

Microbial utilization of poor quality biomass

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INTRODUCTION

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The term "biomass", which originally refers to biological quantity, can be defined as any organic substances directly or indirectly synthesized from an inorganic substances by solar energy. It encompasses all organic organism-related substances incorporated in materials such as plants, animals and microorganisms, in which inorganic substances undergo biomorphological changes, and return to inorganic substances again. The most abundant biomass material is phytomass. In recent years, however, the term "biomass" has been used to indicate substances which accumulate during biological activities and can be utilized as resources (energy source, industrial raw materials, and foodstuff) (1, 2). These substances can be roughly divided into two types: (I) high-quality organic substances which are produced for specific purposes (e. g., plants like sugar cane and cassava) and (II) low-quality organic substances (by-products) of biological origin (agricultural, stockbreeding and industrial by-products) which are produced chiefly during human activities without any prior plan for utilization (1, 2).

In the past, major utilization of biomass as a resource was for energy production such as alcoholic fermentation (3) and methane fermentation (4). For this purpose, plants which can be produced on a large scale by an intensive method have been utilized, and the technology for such purposes has markedly progressed. On the other hand, utilization of poor quality biomass (by-products of biological origin) as resources has not advanced.

Therefore, the author studied utilization as resources and

effective utilization of the following poor quality biomass materials using fermentation microorganisms: (I) surplus sludge (industrial or home by-products), wheat bran (a by-product from food processing industry), (III) fish sperm (by-product from fisheries), and (IV) adenine and nucleosides (unutilized products from the nucleic acid fermentation industry). In this thesis, fermentation microorganisms were frequently used for the following reasons: (I) physiological and biochemical characteristics of various fermentation microorganisms have been utilized for the production of fermented food; for example, *Aspergillus oryzae*, fungus which decomposes protein and sugars, is used for manufacturing of *miso* (bean paste) and soy sauce, and yeast which is used for the fermentation of alcohol; and (II) The safety of these microorganisms has been established. For these reasons, fermentation microorganisms are useful for the utilization of biomass as a raw material or as a additive for food processing and medicine, and for application to food processing.

Surplus sludge is a by-product from the treatment of industrial and home waste water with active sludge. A very small portion of surplus sludge has been utilized for methane fermentation (5) or post-compost soil-conditioning (6). However, most of the surplus sludge has been disposed of on reclaimed land after burning (7). Surplus sludge is made of various microorganisms and low-level animals (for example, protozoa) (8). It contains a large amount of protein and sugars and can be utilized as a source of useful biomolecules.

In this thesis, the author first presents the method for the utilization of products of the acidic hydrolysis of surplus sludge (sludge hydrolyzate) as a cultivation medium for

microorganisms. Then, the author examined the possibility of producing useful enzyme with this medium. These results indicated that hydrolyzate from active sludge is useful not only as a cultivation medium of microorganisms but also for the isolation of microorganisms which produce specific enzymes and for induction of these enzymes.

Next, the author also examined the production of useful enzymes for fermentation by the *koji* cultivation method that uses wheat bran which is known as a good cultivation medium for fungus (a by-product from the food processing industry that has been utilized only as feeding-stuffs) (9). In the production of proteinous fermented foods such as *miso* and soy sauce, a high concentration of NaCl is added to prevent bacterial contamination (10). However, the addition of NaCl prolongs the period required for fermentation because NaCl inhibits fermentation to some degree. To solve this problem, various enzymes that participate in the production of fermented foods are added, or bioreactors using these enzymes are utilized for continuous fermentation (11, 12). In any event, sufficient knowledge as to the properties of these various enzymes is essential. In addition, there is little information about glutaminase, which is an enzyme strongly associated with the taste of fermented foods (13), as compared to the information on proteinase (14) and leucine aminopeptidase (LAP) (15). For this reason, the author studied glutaminase, which is produced by the solid *koji* cultivation method using wheat bran.

The solid *koji* cultivation method is useful for enzyme production with fungus. However, because separation of mycelia from the cultivation medium is difficult with this method, it is not suitable for the analysis of the relationship between fungus

growth and enzyme production, and for the analysis of enzyme localization. Although various methods have been devised to overcome these problems (16~20), the results have not been satisfactory. Therefore, the author devised a "soft gel cultivation method" by which mycelia are grown on the surface of bran-added soft agar medium (agar concentration 0.2~0.4 %, wheat bran 10 %). The author demonstrated that this method can be used for the analysis of enzyme production. Because this method allows the easy isolation of the mycelial mat from the medium, it seems to be useful for the analysis of the relationship between fungus growth and enzyme production, and purification of the intracellular enzyme.

As mentioned above, the addition of a high concentration of NaCl for the prevention of bacterial contamination prolongs the period required for the production of proteinous fermented foods and can cause an insufficient decomposition of raw material protein. To solve this problem, the author observed the effects of ethanol which has a bacteriostatic action like NaCl (21, 22). Instead of adding ethanol directly to the fermentation system, the author devised a method, "sugar-yeast addition method", in which glucose and yeast were added at the start of fermentation, and which utilized the prevention ability of microbial contamination by ethanol produced by yeast, and the reducing ability of sugar fermentation. The author examined the usefulness of this method.

Finally, regarding the utilization of fish sperm (a by-product from the marine industry), and adenine and nucleosides such as inosine and uridine (products from the nucleic acid fermentation industry), the author studied the production method of useful materials using the ATP energy produced by yeast's

alcoholic fermentation which was a method established for energy-saving and pollution-free chemical production (23, 24). And, deoxyribonucleoside triphosphates and ATP were produced from fish sperm, and adenine and nucleosides, respectively.

In brief, the present study indicates the effective utilization of poor quality biomass of biological origin, such as agricultural, stockbreeding and industrial by-products, as the raw materials of food processing and medicine and in the development of a new manufacturing techniques for food processing, which chiefly use fermentation microorganisms.

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Chapter I. Utilization of surplus active sludge

Section 1. Microbiological utilization of surplus active sludge as a new resource

Introduction

Utilization of the large amount of sludge resulting from biological treatment of liquid wastes discharged from various factories and households has been attempted partly through methan fermentation (1, 2) or conversion to compost for soil enrichment (3). However, a number of problems must be overcome before its realization, and most of the sludge is disposed of by burning, or incineration without pretreatment (1). These methods, however, are inadequate for disposal of sludge, which is increasing with the expansion of our life style, and may produce sources of serious secondary environmental pollution (1). The development of more efficient methods for sludge disposal is needed. Active sludge, which may differ somewhat according to the kind of the waste water treated or conditions of the treatment, is a mass containing a variety of microorganisms including bacteria, yeasts, molds, and protozoans and may be regarded as a mixed culture of microorganisms (4). It is also known to contain large amounts of polysaccharides and proteins involved in flock formation (5~8).

The author noted the property of active sludge as a mixed microbial system and carried out this study in an attempt to establish a method for its efficient utilization as a biological resources.

In this section, the possibility of extraction of useful substances such as amino nitrogen compounds and polysaccharides and their utilization in media of microbial cultures and as fermentation substances was examined. This method is considered to be effective not only for recovery of useful substances but also for reduction of the amount of sludge to be disposed of.

Materials and Methods

Sludge.

In this study, sludge primarily resulting from treatment of water used for washing rice in a sake brewery was collected by the aggregation-sedimentation method using a surfactant. The water content of the sludge was about 85 %.

Extraction of useful substances from active sludge.

Sludge (250 g) placed in a 500 ml sterilized bottle was mixed with various solvents (250 ml), heated, and centrifuged. The supernatant was used as the extract.

Assay.

Total amino-substances in the sludge extract were assayed by the method of Lowry *et al.* (9) using ovalbumin as the standard. Total carbohydrates were assayed by the phenol-sulfuric acid method (10) using glucose as the standard. The extract was dialyzed against water and assayed similarly, and the values obtained were regarded as protein and polysaccharide contents. Reducing sugars were assayed by Somogyi-Nelson method (11, 12) using glucose as the standard, and glucose was determined by the

enzymatic method using glucose oxidase (13). Ethanol was assayed by the enzymatic method using alcohol dehydrogenase (14). The amino acid analyzer and gas chromatograph were employed for differential assays of amino acids and carbohydrates in lyophilized specimens of the extract.

Determination of the amount of microbial growth.

The amount of microorganisms that have grown in cultures was expressed as the turbidity at 610 nm or the dry weight.

Results and Discussion

Extraction and recovery of carbohydrates and amino-substances from sludge.

The extraction ratio of useful substances from sludge using diluted acid or alkali was compared by boiling and autoclaving procedures (Table I). When the sludge was boiled (100 °C for 60 min) with 0.3 N NaOH (final concentration), more amino-substances than carbohydrates were extracted, and recovery ratio of amino-substances was about 18 % of the dry sludge weight. With 0.3 N acid, more carbohydrates than amino-substances were extracted, and a part of them had been hydrolyzed. On the other hand, when the sludge was autoclaved with 0.3 N HCl, 1.5 times as much carbohydrates (30 % of dry sludge weight) as by boiling were extracted, and most of them were reducing sugars. About 50 % of the reducing sugars was glucose, but they also included galactose, mannose, xylose, fucose, and ribose (Table II). Most of amino-substances had also been hydrolyzed, and were found as amino acids and micromolecular peptides. The amount of

Table I. Recovery of amino-substances and carbohydrates from surplus sludge with various treatment.

A*: extract, B**: dialyzed solution.

Treatment and solvent	Recovery (% on dry sludge)					
	Amino- substances		Total sugar		Reducing sugar	
	A*	B**	A*	B**	A*	B**
Boiling at 100 °C for 60 min						
0.3 N NaOH	18.2	13.0	12.0	9.2		
0.3 N HCl	13.3	4.2	20.0	6.7	8.3	0
0.3 N H ₂ SO ₄	11.2	4.4	14.3	10.8	4.0	0
Autoclaving at 120 °C for 30 min						
0.3 N NaOH	18.5	13.4	12.2	9.4		
0.3 N HCl	19.2	6.6	31.8	5.1	25.2	0
0.3 N H ₂ SO ₄	17.6	8.9	24.2	8.2	17.0	0

Table II. Sugar component of 0.3 N NaOH extract of sludge.

Sugar	Content (% on dry weight)
Glucose	9.7 %
Galactose	2.7
Mannose	1.3
Rhamnose	1.6
Ribose	0.6
Fucose	0.4
Arabinose	NC*
Fructose	NC*
Xylose	NC*

NC : not calculated

Table III. Growth of microorganisms in 0.3 N HCl extract of sludge.

Cultivation was carried out at 28 °C for 3 days with shaking.

Microorganisms	Growth (ODat 610 nm or mg/5 ml)
<i>Candida lipolytica</i>	10.0
<i>tropicalis</i>	8.4
<i>Hansenula anomala</i>	9.4
<i>miso</i>	11.8
<i>Rhodotorula mucilaginosa</i>	11.3
<i>Debaryomyces hansenii</i>	13.0
<i>japonicus</i>	12.5
<i>Torulopsis candida</i>	10.3
<i>Flavobacterium arborescens</i>	22.3
<i>Micrococcus glutamicus</i>	9.0
<i>Pseudomonas aeruginosa</i>	5.4
<i>Aspergillus oryzae</i>	73 mg/5 ml
<i>niger</i>	81
<i>Penicillium chrysogenum</i>	86
<i>oxalicum</i>	60
<i>Rhizopus nigricans</i>	63
<i>Neurospora sitophila</i>	70
<i>Monascus anka</i>	73
<i>Mucor jansseni</i>	33
<i>Trichoderma viride</i>	16

amino-substances extracted was similar when 0.3 N NaOH was used.

Cultivation of microorganisms in sludge extract.

(1) Growth of microorganisms in acid extract.

To explore the possibility of utilizing HCl extract of sludge as a cultivation material, the extract was neutralized, and microorganisms were screened for those that show sufficient growth in it. Table III summarizes part of the results obtained. Yeasts such as *Candida lipolytica*, *C. tropicalis*, *Hansenula anomala*, *H. miso*, *Rhodotorula mucilaginosa*, *Debaryomyces japonicus*, *D. hansenii*, and *Torulopsis candida*, bacteria such as *Flavobacterium arborescens*, *Micrococcus glutamicus*, and *Pseudomonas*, and fungi such as *Aspergillus*, *Penicillium*, *Rhizopus*, *Mucor*, *Neurospora*, and *Monascus* showed good growth. These results suggest that the acid extract of sludge contains inorganic salts and trace nutrients as well as sources of carbon and nitrogen, which are primary nutrients of microorganisms, but no growth inhibitory factors. This result suggests that recovery of pollutants and their use as a resource are simultaneously possible. Since the nutritional composition was found to differ between the acid extract and alkali extract, cultivation study was performed using a mixture of HCl extract and NaOH extract as the cultivation medium. The growth yields such as *Flavo. arborescens* and *Rh. mucilaginosa* were higher in the mixture than in the HCl extract or NaOH extract alone (Fig. 1). From the results shown in Table IV, the carbohydrate concentration was lower in the mixture than in the HCl extract alone, and this high growth in the mixed medium is considered to be due to supplementation of other nutrients. Therefore, cultivation in the mixed medium is considered to be advantageous in the growth

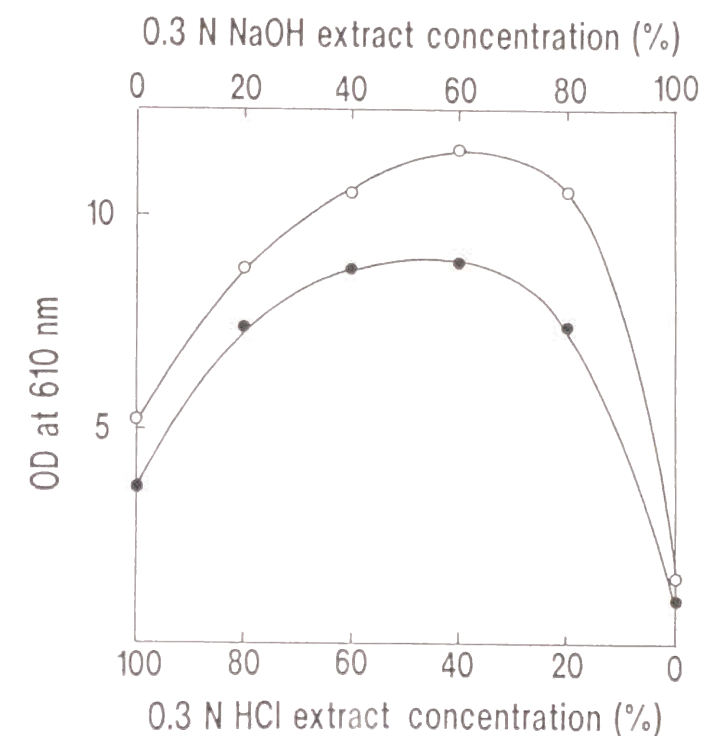


Fig. 1. Growth of microorganisms in the mixture HCl-extract and NaOH-extract of sludge.

HCl extract and NaOH-extract were mixed with ratio shown in Figure. Cultivation was carried out at 28 °C for 3 days with shaking.

Symbols: ○; *Flavo. arborescens*, ●; *Rh. mucilaginosa*.

Table IV. Composition of mixture of acid and alkali extracts of sludge.

Proportion		Initial concentration (mg/ml)				
0.3 N HCl extract	0.3 N NaOH extract	Amino-substances	Amino acid	Total sugar	Reducing sugar	Glucose
5	0	20.0	4.2	17.5	11.5	5.2
2	3	19.3	3.1	14.4	4.9	2.2
0	5	18.9	2.3	12.3	0.5	0.1

of some microorganisms.

(2) Alcoholic fermentation using acid extract of sludge.

The author noted that a considerable amount of fermentable carbohydrates are contained in the acid extract of sludge and examined whether it can be used a material of alcoholic fermentation. The H_2SO_4 extract of sludge was neutralized, concentrated 7 fold, and fermented by adding fresh bread yeast. Figure 2 shows the time course of fermentation. No marked inhibition of fermentation was observed, and ethanol production proceeded with a decrease in glucose. About 5 % of ethanol could be recovered from the fermentation fluid by distillation. Ethanol production was confirmed to proceed without inhibition also when acid-treated sludge was neutralized without filtration and allowed to ferment with sludge residues (Table V). These results suggest that acid-treated sludge with a high fermentable carbohydrate content may be used as a material of alcoholic fermentation.

