured under the standard assay conditions using the following buffer: potassium phosphate - citric acid buffer (△), Potassium phosphate buffer (▲), boric acid - NaOH buffer (●) at various pH values.

(b): The enzyme, in each buffer described in (a), was preincubated at 30°C for 1 hr. The remaining activity was measured under the standard assay conditions.

(5 μM) and was also inactivated with Cu^{2+}, 80%; Hg^{2+}, 100%; or Pb^{2+}, 45% by the respective percentage figure. There was no effect on the enzyme activity with EDTA, *o*-phenanthroline, 8-hydroxyquinoline, or α,α'-dipyridyl. There was also no effect with Ni^{2+}, Cd^{2+}, Zn^{2+}, Co^{2+}, Ca^{2+}, Ba^{2+}, Ag^{+}, Sr^{2+}, Mo^{6+} or Al^{3+}. The enzyme was not activated by ATP, AMP or reduced glutathione in 1 mM concentration.

DISCUSSION

The two different formate dehydrogenases have so far been described. One of these, cytochrome b_1-linked dehydrogenase, has been found in several bacteria (168 ~ 171) and shown that the reaction proceed anaerobically. The other one, NAD-linked dehydrogenase, has been found in higher plants (172, 173), animals (174, 175) or microorganisms (127, 129, 141, 167). In the methanol-utilizing organisms reported, the oxidation of formate was catalyzed by the latter dehydrogenase (127, 129, 141). It is thought that the last step of the succesive methanol oxidations of *Kloeckera* sp. No. 2201 was also catalyzed by the NAD-linked dehydrogenase, since the enzyme was inducibly formed in response to methanol.

Few information on purification of the NAD-linked formate dehydrogenase from microorganism has so far been obtained, although the enzymes from plants (172, 173) or animals (175) have been highly purified. In present investigation, the purification of formate dehydrogenase from *Kloeckera* sp. No. 2201 was demonstrated. The enzyme was purified about 35 fold from the cell extract, and the purified enzyme was shown to be homogeneous by analyses with electrophoresis and ultracentrifugation.

Experiments with purified enzyme showed that the enzyme was specific for formate and NAD as substrate and hydrogen acceptor, respectively. Mathews and Vennesland (174) showed that NAD-linked formate dehydrogenase from rat kidney and liver were stimulated by ATP and AMP. Strittmatter and Ball (175) reported that the enzyme from liver of chicken and bovine required reduced glutathione as well as NAD. On the enzyme from *Kloeckera* sp. No. 2201, these stimulations or requirements were not observed. It is shown that the animal and plant enzyme were completely inactivated by 8-hydroxyquinoline (172 ~ 175). While, the *Kloeckera* enzyme was much less sensitive to inhibition by any chelating reagents tested, such as 8-hydroxyquinoline (5 mM), EDTA or α,α'-dipyridyl (1 mM). The behavior of the enzyme against inhibitors were similar to that of *Pseudomonas* AM1 (129), or *Ps. oxalaticus* (167). The enzyme is thought to be a sulfhydryl enzyme because of complete inactivation with 1 mM *p*-CMB.

In this yeast, methanol is oxidized by alcohol oxidase (152, 153) and the produced

formaldehyde is further oxidized by formaldehyde dehydrogenase which has been described in the preceding section. As for the last step of the oxidation, the resultant formate is oxidized to carbon dioxide by the NAD-linked formate dehydrogenase.

The *Km* value of formate for the enzyme was found to be 22 mM. This value was considerably higher than the value for formate dehydrogenase from *Ps. oxalaticus* (167) and the value of methanol for alcohol oxidase or formaldehyde for its dehydrogenase from *Kloeckera* sp. No. 2201. If a carbon atom of methanol is incorporated *via* formaldehyde or formate by the organism, the formate dehydrogenation might be a disadvantageous reaction in the carbon balance. On the other hand, this reaction play an important role in energy reproduction system. Therefore, the implication of this high *Km* value for the metabolism of methanol by the yeast could not be explicable until the pathway of incorporation of methanol carbon into cell constituents are disclosed.