(3) Value of non-fermentable carbohydrates and amino acid compounds as resources.

To evaluate the possibility of utilization carbon sources other than glucose and amino-substances contained in the acid extract of sludge, the growth of major microorganisms in a medium devoid of fermentable carbohydrates by alcoholic fermentation (glucose-free extract) was compared, and the amount of degraded medium components was determined. Sludge extract (unfermented acid extract) was used as the control. As shown in Table VI, molds and yeasts were found to have the ability to utilize amino-substances in addition to non-fermentable carbohydrates.

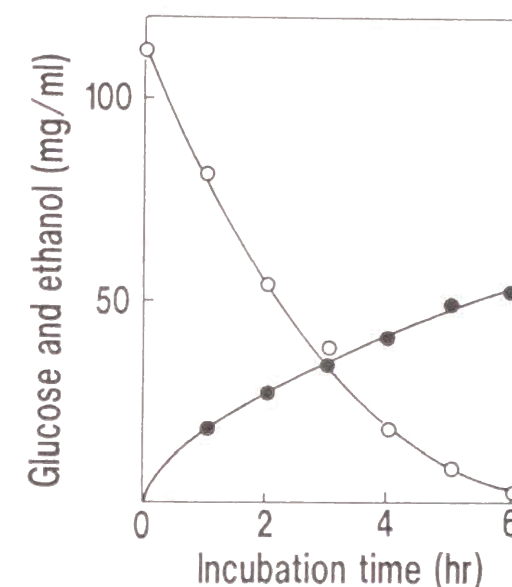


Fig. 2. Alcoholic fermentation using concentrated acid-extract of sludge.

The H_2SO_4 extract of sludge neutralized and concentrated was used as raw materials. Fermentation mixture contained H_2SO_4 -extract of sludge (50 ml) and 5 g of fresh baker's yeast. Fermentation was carried out at 37 °C. Symbols: ○; glucose, ●; ethanol.

Table V. Production of ethanol from acid extract of sludge.

	Initial glucose concentration (mg/ml)	Ethanol concentration (mg/ml)	
		Fermented	Distilled
0.5 N H_2SO_4 extract (concentrated to 1/7)	112	51	49
0.3 N HCl extract containing sludge residue	8.8	4.2	3.7

Table VI. Growth of microorganisms and consumption of nutritional components in 0.3 N HCl extract of sludge and glucose less extract.

Cultivation was carried out at 28 °C for 3 days with shaking. Growth was indicated optical density at 610 nm (yeast) or dry weight of mycelia (mg/5 ml, mold).

	Sludge extract					Glucose less extract			
	Growth	Consumption conc. (mg/ml)				Growth	Consumption conc. (mg/ml)		
		Amino-substances	Total sugar	Reducing sugar	Glucose		Amino-substances	Total sugar	Reducing sugar
<i>Candida lipolytica</i>	10.0	13.1	3.5	2.6	2.1	11.1	14.4	1.3	0
<i>parapsilosus</i>	9.2	2.6	5.7	5.2	2.2	12.3	3.4	3.7	0.2
<i>tropicalis</i>	8.2	2.5	6.5	5.2	2.2	9.4	2.9	3.2	0.6
<i>Debaryomyces hanseii</i>	13.2	0.9	8.4	5.4	2.1	12.1	2.7	2.9	1.4
<i>japonicus</i>	12.3	3.0	7.7	5.2	2.0	12.2	1.2	3.6	1.4
<i>Hansenula miso</i>	12.6	3.3	7.8	5.1	1.9	12.0	5.5	4.8	0.7
<i>anomala</i>	9.4	1.3	6.0	4.9	2.1	10.1	2.5	5.1	0.4
<i>Rhodotorula mucilaginosa</i>	11.1	1.3	7.8	5.1	2.0	11.3	6.8	3.2	1.0
<i>Torulopsis candida</i>	10.4	3.6	5.3	5.6	1.8	10.2	4.0	3.4	1.4
<i>Aspergillus cellulosa</i>	37	5.6	13.9	5.9	1.6	38	8.8	6.3	1.2
<i>oryzae</i>	77	8.2	7.7	5.5	2.0	73	6.8	7.3	1.5
<i>niger</i>	81	10.7	9.3	5.1	1.8	56	6.1	10.0	0.4
<i>awamori</i>	83	9.4	10.6	5.5	2.2	76	10.8	7.1	1.2
<i>Penicillium chrysogenum</i>	85	3.2	9.6	2.8	2.0	72	7.8	8.0	0.9
<i>oxalicum</i>	59	10.2	11.2	6.1	1.8	46	9.6	8.3	1.3
<i>Rhizopus nigricans</i>	63	7.6	7.3	4.9	2.0	39	10.3	4.1	0.4
<i>Mucor jansseni</i>	32	5.9	8.2	4.0	1.8	30	9.4	4.4	0.4
<i>Neurospora sitophila</i>	70	5.2	10.5	5.1	2.0	50	7.7	7.5	0.7
<i>Monascus anka</i>	73	5.3	7.6	4.4	2.0	46	6.5	4.4	0.6

Table VII. Consumption of amino acid by microorganisms in medium using 0.3 N HCl extract of sludge.
Cultivation was carried out at 28 °C for 3 days with shaking.

Amino acid in extract (µg/ml)	Amino acid consumed (µg/ml) by					
	<i>Flavobacterium arborescens</i>	<i>Hansenula miso</i>	<i>Rhodotorula mucilaginosa</i>	<i>Saccharomyces cerevisiae</i>	<i>Aspergillus oryzae</i>	
Asp	1030	750	1030	970	610	680
The	230	230	160	190	110	40
Ser	0					
Glu	210	140	170	160	80	50
Pro	310	310	310	310	310	310
Gly	370	360	350	340	190	230
Ala	530	530	490	500	350	310
Val	290	290	290	240	70	220
Met	210	140	110	210	60	90
Ile	230	190	180	200	70	140
Leu	350	350	320	310	180	240
Tyr	300	210	190	190	130	150
Phe	440	420	240	350	270	20
Lys	80	80	80	50	20	10
His	170	0	50	30	60	40
Arg	140	120	140	140	60	90
Total	4900	4150	4110	4180	1580	1550

The author, moreover, analyzed the amino acid content of the acid extract of sludge and its consumption, using *Saccharomyces cerevisiae* and *Aspergillus* as controls. As shown in Table VII, microorganisms such as *Flavo. arborescens*, *H. miso*, and *Rh. mucilaginoso* readily metabolized and degraded many amino acids in the acid extract.

It was reported that yeast could assimilate amino acid as a nitrogen source (15~17), however, the few studies on the assimilation of amino acid (amino-substances) as a carbon source by yeast was reported (18~20).

Therefore, to examine utilization of amino acid carbon chains by yeast, yeasts were cultivated aerobically in a casamino acid medium (casamino acid; 1.5 %, yeast extract; 0.2 %, KH_2PO_4 ; 0.05 %, K_2HPO_4 ; 0.05 %, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.03 %: pH 6.0) with and without glucose. Yeasts such as *C. lipolytica*, *C. tropicalis*, *H. miso*, *H. anomala*, *Rh. lactosa*, *Rh. rubra*, *Rh. glutinis*, and *Trichosporon cutaneum* were found to be able to utilize carbon of casamino acid for the synthesis of their constituent substances in the absence of glucose (Table VIII). Yeasts of the genus *Saccharomyces* (excluding *Sacch. decipiens*) such as bread yeast, sake yeast, and shoyu yeast exhibited the ability to degrade casamino acid, and their growth was markedly promoted in the presence of glucose. However, they were unable to utilize amino acids as the primary carbon source for their growth.

Next, whether yeasts can utilize various amino acids as the source of carbon and nitrogen for the growth was examined by culturing them with each amino acid alone. Yeasts such as *C. lipolytica*, *Rh. glutinis*, and *Tricho. cutaneum* were shown to derive carbon and nitrogen from a number of amino acids in a medium containing a single amino acid alone as the only source of

Table VIII. Growth of yeasts in the casamino acid medium with or without glucose.

Casamino acid medium contained 1.5 % casamino acid, 0.2 % yeast extract, 0.05 % KH_2PO_4 , 0.05 % K_2HPO_4 , 0.03 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Cultivation was carried out at 28 °C for 3 days with shaking.

	IFO No.	Glucose 0 %		Glucose 1 %
		Growth (OD ₆₁₀)	Consumption of casamino acid	Growth (OD ₆₁₀)
<i>Candida lipolytica</i>	0746	2.9	12.7 mg/ml	7.6
<i>lipolytica</i>	0717	1.2	7.9	2.6
<i>lipolytica</i>	1209	3.9	10.9	8.1
<i>tropicalis</i>	0587	3.1	1.0	7.0
<i>tropicalis</i>	0589	3.2	4.1	8.8
<i>utilis</i>	0396	1.1	0	5.0
<i>Saccharomyces sake</i> No. 6		0.3	3.1	5.8
<i>sake</i> No. 7		0.3	4.1	7.4
<i>cerevisiae</i>	0021	0.5	6.0	5.5
<i>cerevisiae</i>	0259	0.2	2.4	4.7
<i>decipiens</i>	0102	2.5	3.4	2.6
<i>rouxii</i>	0595	0.1	1.7	3.9
<i>Hansenula anomala</i>	0118	2.3	1.3	5.4
<i>anomala</i>	0149	2.8	0.7	6.9
<i>miso</i>	0146	2.6	2.2	8.0
<i>Rhodotorula lactosa</i>	1006	1.9	11.6	6.7
<i>rubra</i>	0892	3.4	6.1	5.1
<i>rubra</i>	0003	2.5	7.5	7.2
<i>rubra</i>	0714	3.8	0	7.4
<i>glutinis</i>	0688	2.6	9.1	9.2
<i>Trichosporon cutaneum</i>	1198	2.8	10.0	6.3

-: Not determined

carbon and nitrogen. Especially, *C. lipolytica* was found to utilize proline, alanine, valine, leucine, isoleucine, lysine, arginine, glutamic acid, and serine as the only source of carbon and nitrogen for growth (Table IX).

With particular attention to this exceptional carbon utilization observed in part of yeasts, the author then examined their growth in a casein mesium. As a result, it was found that considerable species of yeast including *C. lipolytica* can grow in a glucose-casein medium and that *C. lipolytica* can grow within a short period using casein as the only source of carbon and nitrogen (Tables X, and XI). The finding that these particular yeasts can utilize amino acids and macromolecular peptides as a primary source of carbon for growth (the synthesis of essential components) is considered to pose new important problems in classification of yeasts and in the fields of physiology and biochemistry. At the same time, the use of this new ability of carbon utilization may enable efficient manufacturing of yeasts from waste water of food processing factories containing amino acids and peptides as the primary components. Advancement of studies in this respect is anticipated for the development of environmental cleaning techniques and new food and nutritional resources.

From the above observations, acid extract of sludge is considered to contain nitrogen-containing nutrients and inorganic salts as well as carbohydrates but no growth inhibitory factors so that it may be used as a material for production of useful substances such as single cell protein. Also, the ability of amino acid and peptide utilization observed in certain yeasts, bacteria, and molds may be used generally to reduce BOD of organic waste water from various factories and/or to exploit it

Table IX. Growth of yeast in the medium contained each amino acid alone.

Amino acid medium contained 1 % amino acid, 0.2 % yeast extract, 0.05 % KH_2PO_4 , 0.05 % K_2HPO_4 , 0.03 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Cultivation was carried out at 28 °C for 3 days with shaking.

	<i>Candida lipolytica</i>		<i>Rhodotorula glutinis</i>		<i>Trichosporon cutaneum</i>	
	Growth (OD ₆₁₀)	Consumption of amino acid (%)	Growth (OD ₆₁₀)	Consumption of amino acid (%)	Growth (OD ₆₁₀)	Consumption of amino acid (%)
None	0.3	—	0.3	—	0.5	—
Asp	0.8	—	0.8	—	1.6	30
The	0.9	—	0.6	—	2.4	42
Ser	2.4	98	1.3	18	2.2	64
Glu	2.9	87	1.3	32	2.4	68
Pro	4.2	100	1.3	64	3.9	82
Gly	1.0	—	0.8	—	1.3	—
Ala	2.7	95	0.8	—	2.3	42
Val	4.3	100	1.1	12	1.3	23
Met	0.3	31	0.5	6	0.8	5
Ile	5.7	100	1.9	15	3.0	27
Leu	3.5	100	1.2	—	3.6	31
Tyr	0.6	—	1.0	—	2.3	—
Phe	0.8	36	1.7	33	3.6	75
Lys	3.2	97	0.4	—	1.7	26
His	0.2	—	0.5	—	1.5	—
Arg	2.3	100	0.8	—	1.4	24
Try	0.4	7	0.5	—	1.4	—

—: Not determined

Table X. Growth of yeast in the casein medium with or without glucose.

Casein medium contained 1 % casein, 0.2 % yeast extract, 0.05 % KH_2PO_4 , 0.05 % K_2HPO_4 , 0.03 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Cultivation was carried out at 28 °C for 3 days with shaking.

	Glucose 0 %		Glucose 1 %	
	Growth (OD_{610})	Consumption conc. of Protein (mg/ml)	Growth (OD_{610})	Consumption conc. of protein (mg/ml)
<i>Candida lipolytica</i>	2.0	5.0	5.1	6.1
<i>Rhodotorula lactosa</i>	0.6	-	6.2	-
<i>glutinis</i>	0.7	-	5.4	-
<i>mucilaginosa</i>	0.5	0.4	6.4	1.0
<i>Trichosporon cutaneum</i>	1.0	0.3	5.4	0.7
<i>Hansenula miso</i>	0.2	0.2	4.6	1.2
<i>Debaryomyces subglobosus</i>	0.5	-	7.4	-
<i>Saccharomyces cerevisiae</i>	0.1	0.1	1.9	0.5
<i>Torulopsis dattila</i>	0.1	-	3.9	-

-: Not determined

Table XI. Microbial growth in the casein medium with or without glucose.

Casein medium contained 1 % casein, 0.2 % yeast extract, 0.05 % KH_2PO_4 , 0.05 % K_2HPO_4 , 0.03 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Cultivation was carried out at 28 °C for 3 days with shaking.

	Glucose (%)	Growth (OD_{610})	Consumption conc.(mg/ml)	
			Protein	Reducing sugar
<i>Flavobacterium arborescens</i>	0	5.6	6.4	
	2	19.0	3.4	3.4
<i>Candida lipolytica</i>	0	2.0	5.0	
	2	9.8	4.8	11.4
<i>Hansenula miso</i>	0	0.6	0.2	
	2	8.2	0.2	11.4

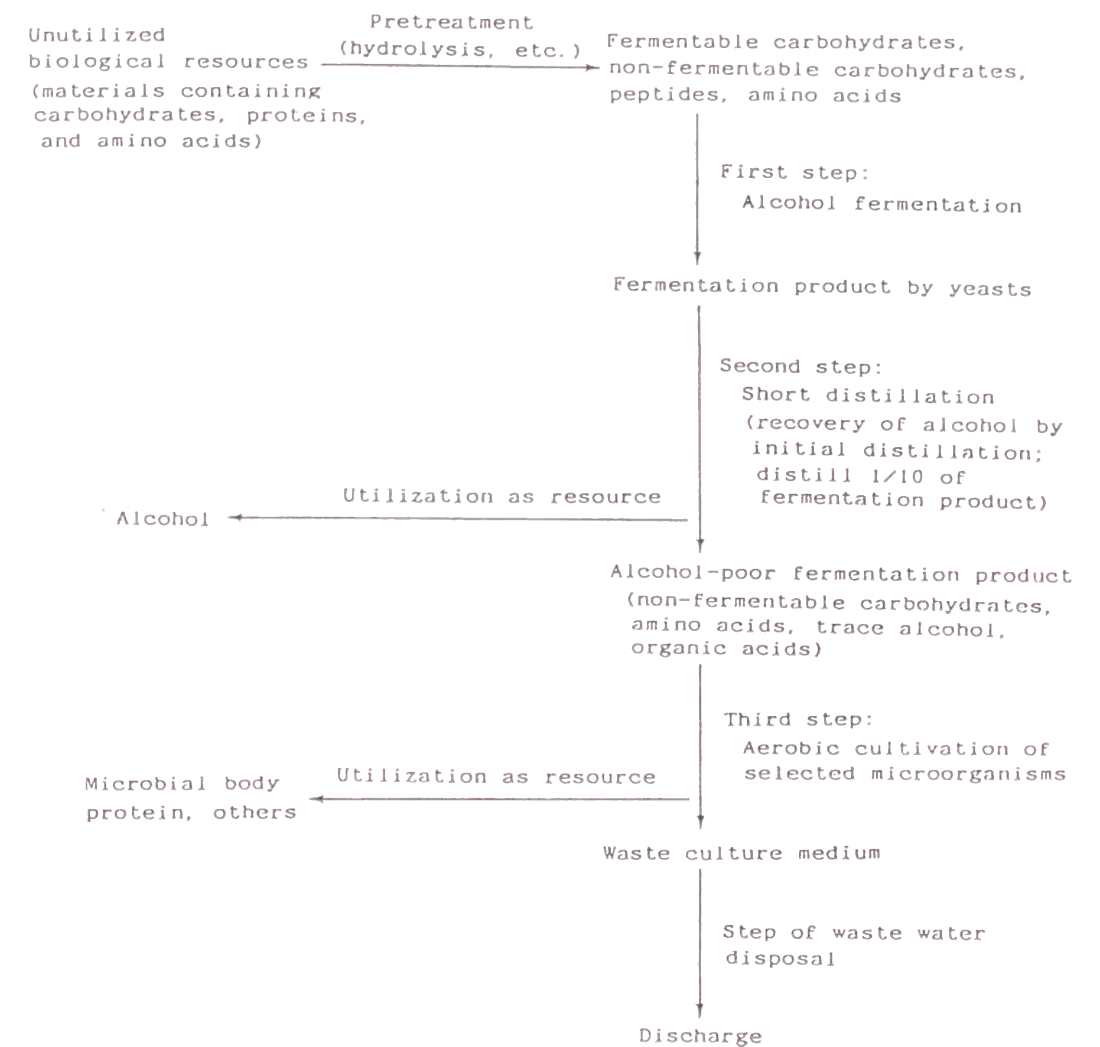


Fig. 3. Step-wise utilization of unutilized biological resources.

as a resource.

Moreover, from the analysis of acid extract of sludge, the following system of its microbiological utilization is suggested as a principle of development of generally unutilized biological resources with a low fermentable carbohydrate content but high contents of non-fermentable carbohydrates and amino-substances. That is, in the first step, alcoholic fermentation is performed with yeast. In the second step, alcohol is recovered by first distillation of the coarse fermentation product (fluid containing a few percentage of alcohol is collected by distilling about one-tenth the volume of the fermentation product). In the third step, single cell protein and other substances are obtained from the fermentation product after collection of alcohol by the first distillation (aerobic cultivation of microorganisms that can utilize non-fermentable carbohydrates, amino-substances, trace amounts of alcohol, and organic acids). The author would like to propose this stepwise system for microbial purification of sludge and its utilization as resources, which is generalized as in Fig. 3.

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Summary

Conditions for efficient extraction of amino-substances and carbohydrates from sludge were determined. Amino-substances and carbohydrates, about 30 % of the dry weight of sludge, respectively, were obtained by adding acid or alkaline to a final

concentration of 0.3 N and heating it at 120 °C for 30 min. The weight of the sludge after extraction was reduced to about 70 % of the initial weight. When this acid extract was neutralized and used as cultivation medium, it allowed rich growth of a number of microorganisms. When alcoholic fermentation was also carried out using this extract to examine its usefulness as a fermentation material, alcoholic fermentation proceeded. These results suggested that the extract contained no inhibitory factors of microbial growth or fermentation. The distillation residue of the extract after alcoholic fermentation also allowed the growth of many microorganisms. From these results, the author designed a general method for utilization of unutilized biomass as new resources.

Section 2. Purification and characterization of a novel α -L-fucosidase from *Fusarium oxysporum* grown on sludge

Introduction

At present, increasing surplus active sludge which is produced on the treatment of city sewage and plant drainage is becoming a social problem, because it is one of the great unutilizable biomasses. The author has investigated ways of reusing surplus sludge with microorganisms. The author took advantage of the abundant glycoconjugates in the sludge and tried to obtain useful glycosidases produced by microorganisms able to assimilate the sludge as a medium. During the screening of glycosidases capable of degrading complex carbohydrates, the author found that a strain of fungus isolated from a soil sample produced a novel α -L-fucosidase in the culture fluid. The fungus was identified as *Fusarium oxysporum* Schlechtendahl emend. Snyder and Hansen.

L-Fucose is a very important component of various oligo- and polysaccharides, and glycoconjugates. It is usually located on the nonreducing termini of the carbohydrate moieties of several biologically active molecules such as serum glycoproteins, immunoglobulins, submaxillary mucins and blood group substances (1). It is also present in oligosaccharides of human milk, marine algae polysaccharides and plant gums (2). This sugar moiety of some complex carbohydrates plays an important role in cellular recognition, the immune response, etc.

α -L-Fucosidase (EC 3. 2. 1. 51) has been found to release L-fucose from these molecules by splitting the terminal

α -L-fucosidic linkage. All enzymes from various sources reported generally showed a narrow aglycon specificity. They seem to be classifiable into two types. One type comprises the microbial enzymes. The α -L-fucosidases from *Trichomonas foetus* (3), *Clostridium perfringens* (4), *Aspergillus niger* (5) and *Bacillus fulminans* (6) were found to release fucose from blood group substances but did not hydrolyze simple nitrophenyl fucosides. They may be α -(1 $\rightarrow$ 2)-specific L-fucosidases. The enzyme from almond emulsin (7) also showed this type of specificity. On the other hand, the α -L-fucosidases from mammalian tissues (8~10) abalone (11) and a marine gastropod (12) readily hydrolyzed *p*-nitrophenyl α -L-fucoside and also liberated fucose slowly from mucin, but seemed not to hydrolyze blood group substances.

The fungal α -L-fucosidase showed a novel substrate specificity in that it could hydrolyze both *p*-nitrophenyl α -L-fucoside and blood group substances. This section describes the purification and properties of the unique α -L-fucosidase from *Fusarium oxysporum* isolated from soil which was grown on active sludge hydrolyzate as a medium.

Materials and Methods

Preparation of the sludge hydrolyzate.

Active sludge was obtained after waste treatment from a Japanese sake company. Its water content was about 85 %. The hydrolyzate of the active sludge as a specific medium for microorganisms was prepared according to the following procedure. Active sludge was added to an equal volume of 0.6 N NaOH solution, and then the mixture was hydrolyzed by autoclaving it

at 120 °C (1.2 atm) for 30 min. It should be hydrolyzed with 0.3 N NaOH. After centrifugation at 10,000 x g for 30 min, the supernatant was dialyzed against tap water overnight and then lyophilized. 0.3 N HCl solution was added to the lyophilized powder, and then the mixture was hydrolyzed by autoclaving it for 30 min again. The hydrolyzate was neutralized by adding NaOH.

Microorganisms.

The fungal strain, S252, which was isolated from a soil sample under *Musa basjoo* sieb. in the botanical gardens of Kyoto University, was used throughout this investigation.

Cultivation.

The fungus was cultured in a liquid medium composed of 2 % sludge hydrolyzate, pH 7.0. A loop of the spore suspension was inoculated into 5 ml of the medium in a test tube. Cultivation was carried out at 28 °C with shaking. The fungus was maintained on 2 % sludge hydrolyzate-agar slants.

Enzyme assay.

α -L-Fucosidase activity was assayed using *p*-nitrophenyl α -L-fucoside as substrate. The enzyme solution (30 to 50 μ l containing 5~10 μ g protein) was added to 0.25 ml of 2 mM substrate dissolved in 50 mM sodium citrate buffer (pH 4.5). After incubation for an appropriate time at 37 °C, 1.75 ml of 0.2 M sodium borate buffer (pH 9.8) was added to terminate the reaction and the release of *p*-nitrophenol was determined at 400 nm. With these conditions, an extinction coefficient of $1.77 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (13) was used to calculate the concentration of *p*-nitrophenol in assay mixtures. Other glycosidase activities

were also assayed using the corresponding *p*-nitrophenyl glycosides as substrates according to the same procedure as for α -L-fucosidase activity. One unit of the enzyme activity was defined as the amount of the enzyme which released 1 μ mol of *p*-nitrophenol per min. The specific activity was expressed as units per mg of protein.

Materials.

p-Nitrophenyl derivatives of sugars were purchased from Nakarai Chemicals Ltd. Hydroxylapatite was prepared according to the method of Tiselius *et al* (14). Sephadex G-150 and G-200, and Concanavalin A-Sepharose 4B were obtained from Pharmacia Fine Chemicals Co. Porcine gastric mucin was obtained from Nakarai Chemicals Ltd. All other chemicals used were of the highest grade commercially available.

Analyses.

Protein was determined by the method of Lowry *et al.* (15) with crystalline egg albumin as the standard. For chromatographic procedures, the protein content was estimated by measuring the absorbance at 280 nm. The amount of free fucose was determined by the microdiffusion method (16) using a Conway chamber.

Gel electrophoresis.

Polyacrylamide disc gel electrophoresis was performed by the method of Davis (17) with a 7.5 % polyacrylamide gel and Tris-glycine buffer, pH 8.3. Electrophoresis was carried out at a current of 2 mA per column and gels were stained for protein with Amido black.

Molecular weight determination.

The molecular weight of the enzyme was estimated by gel filtration on a column of Sephadex G-200 (1.0 x 120 cm) by the method of Andrews (18). Elution was carried out with 10 mM potassium phosphate buffer (pH 7.0). Catalase (MW, 232,000), aldolase (MW, 158,000), bovine serum albumin (MW, 67,000), ovalbumin (MW, 45,000), chymotrypsinogen (MW, 25,000) and ribonuclease (MW, 13,700) were used as standard proteins.

Hemagglutination inhibition test for serological activity.

The soluble H blood group substance of human saliva was prepared as follows: human group O saliva was centrifuged and the supernatant was boiled for 10 min. After centrifugation, 99 % ethanol was added to the supernatant to 80 %. The precipitate was lyophilized and the resulting powder used as the soluble H group substance. The group O cell suspension was prepared as follows: O cells were washed with saline three times. After the last wash, the pellet was suspended to 3 % in saline. The hemagglutination-inhibition test was carried out as follows. To 0.1 ml of each serial dilution of an *Ulex europaeus* (anti-H lectin) extract solution was added 0.1 ml of the H substance solution treated with varying numbers of units of the α -L-fucosidase at 37 °C for 2 hr, and the solutions were then incubated for 10 min at room temperature. Then, 0.2 ml of the 3 % O cell suspension was added to each of the solutions. The H substance after decomposition by the enzyme no longer inhibited the anti-H activity of the lectin. Therefore, the author could determine the degree of decomposition of the H substance by means of the hemagglutination-inhibition test.

Results

Morphological characteristics of the fungus.

The isolated fungus grew rapidly as a white aerial mycelium which soon became tinged with purple when it was grown on malt extract-agar (MA) medium containing 2 % of malt extract, 2 % of glucose, 0.1 % of peptone and 2 % of agar. The fungus grown on the above medium abundantly produced only single-celled, ellipsoidal, oval to kidney-shaped microconidia in false heads. On the other hand the fungus produced abundant sickle-shaped, thin-walled, 3~4 septate macroconidia, with an attenuated apical cell and a prominent foot cell, when it was grown on Czapek agar medium or corn meal agar medium. Sometimes chlamydospores were formed singly or in pairs in aerial hyphae and macroconidia, being either terminal or intercalary. Conidiophores were sparsely branched or unbranched, as short, lateral monophialides on hyphae (Fig. 1). The average size of microconidia was 5~14 x 1.6~4 μm . The 3-septate macroconidia averaged 24 x 4~5.6 μm in size. The growth rate on MA-agar medium plate was fairly rapid (about 6 mm/day) at 28 °C, but the fungus grew only slightly at 37 °C. The presence of chlamydospores, microconidia borne in false heads on simple short lateral monophialides and thin-celled, sickle-shaped. 3~4 septate macroconidia are the most distinguishable characteristics of *Fusarium oxysporum* Schlechtendahl (19~21) with which the fungus described above was identified.

Production of α -L-fucosidase.

After the fungus had been cultivated in a liquid medium composed of 2 % sludge hydrolyzate for 2 days, various

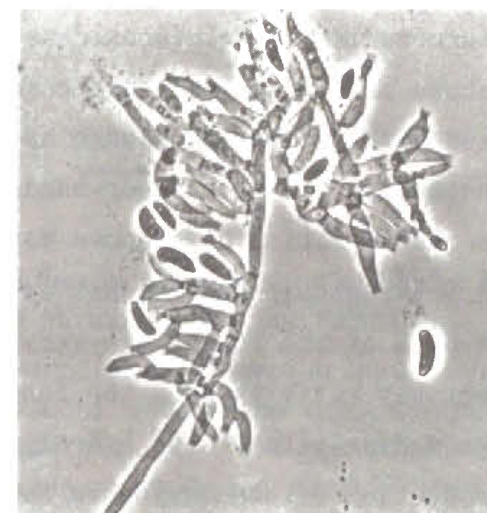


Fig. 1. Micrograph of *Fusarium oxysporum* strain S252 isolated from soil.

Simple or sparsely branched, short, lateral monophialides on a mycelium on sludge hydrolyzate agar medium.

glycosidase activities in the culture fluid were investigated. The author found high α -L-fucosidase activity and slight β -glucosidase and β -galactosidase activities. The latter was about one-eighth of the former. Then, the effect of the concentration of sludge hydrolyzate used as the sole nutrient source for the culture medium on α -L-fucosidase activity was investigated. As shown in Fig. 2, the enzyme activity on 2 days' cultivation increased with increasing sludge hydrolyzate concentration in the medium. On the other hand, no activity was found even when the concentration of glucose was increased in a medium containing 0.5 % peptone, 0.5 % yeast extract and 0.5 % NaCl although the growth of the fungus was very good. The author also could not detect any activity when the fungus was cultivated in the active sludge medium without the hydrolysis treatment.

For maximum production of the enzyme in case of cultivation on 2 % sludge hydrolyzate medium, the author determined the enzyme activity in the culture fluid at different stages of growth of the fungus. As shown in Fig. 3, the enzyme activity was highest at 3 days, when the mycelial weight and pH of the medium became constant.

Purification of α -L-fucosidase.

Purification was conducted at 5 °C. Potassium phosphate buffer (pH 7.0) was used throughout the purification procedure.

Crude enzyme in the culture fluid.

After 3 days' cultivation with 2 % sludge hydrolyzate, the culture broth in 39 test tubes, each containing 5 ml of medium, was combined and centrifuged at 10,000 x g for 30 min to remove mycelia and the sludge hydrolyzate precipitates. The resulting

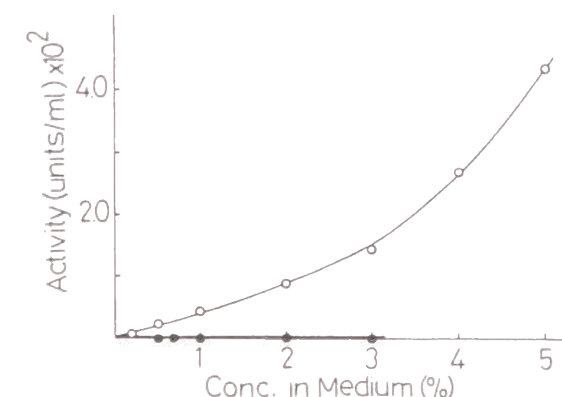


FIG. 2. Effect of Concentration of Sludge Hydrolyzate as Culture Medium on Enzyme Activity.

Enzyme activities were assayed in culture filtrates after 2 day cultivations with various concentrations of sludge hydrolyzate (O) and various concentrations of glucose in medium containing 0.5% peptone, 0.5% yeast extract and 0.5% NaCl (●).