SUMMARY

An NAD-linked formate dehydrogenating enzyme which catalyzed the last step of methanol oxidation system was extracted from the methanol grown *Kloeckera* sp. No. 2201. The specific activity of the enzyme in the extract of methanol-grown cells was found to be considerably higher than that of the glucose-grown cells. The enzyme was purified about 35-fold from the extract of methanol grown cells by heat treatment, column chromatographies on DEAE-cellulose and on hydroxylapatite, and Sephadex G-200 gel filtration. The purified enzyme was shown to be homogeneous by analyses with electrophoresis and ultracentrifugation. The purified enzyme was a kind of NAD:formate oxidoreductase (EC, 1.2.1.2) which catalyzed specifically the oxidation of formate to carbon dioxide. The *Km* values were 22 mM for formate, and 0.1 mM for NAD. The enzyme was inactivated by potassium cyanide, sodium azide, and *p*-CMB but not by any metal-chelating reagents tested. Other general properties of the enzyme were also investigated.

SECTION 3

ENZYME SYSTEM FOR METHANOL OXIDATION IN YEASTS[k)]

INTRODUCTION

The enzyme responsible for each step of methanol oxidation has so far been well known in methane- and methanol-utilizing bacteria. Especially, a number of studies have been made on the first step of methanol oxidation. In those cases, three types of enzymes, namely NAD-linked methanol dehydrogenase (127), catalase-linked peroxydase (128) and primary alcohol dehydroganse containing a pteridine cofactor (129, 133, 156, 176, 177), have been described in different bacteria. In a methanol-utilizing yeast, *Kloeckera* sp. No. 2201, the three enzymes, alcohol oxidase (152, 153), formaldehyde dehydrogenase and formate dehydrogenase were detected in high activities in the cell extract. The same evidences were also shown in *Candida, Saccharomyces* and *Torulopsis* by Fujii and Tonomura (141).

The present studies deals with the enzyme system which catalyzes the oxidation of methanol in several strains of methanol-utilizing yeasts.

MATERIALS AND METHODS

Organisms. Seven types of yeasts were used. Among the yeast, *Candida methanolica* and *Torulopsis methanolovescens* were isolated from rotten plants by Oki *et al.* (142), and four strains of yeasts, *Torulopsis pinus* IFO 0741 (CBS 970), *Hansenula capsulata* IFO 0974, *Pichia pinus* IFO 1342 (CBS 744) and *Pichia trehalophile* IFO 1282 (CBS 5361) were found to be able to assimilate methanol by Hazeu *et al.* (143).

Medium and cultivation. A cultural medium of the organisms contained carbon source 1.0 g, NH_4Cl 0.4 g, K_2HPO_4 0.1 g, KH_2PO_4 0.1 g, $MgSO_4 \cdot 7H_2O$ 0.05 g and yeast extract 0.05 g in 100 ml of tap water, pH 7.0. The cultivation was carried out at 28°C on a reciprocal shaker at 130 rpm. In the case of *Torulopsis pinus* IFO 0741, the cultivation was performed at 20°C, since the organism was anable to grow above 25°C under the conditions employed.

Enzyme Assay. The dialyzate of cell extract was prepared as described in the preceding sections. The method of analysis of alcohol oxidase was carried out according to Tani *et al.* (152, 153), and formaldehyde dehydrogenase and formate dehydrogenase were essentially the same as that used previously. The determinations of alcohol dehydrogenase and catalase were performed according to Racker (178) and Chance and Maehly (179), respectively. Protein was estimated by the method of Lowry *et al.* (63).

RESULTS AND DISCUSSION

Oxidation of methanol to formaldehyde

Table I lists the activities of alcohol oxidase in the identically prepared cell extracts of seven strains of yeast, grown individually on methanol, ethanol, glycerol, or glucose. It was evident in all yeasts tested that the activities of hydrogen peroxide formation from methanol in methanol-grown cells were higher than in cells grown in the other substrates.

TABLE I. SPECIFIC ACTIVITY OF ALCOHOL OXIDASE IN CELL EXTRACT OF YEASTS

Alcohol oxidase was determined by measuring the produced H_2O_2 according to Janssen and Ruelius (180) (A), and formaldehyde in the reactant was estimated as described previously (153) (B). The specific activity was defined as μmoles of H_2O_2 or formaldehyde/min/mg protein.