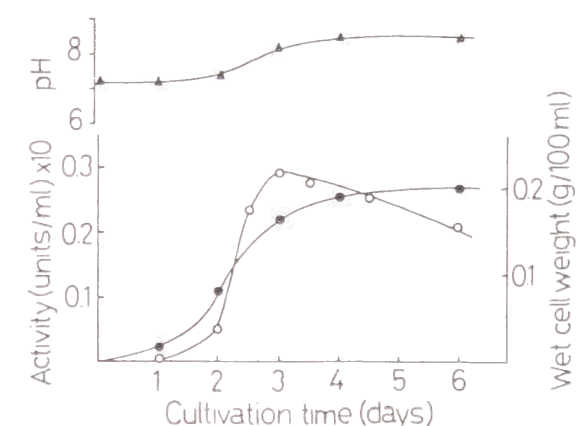


FIG. 3. Time-courses of Growth and Enzyme Production in 2% Sludge Hydrolyzate Cultures of *F. oxysporum*.

Wet cell weight (●), α -L-fucosidase (O) and pH (▲) in culture broth.

brown supernatant was used as the crude enzyme preparation for purification.

Ammonium sulfate fractionation.

To about 180 ml of the supernatant, solid ammonium sulfate was added to 75 % saturation. After standing overnight, the precipitate was collected by centrifugation and dissolved in 10 mM phosphate buffer. The solution was dialyzed overnight against the same buffer.

DEAE-Sephadex A-50 column chromatography.

The dialyzed enzyme solution (40 ml) was charged on a DEAE-Sephadex A-50 column (2.2 x 17 cm) previously equilibrated with 10 mM phosphate buffer. The column was thoroughly washed with the same buffer and the enzyme was eluted with a linear gradient of NaCl of from 0 to 0.8 M in the same buffer. The active fractions were pooled and made to 80 % saturation with ammonium sulfate. The resulting precipitate was dissolved in 1 mM phosphate buffer and then dialyzed against the same buffer overnight.

Hydroxylapatite column chromatography.

The dialyzed enzyme solution (28 ml) was charged on a hydroxylapatite column (2.3 x 12.5 cm) previously equilibrated with 1 mM phosphate buffer. The column was washed with the same buffer. The adsorbed enzyme was eluted with linear gradient of phosphate buffer of from 1 mM to 0.5 M. The active fractions were pooled and concentrated by the addition of ammonium sulfate to 80 % saturation. The precipitate obtained was dissolved in a small volume of 10 mM phosphate buffer.

Sephadex G-150 column gel filtration.

The concentrated enzyme solution (4 ml) was applied on a Sephadex G-150 column (1.5 x 105 cm) previously equilibrated with 10 mM phosphate buffer and eluted with the same buffer. The active fractions were pooled and precipitated by 80 % saturation with ammonium sulfate. The precipitate was dissolved in 50 mM phosphate buffer and then dialyzed overnight against the same buffer.

Con A-Sepharose 4B column chromatography.

The dialyzed enzyme solution (6 ml) was applied to a column (0.8 x 6 cm) of Con A-Sepharose 4B equilibrated with 50 mM phosphate buffer. After the column had been washed with 50 mM phosphate buffer and the same buffer containing 0.1 M NaCl, the enzyme was eluted with a linear gradient of 0 to 0.5 M methyl α -D-mannoside in the above buffer containing 0.1 M NaCl (Fig. 4). The active fractions were pooled and concentrated with a collodion bag. The concentrated enzyme solution was used as the purified enzyme.

The results of the enzyme purification are summarized in Table I. The enzyme was purified about 360-fold with a recovery of 20 %. The purified enzyme was shown to be almost homogeneous by disc gel electrophoresis on polyacrylamide gel as shown in Fig. 5.

Molecular weight.

The molecular weight of the enzyme was estimated by gel filtration on Sephadex G-200 to be about 80,000.

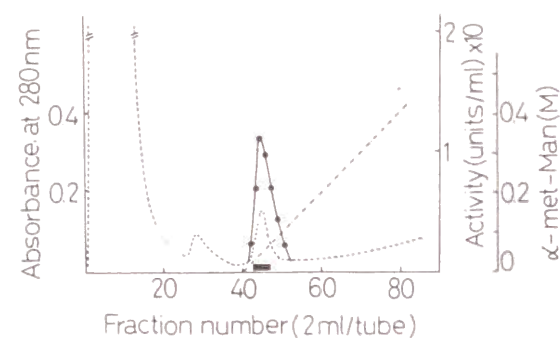


FIG. 4. Con A-Sepharose 4B Column Chromatography of α -L-Fucosidase.

The enzyme solution (ca. 5.4 mg of protein) was applied to a column (0.8×6 cm) of Con A-Sepharose 4B equilibrated with 50 mM phosphate buffer (pH 7.0). The column was eluted with the same buffer and the buffer containing 0.1 M NaCl, followed by with a linear gradient of methyl α -D-mannoside in the same buffer. ●, α -L-fucosidase; -----, absorbance at 280 nm; - - - - -, methyl α -D-mannoside concentration; ■■, fractions pooled.

TABLE I. PURIFICATION OF α -L-FUCOSIDASE FROM *F. oxysporum*

	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Culture filtrate	538	3.82	0.0071	100
Ammonium sulfate precipitation	376	3.68	0.0098	96
DEAE-Sephadex A-50 column chromatography	51	2.19	0.043	57
Hydroxylapatite column chromatography	26	2.02	0.078	52
Sephadex G-150 column chromatography	5.4	1.56	0.29	40
ConA Sepharose 4B column chromatography	0.3	0.77	2.58	20



Fig. 5. Disc gel electrophoresis of the purified α -L-fucosidase.

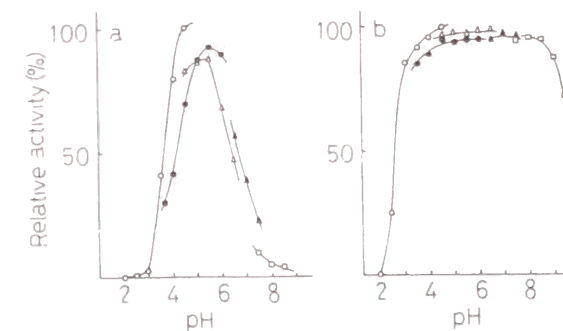


FIG. 6. Effects of pH on the Activity and Stability of the Enzyme.

(a) Effect of pH on the activity. The activity at various pHs was measured under the standard assay conditions ($7.5 \mu\text{g}$ of protein) using various buffer system (50 mM) and expressed as percentage of the maximum activity.

(b) Effect of pH on the stability. The enzyme was kept at various pHs at 4°C for 2 days using various buffer systems (20 mM). The remaining activity was determined by the standard assay method ($7.5 \mu\text{g}$ of protein) and expressed as a percentage of the original activity.

Buffers: ○, sodium citrate-HCl; ●, acetate; △, citrate; ▲, potassium phosphate; □, Tris-HCl.

Effects of pH on the activity and stability.

The enzyme showed maximum reactivity in the pH range of from 4.5 to 5.5, as shown in Fig. 6a. The enzyme was stable over the wide pH range of 4~8, as shown in Fig. 6b.

Effects of temperature on the activity and stability.

The optimum temperature for the enzyme reaction was about 50 °C under the standard assay conditions (pH 4.5). The heat stability of the enzyme was examined by heating it at various temperatures for 10 min at pH 7.0. The enzyme was found to be stable up to about 45 °C.

Effects of various substances on the enzyme activity.

The effects of various metal ions and SH-reagents on the enzyme were investigated. The enzyme (10 µg protein) was preincubated with 20 mM of a metal ion or with 10 mM of a SH-reagent in 10 mM potassium phosphate buffer (pH 7.0) at 37 °C for 15 min, and then the residual activity was assayed under the standard conditions. Though marked inhibition by Hg^{2+} was seen, the following ions had no significant effect on the enzyme activity: Mg^{2+} , Mn^{2+} , Co^{2+} , Cu^{2+} , Zn^{2+} , Fe^{2+} and Ca^{2+} . SH-reagents such as dithiothreitol, 2-mercaptoethanol, *p*-chloro-mercuric benzoate and *N*-ethylmaleimide did not have a significant effect on the activity.

Effects of various sugars on the enzyme activity.

The effects of various sugars on the enzyme activity were examined by preincubating the enzyme with 2.5 mM of each sugar at 37 °C for 10 min, and then the residual activity was assayed. The enzyme activity was reduced to 85 % and 95 % by L-fucose and

D-arabinose, which has a similar structure to L-fucose, respectively. Other sugars including D-fucose and L-arabinose had no effect on the enzyme activity.

Effect of the substrate concentration.

The effect of the substrate concentration on the enzyme activity was examined at 37 °C using *p*-nitrophenyl α -L-fucoside. The Michaelis constant, calculated from a Lineweaver-Burk plot, was 8.7×10^{-4} M.

Substrate specificity.

The purified enzyme was investigated as to other glycosidase activities by assaying with appropriate *p*-nitrophenyl glycosides: β -D- and β -L-fucosides, α - and β -D-glucosides, α - and β -D-galactosides, α - and β -D-mannosides, α - and β -D-*N*-acetylglucosaminides, α - and β -D-*N*-acetylgalactosaminides, and β -D-glucuronide. The enzyme showed only α -L-fucosidase activity, no other glycosidase activities being found.

The ability of the enzyme to split L-fucose off from naturally occurring substrates was examined using porcine gastric mucin. As shown in Fig. 7, the enzyme was able to hydrolyze porcine gastric mucin and liberated L-fucose residues.

Change in the serological activity of a salivary blood group substance caused by the enzyme.

The H activity of the secretor in saliva, before and after treatment with the α -L-fucosidase, was examined by means of the hemagglutination inhibition test as shown in Table II. The hemagglutination inhibition test was carried out using O cells-anti H lectin of *Ulex europaeus* system. Before the

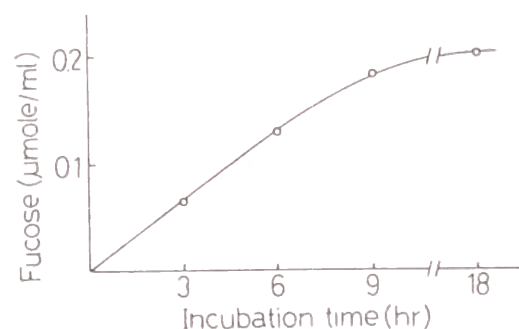


FIG. 7. Liberation of Fucose from Porcine Gastric Mucin by α -L-Fucosidase.

Porcine gastric mucin (30 mg/ml) in 0.05 M sodium citrate buffer (pH 4.5) was incubated with the enzyme solution (0.007 units/ml) at 37°C. Aliquots were analyzed for liberated fucose.

TABLE II. INHIBITION OF ANTI-H LECTIN BY α -L-FUCOSIDASE-TREATED GROUP O SALIVA

Details of the hemagglutination inhibition test were given under MATERIALS AND METHODS. Figures represent the degree of hemagglutination of O cells and anti-H lectin. 4 indicates strong hemagglutination and 1 weak hemagglutination. A minus sign indicates no hemagglutination.

	Dilution of anti-H (<i>Ulex europaeus</i>)							
	1,	2,	4,	8,	16,	32,	64,	128,
Untreated O saliva	—	—	—	—	—	—	—	—
0.013 units fucosidase treated O saliva	3	1	—	—	—	—	—	—
0.04 units fucosidase treated O saliva	3	3	2	—	—	—	—	—
Saline	4	4	4	4	4	3	—	—

treatment, the H substance of saliva completely inhibited the hemagglutination of O cells by the anti-H lectin. After enzyme treatment of the salivary blood group substance, the inhibition was significantly reduced. The conversion of the H substance to an inactive form would be the result of the removal of the terminal linked α -L-fucoside of the H group substance by the enzyme.

Discussion

Recently, as the problem of treatment of surplus sludge has increased, it has been expected that the surplus sludge can be reused in some way. The author used it as a medium for microorganisms and tried to obtain useful enzymes produced by the microorganisms. It was reported that active sludge contains rhamnose, fucose, arabinose, xylose, mannose, galactose and glucose, as neutral sugar components (22, 23). These sugars seemed to exist in the form of complex carbohydrates. The author found various glycosidases produced by many microorganisms grown on a medium of active sludge hydrolyzate (data not shown). This may indicate that the microorganisms produced various glycosidases in order to utilize the sludge hydrolyzate.

During the progress of the study on sludge, a strain of *Fusarium oxysporum* isolated from soil was found to produce a unique α -L-fucosidase in the culture fluid only when it was cultivated on the active sludge hydrolyzate medium. α -L-Fucosidase is very useful for elucidating the structures of glycoconjugates which have L-fucose as a nonreducing terminal residue with binding through an α -glycosidic linkage. It was

reported by Bahl (5) that α -L-fucosidase can be broadly classified into two groups: those which are specific for the L-fucopyranosyl group and the α -anomeric configuration of the fucosidic linkage, and those which require, in addition, the presence of the specific α -1,2 linkage of fucose to the next sugar residue in the carbohydrate chain. The former class of fucosidases hydrolyzes alkyl or aryl fucosides and also liberates fucose from the nonreducing ends of oligo- or polysaccharide chains, but do not hydrolyze blood group substances. The fucosidases of mammalian tissues (8~10), abalone (11) and a marine gastropod (12) which are able to hydrolyze *p*-nitrophenyl α -L-fucoside belong to this class. The latter class of fucosidases does not hydrolyze alkyl or aryl fucosides, but hydrolyzes only specific α -1,2-fucosidic linkages in oligo- and polysaccharides. Microorganisms (3~6) and almond (7) fucosidases show this type of specificity. Although they do not hydrolyze *p*-nitrophenyl α -L-fucoside, they have attracted much attention because of their specificity as to the hydrolysis of blood group substances having specific fucosyl linkages. Their activities were assayed on the basis of the amount of fucose liberated from natural substrates such as blood group substances by using a Conway chamber, gas-liquid chromatography or a serological method. The enzyme the author found could hydrolyze *p*-nitrophenyl α -L-fucoside and also liberated fucose from porcine gastric mucin and a blood group substance in saliva. So, the author can easily determine the enzyme by using *p*-nitrophenyl α -L-fucoside as a substrate. Moreover, since this fungal enzyme is an extracellular one, in contrast with the *Bacillus* and *Trichomonas* enzymes which are intracellular (3, 6), it is also easier to obtain it. This is the first report of such a novel

enzyme being found in a microorganism.

The author concluded that this fungal enzyme is an inducible one, on consideration of some information on the catabolite repression by glucose (the detailed data are not shown). This may be the first report concerning an inducible α -L-fucosidase. However, some properties of this enzyme except substrate specificity, such as optimum pH and stability, were not so different from those of the two types of enzyme mentioned above.

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Summary

A novel α -L-fucosidase was found in the culture broth of *Fusarium oxysporum* isolated from a soil sample when the fungus was cultivated on a liquid active sludge hydrolyzate medium. The enzyme was not found in the culture broth of the fungus grown on glucose medium. The α -L-fucosidase from the fungus was purified to homogeneity by polyacrylamide gel electrophoresis after ammonium sulfate fractionation and successive column chromatographies on DEAE-Sephadex A-50, hydroxylapatite, Sephadex G-150 and Con A-Sepharose 4B. The molecular weight was estimated to be about 80,000 by gel filtration, and the optimum pH was found to be 4.5. The enzyme was relatively stable in the pH range of 4~8 and up to 45 °C on 10 min incubation. The K_m value for *p*-nitrophenyl α -L-fucoside was 0.87 mM. The enzyme showed a novel substrate specificity in that it could hydrolyze porcine mucin and blood group substances of human saliva besides nitrophenyl compounds. Such a specificity has not been found for any other α -L-fucosidase from various sources.

Chapter II. Production of enzymes for fermentation using wheat
bran and development of a soft gel cultivation method

Section 1. Purification and properties of glutaminase from
Aspergillus oryzae

Introduction

In soy sauce fermentation, it is important to increase the amount of glutamic acid in the mash for a delicious taste. Glutamic acid is formed through two pathways during brewing; firstly, the release from material proteins, in which proteinases and peptidases play roles, and secondly, the hydrolysis of free glutamine catalyzed by glutaminase (L-glutamine amidohydrolase, EC 3. 5. 1. 2). Nakadai and Nasuno (1) demonstrated that the proteinase did not directly participate in the release of glutamic acid and that leucine amino peptidase greatly contributed to it. As for glutaminases of soy sauce fermenting *Aspergillus* strains, some properties of partially purified preparations were investigated by Kuroshima *et al.* (2), Yamamoto and Hirooka (3, 4), Shikata *et al.* (5, 6), and Teramoto *et al.* (7), but no report on a purified glutaminase has been found. Therefore, it can be said that though the glutaminases of various species of bacteria (8~12) and animals (13~17) have been well studied, many points about *Aspergillus* glutaminases still remain to be elucidated in detail.

Two forms of glutaminases, a free form and binding form, are known to exist in *Aspergillus oryzae* (5~7). In this work, the author selected the free form of glutaminases from the viewpoint

of ease of purification. The intracellular and the extracellular glutaminases were purified to a single protein from *A. oryzae* MA-27-IM, and their characteristics were studied with the aim of adapting the results for the brewing of a high quality soy sauce with a suitable glutamic acid concentration.

Materials and Methods

Microorganisms.

In this study, *A. oryzae* MA-27-IM was mainly used, isolated from a commercial *koji* seed, "Asahi-1", used for soy sauce fermentation. Asahi-1 contains three kinds of *Aspergillus oryzae* strains, MA-27-IM, Lee-1 and H-16.

Medium and cultivation.

Seed culture: Each strain was grown at 28 °C for a few days, until spores were well formed, on an agar slant containing 1 % malt extract and 0.1 % yeast extract, pH 6.5. After pouring 5 ml of sterilized water into the slant tube, it was stirred vigorously to prepare a spore suspension.

Koji culture: A solid *koji* medium composed of 30 g wheat bran moistened with 30 ml of potassium phosphate buffer (0.17 M, pH 7.2) was prepared in a 15 cm-diameter petri dish. After autoclaving at 120 °C for 30 min, the spore suspension was inoculated into the wheat bran and mixed in well. Cultivation was performed at 28 °C for 72 hr. After 24 hr cultivation, the culture was mixed again for aeration.

Extraction of enzymes.

All procedures were carried out at 4 °C. The solid *koji* culture was extracted with twice its weight of distilled water for 12 hr and then filtered through cotton cloth. The filtrate was used as the crude preparation of extracellular glutaminase. The debris containing mycelia was washed twice with sufficient distilled water to remove remaining extracellular enzymes and the washing solution was discarded. Then, each 100 g of the debris was disrupted with 100 g of sea sand C (Nakarai Chemicals Ltd.) and 250 ml of 10 mM potassium phosphate buffer (pH 7.2) in a mill for 1 hr, followed by centrifugation at 10,000 x g, for 20 min. The supernatant was used as the crude intracellular glutaminase preparation.

Determination of enzyme activity.

Glutaminase activity was assayed by a modified method of Suzuki (18). A reaction mixture (final volume, 1.0 ml) containing Tris-HCl buffer (pH 7.2, 100 mM), 8 mM L-glutamine and 100 µl of the enzyme preparation was incubated for 60 min at 30 °C. If necessary, the enzyme preparation was diluted with 100 mM Tris-HCl buffer (pH 7.2). The reaction was stopped by immersing the test tube in boiling water for 3 min. After removal of precipitated proteins by centrifugation, the volume of the supernatant was adjusted to 1 ml with distilled water, followed by mixing with 2 ml of a glutamate dehydrogenase solution consisting of potassium phosphate buffer (pH 7.2, 100 mM), 10 mM EDTA-Na, 4 mM NAD, 400 mM hydroxylamine-HCl and 0.32 mg glutamate dehydrogenase from beef liver. After incubation at 30 °C for 75 min, the absorbance of the mixture at 340 nm was measured. One unit of glutaminase activity was defined as the amount of enzyme that catalyzed the release of 1 µmol of L-glutamic acid per min

from L-glutamine under this condition. The specific activity was expressed in units per mg protein.

Determination of protein.

The protein concentration was determined by the method of Lowry *et al.* (19) or measuring optical density at 280 nm. The latter method was used for monitoring protein in column chromatographies.

Electrophoresis.

Disc gel electrophoresis was carried out in 10 % polyacrylamide gels at pH 9.4, according to the method of Davis (20).

Amino acid analysis.

Amino acids were analyzed with an amino acid analyzer (Kyowa Seimitsu KLA-101S) according to the method of Speckman *et al* (21).

Chemicals.

Glutamate dehydrogenase from beef liver was purchased from Oriental Yeast Co., Ltd., and DL-theanine was the product of Takara Kohsan Co., Ltd. The *koji* seed, "Asahi-1", was obtained from Hisiroku Co., Ltd. Chymotrypsinogen, bovine serum albumin, catalase, DEAE-Sepharose CL-6B, Sepharose 6B and Sephadex G-150 were the products of Pharmacia Fine Chemicals. Alcohol dehydrogenase was obtained from Sigma Chemicals Ltd.

Results

Glutaminase activities of A. oryzae.

Glutaminase activities of *A. oryzae* strains Lee-1, H-16 and MA-27-IM were examined in both intracellular and extracellular fractions. Their extracellular fractions contained about twice as much total activity as their intracellular fractions, respectively (Table I). The intracellular and extracellular fractions of strain MA-27-IM contained the highest glutaminase activities among the three strains. Therefore, this strain was used in further studies.

Time course of glutaminase production.

The time course of glutaminase production by strain MA-27-IM in a wheat bran *koji* culture is shown in Fig. 1. Higher glutaminase activity was found in the extracellular fraction than in the intracellular fraction. The time course profiles of the two fractions, however, resembled each other. The maximum production of the enzymes was found at 70~80 hr incubation.

Purification of the intracellular glutaminase.

Strain MA-27-IM was cultivated in a wheat bran *koji* medium (18 dishes) at 28 °C for 72 hr. After extraction as described under Materials and Methods, the glutaminase in the intracellular fraction was purified as shown in Table II. Approximately 730-fold purification was achieved with recovery of 6.2 %.

Purification of the extracellular glutaminase.

Wheat bran *koji* cultures in 36 dishes were used. Extracellular glutaminase was purified in almost the same way as

Table I. Glutaminase production in wheat bran *koji* culture.

Strain		Enzyme activity (units)
Lee-1	Intracellular	13.9
	Extracellular	25.1
H-16	Intracellular	10.4
	Extracellular	22.9
MA-27-IM	Intracellular	20.1
	Extracellular	40.2

After 72 h cultivation, the intracellular and extracellular fractions were prepared as described under Materials and Methods. Enzyme activities are expressed per 100 g of wheat bran *koji*.

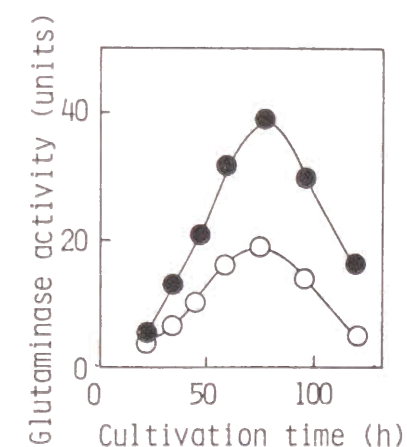


Fig. 1. Time course of glutaminase production in a wheat bran *koji* culture *A. oryzae* MA-27-IM. The culture conditions and enzyme activity measurement method are given under Materials and Methods. Enzyme activity is expressed per 100 g of wheat bran *koji*. ○, intracellular glutaminase; ●, extracellular glutaminase.

Table II. Purification of the intracellular glutaminase.

Purification steps	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
1. Crude extract	6610	55.1	0.008
2. Freezing and thawing	2890	39.3	0.014
3. $(\text{NH}_4)_2\text{SO}_4$ ^a	1040	22.2	0.021
4. DEAE-Sephadex CL-6B	74.5	11.4	0.153
5. Sephadex 6B	36.6	9.13	0.249
6. Hydroxyl apatite	1.39	4.19	3.01
7. Sephadex G-150	0.58	3.40	5.86

^a 45–75% saturated.

Table III. Purification of the extracellular glutaminase.

Purification steps	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
1. Crude extract	100600	389	0.004
2. Freezing and thawing	67400	346	0.005
3. $(\text{NH}_4)_2\text{SO}_4$ ^a	11000	295	0.027
4a. DEAE-Sephadex CL-6B (i)	2470	82.4	0.033
4b. DEAE-Sephadex CL-6B (ii)	853	38.3	0.045
5. Sephadex 6B	177	33.1	0.187
6. Hydroxyl apatite	17.1	28.1	1.64
7a. Sephadex G-150 (i)	4.29	15.6	3.64
7b. Sephadex G-150 (ii)	2.82	12.4	4.40

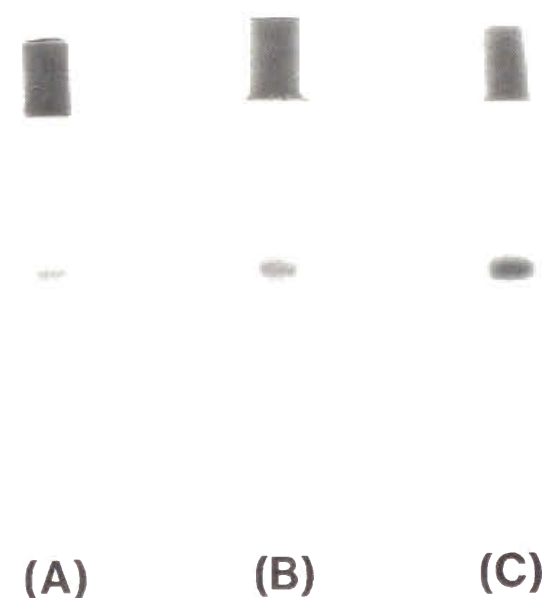
^a 45–75% saturated.

Fig. 2. Polyacrylamide gel electrophoresis of glutaminases. Electrophoresis was carried out at a current of 2 mA for 120 min. Protein was stained with Amido Black 10B. The amount of protein applied was 20 μg in each case. (A) intracellular glutaminase; (B) extracellular glutaminase; (C) a mixture of the intracellular and extracellular glutaminases (20 μg + 20 μg).

intracellular one. A summary of the purification is presented in Table III. These procedures resulted in 1,100-fold purification with recovery of 3.2 %.

Polyacrylamide gel electrophoresis.

Figures 2(A) and 2(B) show polyacrylamide gel electrophoresis of the final preparations of the intracellular and extracellular glutaminases. Both preparations gave single protein bands with almost identical electrophoretic mobilities. A single protein band also appeared when a mixture of both preparations was subjected to electrophoresis (Fig. 2(C)).

Molecular weights of glutaminase.

As standard proteins, chymotrypsinogen (MW 25,000), bovine serum albumin (MW 67,000), alcohol dehydrogenase (MW 148,000) and catalase (MW 232,000) were used. The molecular weights of the intracellular and extracellular glutaminases were estimated to be about 113,000 by gel filtration on a Sephadex G-150 column.

Effects of pH and temperature.

Both the intracellular and extracellular glutaminases showed fairly sharp pH dependence curves and were found to be most active at pH 9.0 (Fig. 3(A) and 3(B)). They were equally most stable at pH 9.0 and relatively stable in a basic pH range (Figs 3(C) and 3(D)). The optimum temperature for both glutaminases was 45 °C (Figs. 4(A) and 4(B)), and both were stable up to 37 °C, but almost completely lost their activities at 55 °C (Figs. 4(A) and 4(B)).

Effects of metallic salts and sulfhydryl reagents.

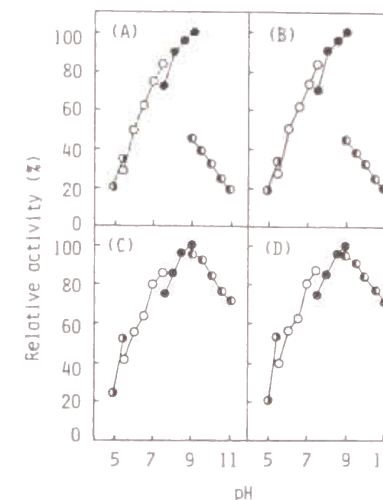


Fig. 3. Effect of pH on glutaminase activity and stability. (A) optimal pH of the intracellular glutaminase; (B) optimal pH of the extracellular glutaminase: The enzyme activity was measured in buffers of various pHs containing 0.5 μ g/ml enzyme preparation; (C) pH stability of the intracellular glutaminase; (D) pH stability of the extracellular glutaminase. The enzyme preparations (5 μ g/ml) were stored in buffers of various pHs (100 mM) at 4°C for 66 h and the residual activities in the mixtures were measured as described in *Materials and Methods*. $\circ$, acetate buffer; $\circ$, potassium phosphate buffer; $\bullet$, Tris-HCl buffer; $\bullet$, glycine-NaOH buffer.

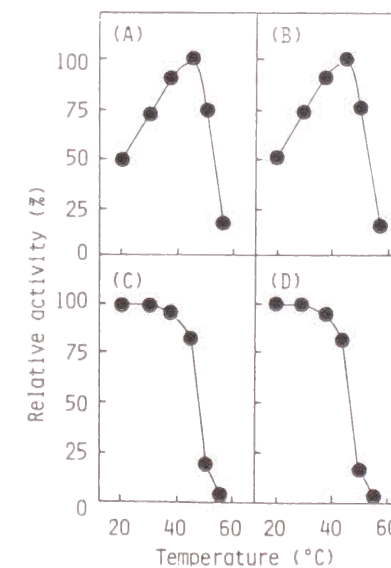


Fig. 4. Effect of temperature on glutaminase activity and stability. (A) optimal temperature of the intracellular glutaminase; (B) optimal temperature of the extracellular glutaminase. The enzyme activity was measured at various temperatures in potassium phosphate buffer (pH 7.2, 100 mM) containing 0.5 μ g/ml enzyme preparations; (C) thermal stability of the intracellular glutaminase; (D) thermal stability of the extracellular glutaminase. The enzyme preparations (5 μ g/ml) were preincubated in potassium phosphate buffer (pH 7.2, 100 mM) at various temperatures for 10 min.

After storage in the presence of Hg^{2+} , Cr^{2+} , Fe^{2+} and Fe^{3+} , the residual activities of both glutaminases decreased to about half the initial levels and about 30 % of their residual activities were lost with Pb^{2+} (Table IV). No sulfhydryl reagents (each 2 mM) affected their stabilities (data not shown). With monoiodoacetate (2 mM), their stabilities were slightly affected, and their remaining activities were reduced to 88 % of their initial levels.

Effect of sodium chloride.

Both glutaminase activities decreased in the presence of sodium chloride, as shown in Figs. 5(A) and 5(B), and they were inhibited about 50 % with 5 % sodium chloride. At a sodium chloride concentration of 18 %, both activities were inhibited to about one-tenth the original levels.

Effect of the L-glutamine concentration.

K_m values of both glutaminases for L-glutamine were investigated. The reaction mixture contained 0.1~10 mM L-glutamine, 100 mM Tris-HCl buffer (pH 7.2) and 2 $\mu\text{g}/\text{ml}$ enzyme. After incubation for 30 min at 30 °C, the amount of glutamic acid formed was measured with an amino acid analyzer. Using Lineweaver-Burk plots (22), the Michaelis-Menten constants for the intracellular and extracellular glutaminases were calculated to be 9.1×10^{-5} M and 9.6×10^{-5} M, respectively.

Substrate specificity.

As shown in Table V, the relative activities of both enzymes towards various substrates were identical. These glutaminases hardly catalyzed the hydrolysis of D-glutamine, or L- or D-

Table IV. Effects of metallic salts on the glutaminase stability.

Compound	Remaining activity (%)	
	Intracellular	Extracellular
None	100	100
HgCl_2	43	38
ZnSO_4	88	90
MnCl_2	94	89
MgCl_2	98	102
CaCl_2	105	100
NiSO_4	98	98
CoCl_2	102	101
CuCl_2	102	98
CrCl_2	48	43
FeCl_3	48	45
FeSO_4	60	52
$\text{Pb}(\text{CH}_3\text{COO})_2$	75	68

After 5 μg of enzyme was stored with each compound (2 mM) at 4°C for 66 h in 1 ml potassium phosphate buffer (pH 7.2, 100 mM), the residual activity in the mixture was measured as described in *Materials and Methods*.

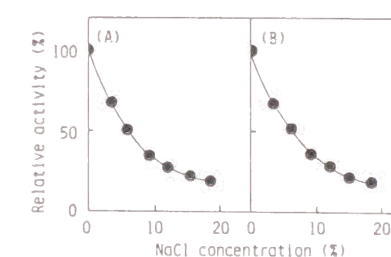


Fig. 5. Effect of the sodium chloride concentration on glutaminase activity. (A) intracellular glutaminase; (B) extracellular glutaminase. The reaction mixture contained 0~18% NaCl, 100 mM Tris-HCl buffer (pH 7.2) and the enzyme preparation (0.5 $\mu\text{g}/\text{ml}$). The enzyme activity was measured as described in *Materials and Methods*.