Strain	Specific activity in extract of cells grown on				
	methanol		ethanol	glycerol	glucose
	A	B	A	A	A
Kloeckera sp.	0.13	0.12	-	-	-
Candida methanolica	0.40	0.37	-	0.05	-
Torulopsis pinus	0.16	0.14	-	-	0.002
Torulopsis methanolovescens	0.08	0.08	0.003	-	-
Hansenula capsulata	0.07	0.06	-	-	-
Pichia pinus	0.24	0.21	-	-	-
Pichia trehalophile	0.48	0.43	-	0.005	0.002

-: Nondetectable.

The value of hydrogen peroxide formed in each case was nearly identical with that of formaldehyde. Thus, the oxidation of methanol may be thought to be catalyzed by alcohol oxidase. When this enzyme reaction proceeds in the living cells, The catalase activity of each extract of cells grown on the four carbon sources were measured (Table II). Those specific activities, in all strains, were greatly increased in the methanol-grown cells, compared with the other cells. This may support the suggestion that the alcohol oxidase actually catalyzes the first step of methanol oxidation during the growth on methanol of the seven types of yeasts.

The activities of NAD-linked alcohol dehydrogenase were able to be detected in all of the cell extracts. However, inducible formation of the enzyme in methanol-grown cells was not observed (Table III).

No activity of phenazine methosulfate-linked alcohol dehydrogenase such as that which was reported by Anthony and Zatman (133, 156, 176) was found in any cell extracts.

TABLE III. SPECIFIC ACTIVITY OF ALCOHOL DEHYDROGENASE IN CELL EXTRACT OF YEASTS

NAD-linked alcohol dehydrogenase was determined according to Racker (178). The specific activity was defined as ΔE_{340}/min/mg protein.

Strain	Specific activity in extract of cells grown on	
	methanol	glucose
Kloeckera sp.	0.100	0.087
Candida methanolica	0.002	0.005
Torulopsis pinus	0.002	0.013
Torulopsis methanolovescens	0.380	0.003
Hansenula capsulata	0.064	0.042
Pichia pinus	0.008	0.020
Pichia trehalophile	0.002	0.004
(Baker's yeast)		(3.11)

Oxidation of formaldehyde to formate

In all the yeasts, except *Pichia pinus*,

TABLE II. SPECIFIC ACTIVITY OF CATALASE IN CELL EXTRACT OF YEASTS

Catalase activity was spectrophotometrically determined according to Chance and Maehly (179). The specific activity was defined as ΔE_{240}/min/mg protein.

Strain	Specific activity in extract of cells grown on			
	methanol	ethanol	glycerol	glucose
Kloeckera sp.	12.9	0.46	5.38	0.64
Candida methanolica	10.7	0.41	5.37	0.20
Torulopsis pinus	6.24	0.27	1.32	0.18
Torulopsis methanolovesces	9.42	0.31	3.42	0.18
Hansenula capsulata	5.65	0.16	0.27	0.09
Pichia pinus	6.58	0.50	1.21	0.35
Pichia trehalophile	3.22	0.21	0.10	0.20

TABLE IV. SPECIFIC ACTIVITY OF FORMALDEHYDE DEHYDROGENASE IN CELL EXTRACT OF YEASTS

The specific activity was defined as ΔE_{340} /min/mg protein.

Strain	Specific activity in extract of cells grown on methanol +GSH	methanol -GSH	glucose +GSH	glucose -GSH
Kloeckera sp.	0.18	-	-	-
Candida methanolica	0.11	-	-	-
Torulopsis pinus	0.03	-	-	-
Torulopsis methanolovescens	0.52	-	0.17	-
Hansenula capsulata	1.18	0.02	0.11	-
Pichia pinus	0.03	-	0.03	-
Pichia trehalophile	0.31	-	0.17	-

-: Nondetectable.

TABLE V. SPECIFIC ACTIVITY OF FORMATE DEHYDROGENASE IN EXTRACT OF YEASTS

The specific activity was defined as ΔE_{340} /min/mg protein.