Table V. Substrate specificity of the glutaminases.

Substrate	Relative activity (%)	
	Intracellular	Extracellular
L-Glutamine	100	100
D-Glutamine	3	2
L-Asparagine	0	0
D-Asparagine	0	0
DL-Theanine	88	87
Glutathione	105	109
L- γ -Glutamyl- <i>p</i> -nitroanilide	126	131

The reaction mixture was composed of each substrate (8 mM), 100 mM Tris-HCl buffer (pH 7.2) and glutaminase (2 μ g/ml). After incubating each mixture at 30°C for 1 h, the glutamic acid or aspartic acid released was measured with an amino acid analyzer.

asparagine. On the other hand, they were active towards γ -glutamyl derivative, *i. e.*, DL-theanine, glutathione and L- γ -glutamyl-*p*-nitroanilide, the relative activities being 90 %, 110 % and 130 %, respectively.

Discussion

Glutaminases exist widely in nature, animal ones especially being regarded as very important from the medical point of view with respect to the metabolism of glutamic acid and ammonia (13~16). As for microorganisms, the glutaminases of *Escherichia coli* have been studied in detail (8, 13).

In the present study the author found that for the three strains tested the glutaminase activities in the extracellular fractions were about two-fold higher than those in the intracellular fractions, though more glutaminase activity is said to exist in the intracellular fraction (7). The extracellular enzymes are advantageous from the point of view of a direct attack upon raw substrate materials.

The author selected strain MA-27-IM as the highest glutaminase producer, and its intracellular and extracellular glutaminases were purified. The results obtained indicate that the intracellular and extracellular glutaminases from strain MA-27-IM may be the same protein.

The molecular weight of the two enzymes were about 113,000, a value resembling the molecular weight, of glutaminases delivered from other microorganisms such as *Aspergillus sojoe* (123,000) (3), *E. coli* (110,000) (8) and *Pseudomonas* (140,000) (9). The optimum pH of these glutaminases was 9.0, which is in a

sharp contrast to the optimum pH value of 5.0 for the glutaminases from *E. coli* (8) and *Clostridium welchii* (10). The Michaelis-Menten constants were calculated to be about $9.1 \sim 9.6 \times 10^{-5}$ M for the hydrolysis of L-glutamine, being smaller than those of glutaminases from *A. sojae* (3.3×10^{-4} M) (3) and hog kidney (4.0×10^{-4} M) (17).

In general, many glutaminases hydrolyze not only L-glutamine but also γ -glutamyl compounds (11). Ohshima *et al.* (12) purified two glutaminase isozymes from *Pseudomonas aeruginosa* and showed that one catalyzed the deamination of asparagine but did not hydrolyze theanine, and that the other hydrolyzed γ -glutamyl derivatives but not asparagine. Ramadan *et al.* (9) isolated a glutaminase from a strain of *Pseudomonas* and found that the enzyme acted as an asparaginase but did not hydrolyze γ -glutamyl compounds. With regard to glutaminases from strain MA-27-IM, like one of the isozymes from *P. aeruginosa*, they hydrolyzed γ -glutamyl compounds but did not show asparaginase activity.

From the view point of practical soy sauce fermentation, an active and stable pH range of the enzyme and its tolerance against sodium chloride are important. Resembling glutaminases from other soy sauce fermenting *Aspergillus*, those obtained from strain MA-27-IM showed weak activities and poor stabilities at pH 5.0, and exhibited only less than 15 % of the total activity at a sodium chloride concentration of 16 %, which are practical conditions for soy sauce production. These conditions are, therefore, very unfavorable for the glutaminases.

As for just the optimum and stable pH range of a glutaminase, improvement of the brewing conditions and/or searches for new types of glutaminases in mutants or other strains might be effective. Furthermore, a reduction of sodium

chloride concentration might also be favorable for increasing the glutamic acid concentration in soy sauce fermentation.

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Summary

Glutaminase activity was found in a water extract of a wheat bran *koji* culture (extracellular fraction) of *Aspergillus oryzae* strains Lee-1, H-16 and MA-27-IM isolated from a commercial *koji* seed for soy sauce fermentation, as well as in their mycelia (intracellular fraction). Both the intracellular and the extracellular glutaminases were purified from strain MA-27-IM. Polyacrylamide gel electrophoresis of each purified preparation gave a single protein band with identical electrophoretic mobility. The molecular weight of the intracellular and the extracellular glutaminases were estimated to be approximately 113,000. Both preparations hydrolyzed various γ -glutamyl compounds besides L-glutamine but did not exhibit asparaginase

activity. Further investigations of these preparations indicated that these glutaminase possessed almost the same properties, suggesting their similarity.

Section 2. Design of a soft gel cultivation method

Introduction

Koji, solid culture of mold with grains, is used not only in traditional fermentation processes but also in recent production of useful enzymes (1, 2) and metabolites (3~5). However, quantitative analysis of enzyme production by *koji* cannot sufficiently be carried out because of difficulty in completely obtaining pure mycelia from solid substrates. Though liquid cultivation is often used for analysis of *koji* cultivation, the features of *koji* are not preserved in liquid cultivation due to differences in aeration, water activity and so on. The surface-culture method is inconvenient because of difficulty and instability in mat formation and in enzyme production. Cultivation methods using asbestos (6, 7), sponge (8) and nylon-paste (9, 10) have been developed to replace *koji* cultivation, but have not been studied sufficiently.

The author designs a new convenient method (soft gel cultivation) of obtaining a pure mycelial-mat. Mold grew on a soft gel medium containing 0.2~0.4 % agar just as well as it did on conventional *koji*, and the mycelial-mat was separated easily from the surface of the medium. Characteristics with respect to the productivity and localization of some enzymes are also indicated.

Materials and Methods

Microorganism and culture conditions.

Aspergillus oryzae MA-27-125 was used. This strain was isolated from Asahi No 1, a commercial seed *koji* for soy sauce fermentation. The basal medium for soft gel cultivation contained 10 % (w/w) wheat bran and 0.4 % Bacto-agar. *A. oryzae* spores were inoculated on the surface of the medium (100 g) in a petri dish (15 cm in diameter). Conventional *koji* cultivation was carried out with wet wheat bran (water content: 50 %, w/w, 60 g), and liquid cultivation with a medium (500 ml) containing 5 % (w/w) wheat bran. They were incubated at 28 °C for 3 days statically (soft gel and *koji* cultivations) or with shaking (liquid cultivation).

Fractionation of enzymes.

(1) *Soft gel cultivation*: After cultivation, the mycelial-mat was peeled from the surface of the medium, and ground in a mill at 5 °C for 1 hr with the same weight of sea sand C and a small amount of 0.01 M potassium phosphate buffer (KPB, pH 7.0). The mixture was suspended in the same buffer and then centrifuged. The supernatant was dialyzed against the same buffer (whole-mycelial-mat fraction, Whole-MF).

The residual agar medium was ground and treated similarly (extra-mycelial-mat fraction, Extra-MF).

Whole-MF and Extra-MF fractions were recognized as intracellular and extracellular fractions, respectively.

(2) *Koji cultivation*: The *koji* was soaked overnight at 5 °C in distilled water (two times the weight of *koji*), and filtered. The filtrate was dialyzed against 0.01 M KPB (extracellular fraction, Extra-CF).

The filter cake was washed with water, and then ground with

sea sand C. The centrifuged supernatant was dialyzed against 0.01 M KPB (intracellular fraction, Intra-CF).

(3) *Liquid cultivation*: The culture was centrifuged. The centrifuged supernatant of the culture was dialyzed against the 0.01 M KPB (extracellular fraction, Extra-CF).

The precipitate was ground with sea sand C, and the extract was dialyzed against the same buffer (intracellular fraction, Intra-CF).

Determination of enzyme activity.

Glutaminase: The reaction mixture containing 8 mM glutamine, 100 mM Tris-HCl buffer (pH 7.2) and enzyme solution was incubated at 30 °C. The reaction was terminated by immersion of the mixture in boiling water for 3 min. The amount of glutamate formed was determined enzymatically by the method of Suzuki (11). One unit of activity was defined as the amount forming 1 μ mole of glutamic acid per min.

Leucine aminopeptidase (LAP): The activity was assayed by measuring leucine formed at 30 °C in a reaction mixture containing 0.25 mM leucylglycylglycine (LeuGlyGly), 50 mM Tris-HCl buffer (pH 8.0) and the enzyme solution. Leucine was determined colorimetrically with ninhydrin (12). One unit of LAP activity was defined as the amount forming 1 μ mole of leucine per min.

Neutral proteinase: Proteinase activity was determined by the modified method of Hagihara (13). The reaction mixture (1.25 ml) containing 65 mg/ml casein, 50 mM KPB (pH 7.0) and enzyme solution was incubated at 37 °C for 30 min. The reaction was terminated by the addition of 1.25 ml of 0.44 M trichloroacetic acid, and the filtrate was assayed by the Lowry's method (14).

One unit of activity was defined as the amount developing color equivalent to 1 mg of egg albumin per min.

α -Amylase: α -Amylase activity was assayed by measuring the decrease in color of the iodine-starch complex (15). The reaction mixture (1 ml) contained 2.4 mg/ml soluble starch, 0.1 M acetate buffer (pH 5.0) and enzyme solution. One unit of enzyme activity was defined as the amount hydrolyzing 10 mg of soluble starch per min at 37 °C.

α -Glucosidase: The reaction mixture (0.2 ml) contained 2 mM *p*-nitrophenyl- α -glucopyranoside, 0.05 M KPB (pH 6.8) and enzyme solution. After incubation for 10 min at 30 °C, the reaction was stopped by the addition of 1.5 ml of 0.2 M borate buffer (pH 9.8), and *p*-nitrophenol released was measured colorimetrically at 400 nm. One unit of activity was defined as the amount releasing 1 μ mole of *p*-nitrophenol per min (molar extinction coefficient $17.7 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) (16).

Determination of growth and protein.

Growth in soft gel cultivation was indicated by wet weight of mycelial-mat.

Protein was determined by the method of Lowry *et al.* (14) with egg albumin as a standard.

Enzyme and chemicals.

Bacto-agar was obtained from Difco Co. Ltd. Glutamate dehydrogenase from beef liver was purchased from Oriental Yeast Co. Ltd. *p*-Nitrophenyl- α -glucopyranoside, alginates and sea sand C were purchased from Nacalai Tesque Co. Ltd. and carrageenans from Sigma Chemical Co. Ltd. LeuGlyGly was obtained from the Institute for Protein Research, Osaka University. Other

chemicals were analytical grade commercial products.

Results and Discussion

Effect of agar concentration on growth and enzyme production.

Figure 1 shows the effect of agar concentration on mycelial growth and enzyme production in soft gel medium. The weight of mycelial-mat (indicating growth) and the amount of Whole-MF and Extra-MF protein hardly varied with agar concentration.

The total activity of glutaminase and LAP in Whole-MF increased with an increase in agar concentration to 0.4 %. On the other hand, the total activity in Extra-MF decreased with an increase in agar concentration, and was scarcely detected in fractions with 0.4 % agar or more.

α -Amylase activity was observed in both fractions, with maximum activity in 0.8 % agar medium. Proteinase activity was also detected in both fractions, but not affected by the agar concentration.

α -Glucosidase, a representative of intracellular enzymes, was hardly found in Extra-MF, and total activity of the enzyme in Whole-MF decreased with an increase in agar concentration to 0.4 %.

The ratio of total activity of each enzyme in Whole-MF to that in Extra-MF varied with the agar concentration differently.

Table I summarizes the ratio of total activity of the enzymes produced by soft gel cultivation using various kinds of gel. The activity ratio of glutaminase and that of LAP increased in parallel with gel hardness, which is consistent with the results shown in Fig. 1, but that of proteinase and α -amylase

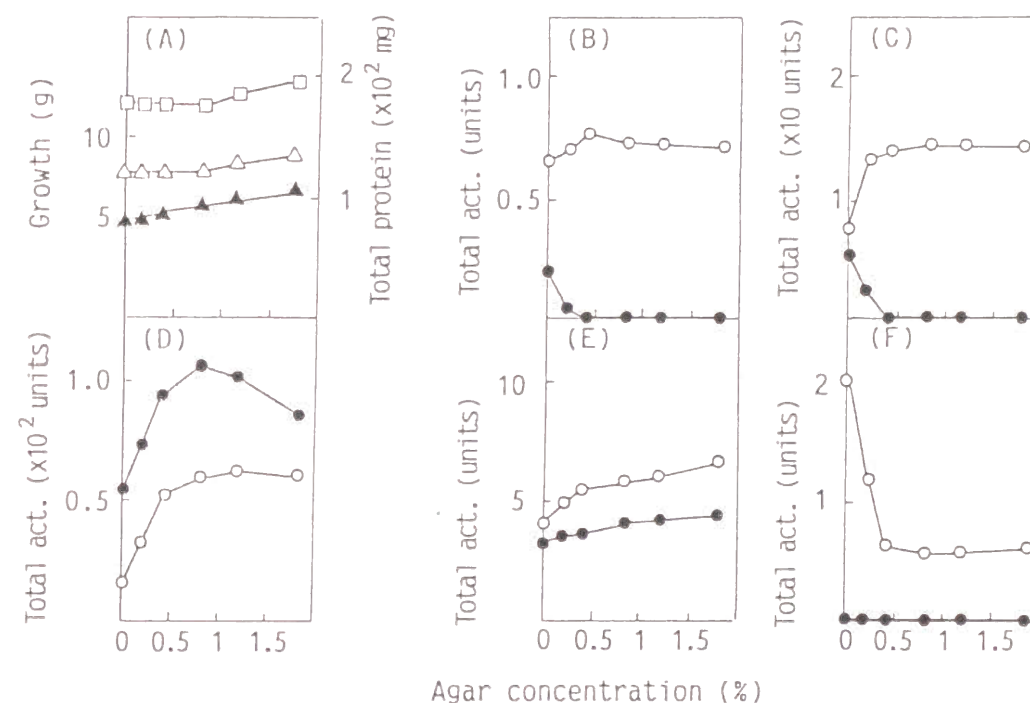


Fig. 1. Effect of agar concentration on enzyme productivity.

Cultivation was carried out under conditions described in Materials and Methods. Agar concentration was varied as indicated. (A) growth (wet weight of mycelia) and total protein, (B) glutaminase activity, (C) LAP activity, (D) α -amylase activity, (E) neutral proteinase activity and (F) α -glucosidase activity.

Symbols: $\square$; growth, $\triangle$; total protein of Whole-MF, $\blacktriangle$; total protein of Extra-MF, $\circ$; total activity in Whole-MF, $\bullet$; total activity in Extra-MF.

Table I. Activity ratio of the enzymes in soft gel cultivation using various kinds of gels.

Cultivation was carried out under the conditions described in Materials and Methods. The kind and concentration of gel changed as indicated in the Table. The viscosity value of alginate in the Table was for 1.0 % gel concentration at 20 °C (commercial information). Type V carrageenan contained pure iota-carrageenan and formed flexible and compliant gels. Type II, a blend of carrageenans, formed flexible gels and contained predominantly iota-carrageenan. Type III contained pure kappa-carrageenan and formed very rigid gels (commercial information).

Gel	Activity in Whole-MF / Activity in Extra-MF				
	Glutaminase	LAP	Proteinase	α-Amylase	α-Glucosidase
0.4 % Agar	35.6	27.3	1.52	0.88	109
0.5 % Alginate					
300 cps	14.7	14.9	1.73	0.83	110
500 cps	21.3	16.4	1.78	0.91	99
1000 cps	31.3	30.8	1.75	0.95	103
0.5 % Carrageenan					
Type V	15.5	6.5	1.83	0.85	106
Type II	21.6	17.7	1.62	0.72	106
Type III	36.9	27.3	1.71	0.71	115

hardly varied.

An agar concentration of 0.4 % was used in the following experiments.

Effect of concentration of wheat bran in the gels.

Figure 2 shows the mycelial growth and enzyme production on gels containing various concentrations of wheat bran. The yields of mycelial-mat and total protein of Whole-MF and Extra-MF increased with the concentration. The total activity of five enzymes in Whole-MF increased in proportion with the increase in total protein. The total activity of proteinase and α-amylase in Extra-MF also increased with wheat bran concentration, and the ratio of total activity of these enzymes in Whole-MF to that in Extra-MF was hardly influenced by wheat bran concentration. Glutaminase, LAP and α-glucosidase were not detected in Extra-MF.

These results indicate that localization of enzymes was not influenced by wheat bran concentration.

Effect of the amount of soft gel medium.

As shown in Fig. 3, mycelial growth increased with the increase in the amount of gel up to 150 g in a dish, and the total protein in Whole-MF increased similarly. The total protein in Extra-MF increased in parallel with the increase in soft gel medium.

Most of the glutaminase and LAP activity (about 95 %) was found in Whole-MF, and their activity in Extra-MF became detectable with culture media of 200 g or more. Proteinase and α-amylase were found in both fractions, independent on gel amount, and the activity ratio of Whole-MF to Extra-MF decreased with the increase in gel amount.

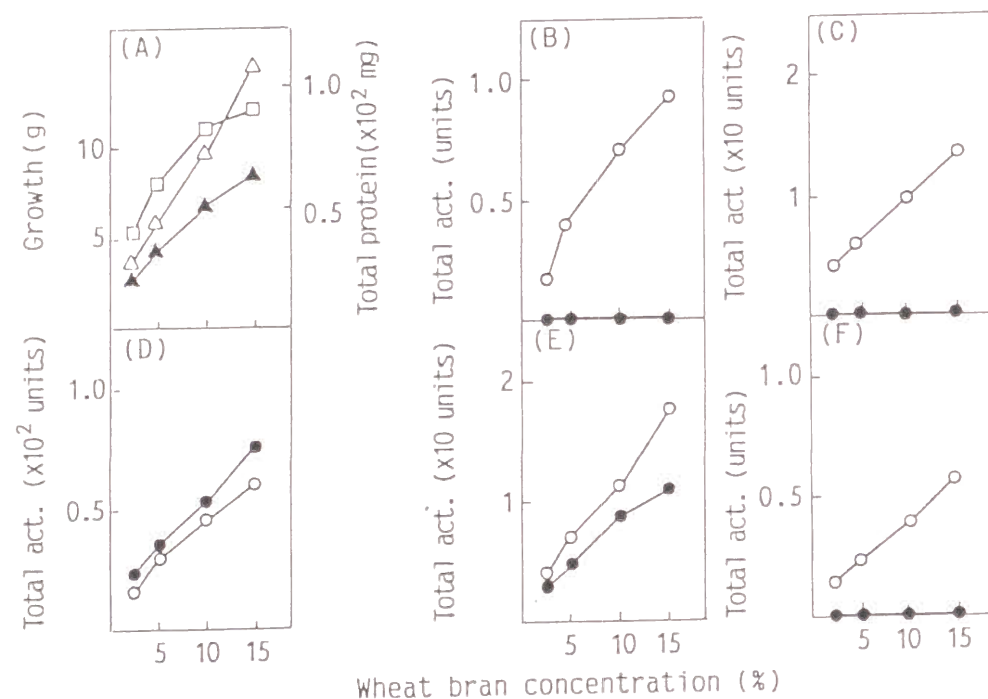


Fig. 2. Effect of wheat bran concentration on the enzyme productivity.

Cultivation was carried out under conditions described in Materials and Methods. The concentration of wheat bran was varied as indicated. (A) growth (wet weight of mycelia) and total protein, (B) glutaminase activity, (C) LAP activity, (D) α -amylase activity, (E) neutral proteinase activity and (F) α -glucosidase activity.

Symbols: $\square$; growth, $\triangle$; total protein of Whole-MF, $\blacktriangle$; total protein of Extra-MF, $\circ$; total activity in Whole-MF, $\bullet$; total activity in Extra-MF.

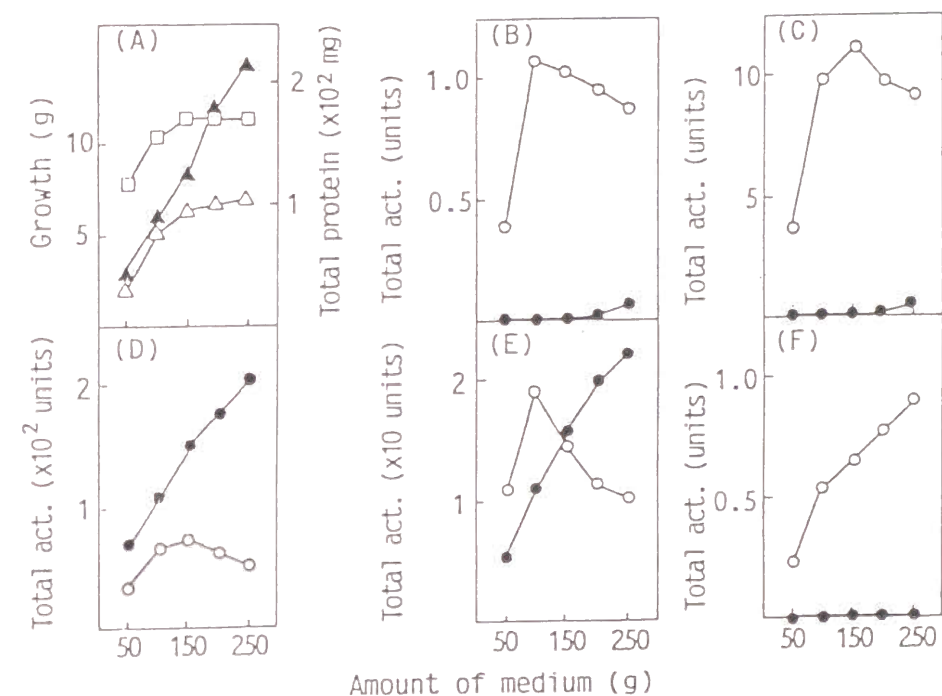


Fig. 3. Effect of the amount of soft gel medium on enzyme productivity.

Cultivation was carried out under conditions described in Materials and Methods. The amount of soft gel medium in a dish was varied as indicated. (A) growth (wet weight of mycelia) and total protein, (B) glutaminase activity, (C) LAP activity, (D) α -amylase activity, (E) neutral proteinase activity and (F) α -glucosidase activity.

Symbols: $\square$; growth, $\triangle$; total protein of Whole-MF, $\blacktriangle$; total protein of Extra-MF, $\circ$; total activity in Whole-MF, $\bullet$; total activity in Extra-MF.

These findings suggest that surface ratio (surface area / amount (volume) of the medium, *i. e.*, depth) of the medium influenced growth, and enzyme production and localization.

Effect of cultivation period.

Figure 4 shows the effect of the cultivation period on enzyme production. The mycelial growth and total protein in Whole-MF and Extra-MF increased for 4 days and then decreased.

The total activity of glutaminase and LAP in Whole-MF also increased for 3 days and then decreased. Glutaminase and LAP activities were hardly detected in Extra-MF on the 4th day of cultivation, but were detected in the fractions after 5 days of cultivation. Glutaminase and LAP in the mycelia may be degraded by autolysis, which was suggested by a decrease in the yield of the mycelial-mat and Whole-MF protein after the 4th day.

The total activity of proteinase and α -amylase in Whole-MF decreased after 3 days of cultivation whereas that in Extra-MF increased for 4 days and then decreased. α -Glucosidase activity in Whole-MF decreased during cultivation.

Comparison of cultivation method.

Enzyme production by the present method was compared with that by conventional methods. As shown in Table II, the productivities of glutaminase, LAP, proteinase and α -amylase in soft gel cultivation were respectively 61 %, 59 %, 29 % and 24 % of that in *koji* cultivation, and 169 %, 226 %, 171 % and 69 % of that in liquid cultivation, respectively.

The total activity of glutaminase and α -amylase of Whole-MF in soft gel cultivation was almost the same as that of Intra-CF in *koji* cultivation. The total activity of LAP and proteinase of

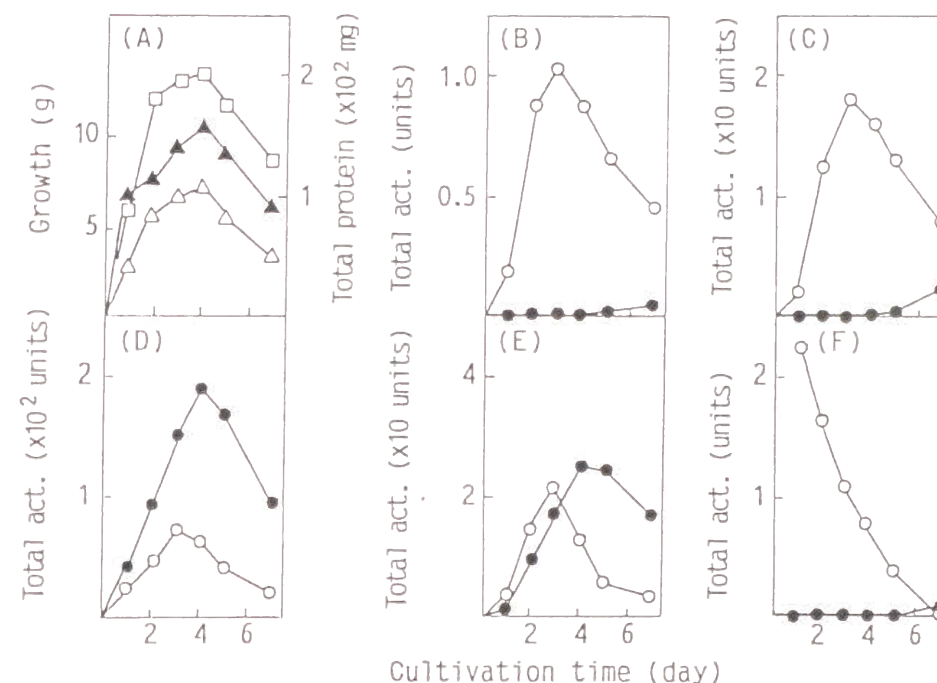


Fig. 4. Effect of cultivation time on enzyme productivity.

Cultivation was carried out under conditions described in Materials and Methods. Cultivation time was varied as indicated. (A) growth (wet weight of mycelia) and total protein, (B) glutaminase activity, (C) LAP activity, (D) α -amylase activity, (E) neutral proteinase activity and (F) α -glucosidase activity.

Symbols: $\square$; growth, $\triangle$; total protein of Whole-MF, $\blacktriangle$; total protein of Extra-MF, $\circ$; total activity in Whole-MF, $\bullet$; total activity in Extra-MF.

Table II. Comparison of cultivation method.

Cultivation was carried out under the conditions described in Materials and Methods. "Productivity" was indicated by the sum of the total activities in Whole-MF (or Intra-CF) and Extra-MF (or Extra-CF) fractions of soft gel cultivation (or *koji* and liquid cultivation). ^zWhole-MF and Extra-MF correspond respectively to Intra-CF and Extra-CF obtained by *koji* and liquid cultivation.

Cultivation	Total activity / 1 g wheat bran (units/g)				
	Glutaminase	LAP	Proteinase	α -Amylase	α -Glucosidase
Soft gel cultivation					
Productivity	0.110	1.89	2.55	15.3	0.110
Total activity in Whole-MF ^z	0.107	1.86	1.41	5.59	0.109
Total activity in Extra-MF ^z	0.003	0.028	1.14	9.67	0.001
<i>Koji</i> cultivation					
Productivity	0.180	3.20	8.93	62.5	0.072
Total activity in Intra-CF	0.111	0.881	0.698	5.19	0.068
Total activity in Extra-CF	0.069	2.32	8.23	57.3	0.004
Liquid cultivation					
Productivity	0.065	0.837	1.49	22.3	0.049
Total activity in Intra-CF	0.013	0.239	0.254	3.59	0.040
Total activity in Extra-CF	0.052	0.598	1.24	18.7	0.009

Whole-MF was 2 times higher than that of Intra-CF in *koji* cultivation and much higher than that of Intra-CF in liquid cultivation.

Koji cultivation yielded the highest total activities of extracellular enzymes (*i. e.*, Extra-MF or Extra CF) and soft gel cultivation the lowest.

About 97 % of glutaminase and LAP, 55 % of proteinase and 37 % of α -amylase activities in soft gel cultivation were found in the Whole-MF, but 62 %, 28 %, 8 % and 8 % of these activities in *koji* cultivation were found in the Intra-CF, and 20 %, 29 %, 17 % and 16 % of these activities in liquid cultivation were found in the Intra-CF, respectively. These findings indicated that these enzymes, especially glutaminase and LAP, were preserved better in soft gel cultivation than in other cultivation methods.

The author originally designed the soft gel cultivation to study the nature of *koji* cultivation; however, the enzyme production and localization of this cultivation were different from those of *koji* and liquid cultivations. However, this method may be better than other methods for studying enzyme production by molds with following reasons: (I) it is easy to analyze the relationship between mycelial growth and enzyme production because there is no contamination of the medium component into the enzyme preparation; (II) mycelial-mat formation can take place more easily but surely by this method to the liquid surface-culture method; (III) preparation and purification of intramycelial (intracellular) enzyme which decreases in liquid cultivation could be easily achieved.

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Summary

Mold mycelia grew on the surface of a soft gel medium containing 0.2~0.4 % agar (soft gel cultivation) just as well as it did on conventional *koji*. Production and localization of several enzymes in soft gel cultivation were studied and compared with those in *koji* and/or liquid cultivations. The total activities of glutaminase, leucine aminopeptidase (LAP), neutral proteinase and α -amylase produced from 1 g of wheat bran in soft gel cultivation were respectively 61 %, 59 %, 29 % and 24 % of those in *koji* cultivation, and localization varied with the cultivation method. Glutaminase and LAP localized mostly in the intracellular and extracellular fractions in *koji* cultivation but only in the whole-mycelial-mat fraction in soft gel cultivation. Only about 8 % of proteinase and α -amylase activities were found in the intracellular fraction from *koji* cultivation, and 55 % of proteinase activity and 37 % of α -amylase activity was in the whole-mycelial-mat fraction in soft gel cultivation.

Section 3. Production and localization of enzymes on soft gel cultivation

Introduction

In section 2 chapter II, the author designed a new mold cultivation method to grow *Aspergillus oryzae* on the surface of soft agar gels (soft gel cultivation), and examined the localization of glutaminase, leucine aminopeptidase (LAP), neutral proteinase and α -amylase in the whole-mycelial-mat (Whole-MF) and extra-mycelial-mat fractions (Extra-MF). The new method was more advantageous for studying microbial growth and enzyme production than conventional *koji*, liquid surface and liquid shaking cultivation methods such as for (I) analysis of the relationship between mycelial growth and enzyme production, (II) preparation of mycelial mat without contamination of medium components and (III) preparation and purification of intracellular enzymes.

The production and localization of glutaminase and LAP obtained by soft gel cultivation were compared with those obtained by *koji* and liquid cultivations.

Materials and methods

Microorganism and media.

Aspergillus oryzae MA-27-125 was used. Media for soft gel, *koji* and liquid cultivations were the same as those described in section 2 of chapter II.

Fractionation of enzymes.

Fractionation of the intracellular (Intra-CF) and extracellular (Extra-CF) enzymes obtained by *koji* and liquid cultivations was carried out as described in section 2 of chapter II.

Four fractions from soft gel cultivation were prepared as follows.

Extra-mycelial-mat fraction (Extra-MF): The agar medium, after removal of mycelial-mat, was ground with sea sand C in the same manner as described in section 2 of chapter II, and this corresponds to the Extra-CF preparation of *koji* and liquid cultivations.

Inter-mycelia fraction (Inter-MF): The mycelial-mat, separated from the surface of the soft gel medium, was treated in 0.01 M potassium phosphate buffer (KPB, pH 7.0) with a blender at 5 °C for 30 sec and centrifuged. The precipitate was resuspended in the same buffer and centrifuged again. The supernatants were combined and dialyzed against 0.01 M KPB. The enzymes in this fraction may be in the spaces separating among the mycelia, and were recognized as a part of the Extra-CF enzymes obtained by *koji* and liquid cultivations.