Strain	Specific activity in extract of cells grown on methanol	glucose
Kloeckera sp.	0.150	0.001
Candida methanolica	0.006	0.001
Torulopsis pinus	0.013	0.003
Torulopsis methanolovescens	0.500	0.001
Hansenula capsulata	0.490	0.001
Pichia pinus	0.010	0.002
Pichia trehalophile	0.006	0.003

NAD-linked and GSH-dependent formaldehyde dehydrogenase had higher specific activities in methanol-grown cells than in glucose-grown cells (Table III). The GSH-independent dehydrogenase was found only in the extract of methanol-grown *Hansenula capsulata*, but its activity was considerably lower than the activity of GSH-dependent dehydrogenase.

Oxidation of formate to carbon dioxide.

Great increases in the activity of formate dehydrogenase were apparent in the extracts of methanol-grown *Kloeckera* sp. No. 2201, *Torulopsis methanolovescens*, and *Hansenula capsulata*. In other cases, the activities in methanol-grown cells were also found to be higher than those in glucose-grown cells (Table IV)

These results suggested that, in yeasts, methanol was successibely oxidized to carbon dioxide by alcohol oxidase, formaldehyde dehydrogenase, and formate dehydrogenase. On the basis of the information so far obtained, a serious difference in methanol-oxidation system between bacteria and yeasts is noted in the first step of the oxidation.

SUMMARY

The enzyme system which catalyzes the oxidation of methanol in seven types of methanol-utilizing yeast, *Kloeckera* sp. No. 2201, *Candida methanolica*, *Torulopsis methanolovescens*, *Torulopsis pinus*, *Hansenula capsulata*, *Pichia pinus* and *Pichia trehalophile*, was investigated. Using the cell extracts of these yeasts, grown on methanol and other carbon source, including ethanol, glycerol or glucose, the activities of alcohol oxidase, catalase, alcohol dehydrogenase, formaldehyde dehydrogenase and formate dehydrogenase were assayed. It was evident in all yeasts tested that the activities of alcohol oxidase and catalase in methanol-grown cells were higher than in cells grown on the other substrates. The NAD-linked alcohol dehydrogenase were not inducibly formed in methanol-grown cells. No activity of phenazine methosulfate-linked alcohol dehydrogenase was found in any cell extract. In the most of the yeasts tested, NAD-linked and GSH-dependent formaldehyde dehydrogenase, and formate dehydrogenase were found to be inducibly formed in methanol-grown cells. Thus, it may be thought that in yeasts, methanol was successibely oxidized to carbon dioxide by alcohol oxidase, formaldehyde dehydrogenase and formate dehydrogenase.

CONCLUSION

From the standpoint of the utilization of microbial activity at low temperatures for microbial productions, following problems have been investigated; a) accumulations of amino acids and organic acids by psychrophilic bacteria, b) formations and characterizations of proteases from a marine-psychrophilic bacterium, c) an ultilization of *n*-paraffin at low temperatures by a marine yeast, d) utilizations of low boiling substance, including methanol, by yeasts.

A psychrophilic bacterium, *Brevibacterium* sp. P145 required methionine for the growth above 20°C. The requirement at the moderate temperatures is explained on the basis of an thermolability of the methionine-biosynthesis system. The organism was found to accumulate the nearly equivalent amount of both L-glutamic acid and L-alanine at the optimum temperature for growth, 15°C. In the cultivation at 5°C, however, L-glutamic acid was predominantly accumulated, wherease, at 28°C, the main product was L-alanine. The alteration of products by temperature is partly attributable to the abnormal cold sensitivity of L-alanine dehydrogenase which catalyzes the last step of the synthesis of this amino acid. Further, it was demonstrated that the deficiency in thiamine, which was required as a growth promoting factor at 28°C, caused the accumulation of L-alanine at this temperature.

The psychrophilic *Pseudomonas* B-71 which was isolated from a water of Lake Biwa accumulated succinic acid at the suboptimum temperatures, below 28°C. On the other hand, 2-ketogluconic acid was accumulated by the cultivations at the superoptimum temperatures. The extreme reduction of the activity of succinic dehydrogenase in the cells grown at low temperature seems to be one of the factors influencing the accumulation of succinic acid at the temperatures. The reduction of the activity at low temperatures is thought to be result of depression of the formation of the enzyme.

Proteolytic activities were found in the culture fluid of numerous marine-psychrophilic bacteria isolated. Among the bacteria, *Pseudomonas* sp. No. 548, which was isolated from sea water showed the highest proteolytic activity. The organism required the salts of sea water for both the growth and the formation of proteases. The extracellular-protease elaboration by this organism was shown to be inversely proportional to the culture temperatures, and the optimum temperature for the accumulation of protease was found to be 5°C. Although no clear account for this phenomenon could be given from the present study, it might be said, at least, that the growth at low temperatues were necessary for an abundant accumulation of the proteases. It is assumed that the variation of the formation or accumulation with culture temperature reflects a substancial difference in the organization and/or the metabolic process at each temperature rather than the thermolability of the protease produced or the variation in the dissolved oxygen with temperature.

The extracellular proteases of this bacterium were fractionated into four components, protease Ia, Ib, IIa and IIb. Among the proteases, Ia was obtained in a crystalline form which was shown to be homogeneous by analysis with electrophoresis. The other three proteases were also highly purified. The temperature ranges for activities of these proteases were found to be relatively lower, comparing with proteases which have been obtained from various microbes. These four proteases were quite different from one another with respects to the behaviors against inhibitors and metal ions, and pH optima. On the basis of the substrate specificity, the alkaline proteases Ia is classified as a serine protease, although the enzyme was inactivated with EDTA as well as DFP. The metal which is chelated by EDTA is thought to

participate in the maintenace the conformation of the enzyme protein. It is thought to be unique that this Gram-negative bacterium produces a serine protease. The protease IIa cleavaged peptide bonds containing amino groups of hydrophobic amino acid residues. The protease is classified as a neutral and metal protease.

It has been known that marine sediments are good samples for the screening of hydrocarbon-utilizing microbes. Further, sea water and marine mud are thought to be favorable environments to low-temperature organisms. The author has isolated a marine yeast, which is able to assimilate *n*-paraffin, from a marine sediment sample of Sagami Bay at depth of 1120 m. The organism was identified as a *Candida* sp. MM313. Although the growth of the yeast was observed in a medium prepared with fresh water, the cell yield increased with increasing the concentration of the salts containing sea water in the medium, with the maximum growth extent being given in the medium containing 75% sea water rather than the full strength. The optimum temperature for growth rate was found to be 28°C. The best cell yield from *n*-paraffin, however, was attained by the cultivation at 10° to 15°C. The cell yields to *n*-paraffin were about 85% at 15°C after 4 days and 56% at 28°C after 3 days under optimum conditions obtained.

Some cultural conditions for cell production of *Kloeckera* sp. No. 2201 from methanol were investigated. The cell yield on the medium containing 0.2% C.S.L as growth factor was about 2.7 times that on the medium containing a vitamin mixture which had been conventionally used. The optimum temperature for growth was found to be 25°C, and at the higher temperatures, the growth was depressed. Using the other six types of methanol-utilizing yeast, the relationships between temperature and growth were investigated. These yeasts had relatively low optimum temperatures. Especially, *Torulopsis pinus* IFO 0741 grew optimally at 20°C and the yeast could not grow at 28°C. Therefore, the organism is able to classify as a psychrophilic yeast.

The two enzymes being responsible for the oxidation of methanol to carbon dioxide were extracted from methanol-grown *Kloeckera* sp. No. 2201 and were highly purified. One of these, a formaldehyde oxidizing enzyme, was NAD-linked and GSH-dependent dehydrogenase and was classified as formaldehyde dehydrogenase (formaldehyde:NAD oxidoreductase, EC 1.2.1.1). This enzyme catalyzes the oxidation of formaldehyde to formate. Another one was NAD-linked formate dehydrogenase (formate:NAD oxidoreductase, EC 1.2.1.2), which catalyzes the oxidation of formate to carbon dioxide. Since these enzymes was inducibly formed in methanol medium, the enzymes were thought to catalyze actually the second and third steps of methanol oxidation during the growth of the yeast on methanol. Furthermore, it is confirmed that, in the other six types of methanol-utilizing yeast, the alcohol oxidase, formaldehyde dehydrogenase and formate dehydrogenase are responsible for the oxidation of methanol to carbon dioxide.

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