Intra-mycelia fraction (Intra-MF): The precipitate described above was mixed with the same weight of sea sand C and a small amount of 0.01 M KPB, and ground with a mill for 1 hr at 5 °C. After centrifugation of the mixture, the supernatant was dialyzed against the same buffer. This corresponded to the Intra-CF preparation obtained by *koji* and liquid cultivations.

Whole-mycelial-mat fraction (Whole-MF): The mycelial-mat was ground with sea sand C in a mill in the same manner as the

intra-MF preparation, and the supernatant was dialyzed as described in section 2 of chapter II. It is the mixture of Inter-MF and Intra-MF.

Determination of enzyme activities.

Glutaminase activity was assayed mainly as described in section 2 of chapter II. In experiments for substrate specificity, a reaction mixture (1 ml) containing 25 mM substrate (L-glutamine, L-asparagine or DL-theanine), 300mM hydroxylamine-HCl (pH 7.0), 40 mM Tris-HCl buffer (pH 7.0) and an appropriate amount of the enzyme solution was incubated at 37 °C for 30 min. The reaction was stopped by addition of a ferric reagent. The amount of γ -glutamylhydroxamate or β -aspartylhydroxamate was determined spectrophotometrically at 540 nm (1).

LAP activity was assayed by two methods. The one using L-leucylglycylglycine (LeuGlyGly) as a substrate was as described in section 2 of chapter II. The other used L-leucine- β -naphthylamine (Leu- β -NA) as a substrate (2): a reaction mixture (1 ml) containing 0.02 % Leu- β -NA, 1 mM CoSO₄, 50 mM Tris-HCl buffer (pH 8.0) and an appropriate amount of enzyme solution was incubated at 37 °C for 30 min. The reaction was stopped by addition of 1 ml of 0.7 % HCl in ethanol and 1 ml of 0.06 % *p*-dimethylamine cinnamaldehyde in ethanol. The mixture was kept at room temperature for 10 min, and the amount of β -naphthylamine was estimated spectrophotometrically at 540 nm (molar extinction coefficient $10.5 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$).

Protein determination.

Protein was determined by the Lowry's method (3) with egg albumin as a standard. During column chromatography, it was

followed by absorbance at 280 nm.

Column chromatography of glutaminase.

The fraction was applied to a DEAE-Sepharose CL-6B column (2.4 x 10 cm) equilibrated with 0.01 M KPB. After washing the column, the concentration of NaCl in the buffer was increased in gradients for chromatography.

Column chromatography of LAP.

The fraction was concentrated with ammonium sulfate (95 % saturation), and dialyzed against 0.05 M KPB (pH 7.0). The enzyme solution was filtered through a Sephadex G-200 column (2.6 x 110 cm) with the same buffer.

Chemicals.

LueGlyGly was obtained from the Institute for Protein Research, Osaka University. Leu- β -NA was purchased from Sigma Chemical Co. DEAE-Sepharose CL-6B and Sephadex G-200 were products of Pharmacia Fine Chemicals.

Results and Discussion

Enzyme production and localization.

Enzyme production in soft gel cultivation was compared with that in conventional *koji* and liquid cultivations (Table I). As shown in section 2 of chapter II, glutaminase and LAP were obtained mainly in the Whole-MF obtained by soft gel cultivation, whereas they were found in both the Intra-CF and Extra-CF obtained by *koji* and liquid cultivations, though the activity

Table I. Localization of enzymes in soft gel cultivation.

The values in parentheses are relative to the activity in the Whole-MF.

Fraction	Total activity / 1 g wheat bran (units/g)	
	Glutaminase	Leucine aminopeptidase
Whole-MF	0.094 (100)	2.05 (100)
Inter-MF	0.022 (25)	1.07 (52)
Intra-MF	0.072 (75)	0.98 (48)
Extra-MF	0.001	0.02

ratios (Intra-CF/Extra-CF) varied. Table I gives further information on the enzyme localization in Whole-MF: some glutaminase and LAP were released to the Inter-MF by mild extraction (blender treatment).

Low activity of glutaminase and LAP in the Extra-MF indicated that either (I) so-called extracellular enzymes were not secreted into the medium even though they were formed by the soft gel cultivation or (II) the extracellular enzymes were not formed at all by the soft gel cultivation.

Analysis of glutaminase by DEAE-Sepharose CL-6B column chromatography.

Whole-MF glutaminase was separated into three components by DEAE-Sepharose CL-6B column chromatography (peaks I, II and III, Fig. 1). Peak I showed higher activity with asparagine than glutamine, peak II theanine (γ -glutamylethylamide) than glutamine, and peak III glutamine than asparagine. Their thermal stabilities were also distinguishable (peaks I and II maintained their activity at pH 7.0 after heat treatment at 50 °C for 10 min and peak III did not). Their reactivity toward the substrates and thermal stability were used for identifying the enzyme species.

Table II summarizes the enzyme species and the amounts of glutaminases in various fractions. Soft gel Intra-MF contained peaks I, II and III, however Inter-MF contained peaks I and II. *Koji* Intra-CF contained peak II and a new species (peak IV) which was eluted at the front of peak II as a shoulder, and peak II was found in Extra-CF. With liquid cultivation, peaks I, II and III were found in Intra-CF, and peaks I and II in Extra-CF.

With soft gel and liquid cultivations, the highest activity

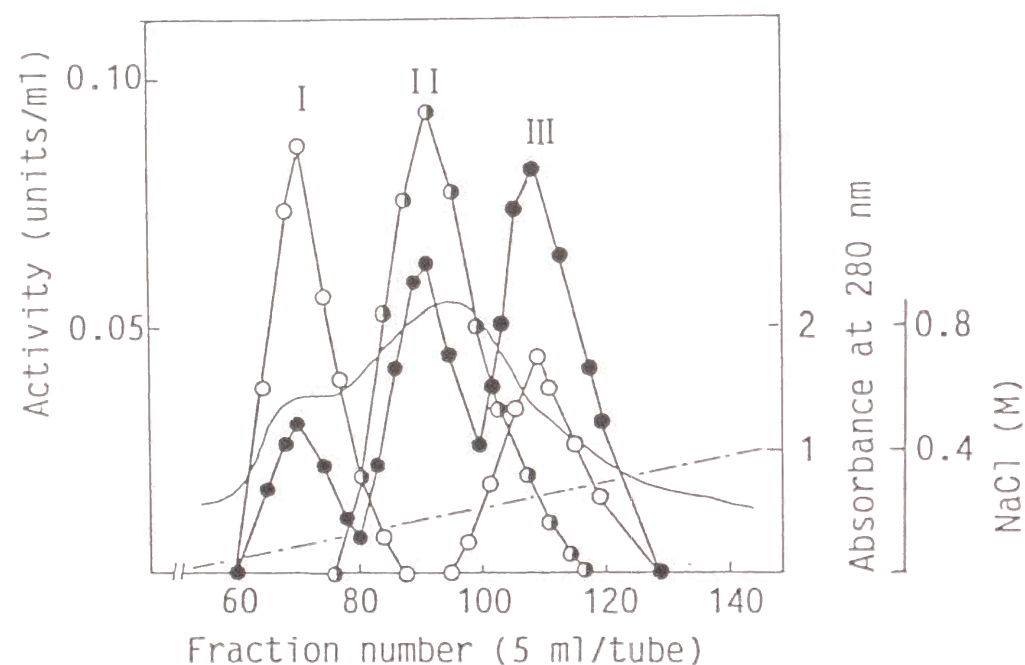


Fig. 1. DEAE-Sepharose CL-6B column chromatography of glutaminase in Whole-MF of *Asp. oryzae* MA-27-125, soft gel cultivation.

The enzyme solution (0.37 g protein) was obtained from 400 g of soft gel medium, and applied to a DEAE-Sepharose CL-6B column (2.4 x 10 cm). The activities were determined by estimating γ -glutamylhydroxamate and β -aspartylhydroxamate concentrations.

Symbols: ●; glutaminase activity, ○; γ -glutamyl-transpeptidase activity, —; absorbance at 280 nm, ---; NaCl concentration.

Table II. Glutaminase species and their amounts in subcellular fractions.

The activity was determined with glutamine as a substrate.

Cultivation and subcellular fraction	Total activity (unit/10 g wheat bran)			
	peak I	peak II	peak III	peak IV
Soft gel				
Whole-MF	0.098	0.32	0.48	ND [#]
Inter-MF	0.031	0.18	ND	ND
Intra-MF	0.068	0.17	0.41	ND
Koji				
Intra-CF	ND	0.96	ND	0.14
Extra-CF	ND	0.85	ND	ND
Liquid				
Intra-CF	0.010	0.021	0.06	ND
Extra-CF	0.07	0.41	ND	ND

[#] Not determined.

was of peak III and the lowest was of peak I. In *koji* Intra-CF, peaks I and III were not detected, some activity of peak IV was found besides that of peak II, and peak IV showed the same substrate specificity as peak III. These findings indicate that peak III is an intracellular enzyme barely releasable from mycelia.

Fractionation of LAP.

Nakadai *et al.* (4) separated LAP from soy sauce *koji* (*Aspergillus sojae*) by Sephadex G-200 column chromatography into seven components (peaks 7, 4, 5, 2, 3, 6 and 1 in order of molecular weight from large to small) with LeuGlyGly as a substrate, and reported that three of them (peaks 1, 3 and 6) were also active with Leu- β -NA.

LAP in the Intra-CF (Fig. 2(A)) and Extra-CF (Fig. 2(B)) of *koji* cultivated *Asp. oryzae* MA-27-125 was chromatographed on a Sephadex G-200 according to the method of Nakadai *et al.*, and was separated into five components (peaks A, B, C, D, E).

Substrate specificity and elution volume indicated that peak A of *Asp. oryzae* corresponded to peak 7 of *Asp. sojae*, B to 5, C to the mixture of 2 and 3, and D to 6. Peak E was not identified and was considered as a new species. Peak 4 reported by Nakadai *et al.* could not be found in *Asp. oryzae*. The activity ratio of each enzyme species (Intra-CF/Extra-CF) was about 1/3, being nearly equal to those in Table I.

LAP from soft gel Inter-MF and Intra-MF was separated into four peaks (Figs. 2(C) and 2(D)), and were identified as peaks A, C, D and E from those in *koji* cultivation (Figs. 2(A) and 2(B)).

Peak C obtained by *koji* cultivation (Figs. 2(A) and 2(B)) had higher reactivity to LeuGlyGly than to Leu- β -NA. However,

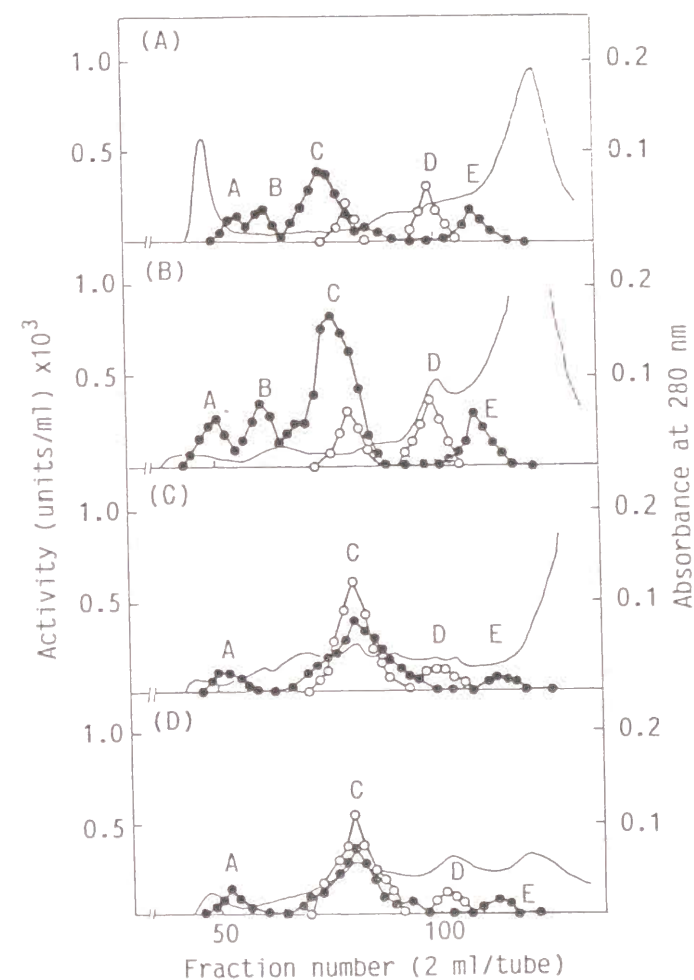


Fig. 2. Sephadex G-200 column chromatography of LAPs in various fractions of *Asp. oryzae* MA-27-125.

(A) *Koji*-cultivated Intra-CF (64 mg protein) from 10 g of *koji* medium, (B) *Koji*-cultivated Extra-CF (53 mg protein) from 10 g of *koji* medium, (C) Soft-gel-cultivated Inter-MF (33 mg protein) from 50 g of soft gel medium and (D) Soft-gel-cultivated Intra-MF (47 mg protein) from 50 g of soft gel medium filtered through a Sephadex G-200 column (2.6 x 110 cm).

Symbols: ●; activity for Leuglygly, ○; activity for Leu- β -NA, —; absorbance at 280 nm.

peak C obtained by the soft gel cultivation showed lower reactivity to LeuGlyGly than to Leu- β -NA (Figs. 2(C) and 2(D)). There are two possibilities as to the nature of peak C: (I) if peak C is a mixture of peaks 2 and 3 of *Asp. sojae*, peak 3 increased in soft gel cultivation, and (II) if peak C consists of a single enzyme, a new enzyme species was formed in soft gel cultivation.

LAP obtained by liquid cultivation could not be analyzed because of low activity.

On both soft gel and *koji* cultivations growth of mold on a solid substance and the enzyme productivity (Table I) were similar. However, as shown in Table II and Fig. 2: (I) peaks I and III of glutaminase obtained by soft gel cultivation were not formed by *koji* cultivation, but were formed by liquid cultivation, (II) peak IV obtained by *koji* cultivation was not found in soft gel and liquid cultivations, (III) peak B of LAP obtained by *koji* cultivation was not detected in soft gel cultivation and (IV) substrate specificity of peak C varied in response to soft gel and *koji* cultivations.

Shikata *et al.* (5~8) and Teramoto *et al.* (9) reported the existence of insoluble (membrane bound) glutaminase in *Asp. sojae* and *Asp. oryzae*. Further studies on membrane-bound enzymes are required.

Enzyme formation might also be related to morphological feature considering the production of L-glutamate oxidase by *Streptomyces* sp. (10, 11) and of mycophenolic acid by *Penicillium* sp. (12~14). After 3 days of soft gel cultivation, horizontal mycelia developed very thickly on the gel, and aerial mycelia and conidiation were observed. On the other hand, development of aerial mycelia was rich, and marked conidiation was observed in

koji cultivation. In liquid cultivation, only vegetative mycelia developed and conidia were not formed. Formation of new enzyme species and disappearance of some enzyme species might be associated with the development of aerial, horizontal and/or vegetative mycelia.

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Summary

Production and localization of glutaminase and leucine aminopeptidase (LAP) in soft gel cultivation were compared with those in *koji* and liquid cultivations. The enzymes were detected only in the whole-mycelial-mat fraction by soft gel cultivation, but in both intracellular and extracellular fractions by the other two methods. The enzyme species of glutaminase and LAP in soft gel cultivation were analyzed by ion exchange and gel filtration column chromatographies. Three species of glutaminase and four (or five) species of LAP were formed in the whole-mycelial-mat fraction. The intracellular and extracellular fractions of the *koji* and liquid cultivations contained different species of enzymes.

Chapter III. Development of food production and preservation method using alcoholic fermentation

Section 1. Peptide production from protein without salt.

Introduction

In Asia, a variety of fermented foods are produced by using *koji*, which is the solid substrate culture of molds. Therefore, *koji* serves as a source of a variety of enzymes, which catalyze the degradation of the solid raw material.

Fermented protein foodstuff, such as *miso*, soy sauce and fermented fish, are produced by the addition of this *koji* to the raw material in the presence of high concentrations of NaCl in order to prevent putrefactive fermentation by contaminating microorganisms. For example, *miso* is produced in the presence of 12 % NaCl (1). However, fermented foods containing lower concentrations of NaCl are now favored for health reasons, and these fermented foods are produced in the presence of 8~10 % NaCl (2). Moreover, the ethanol addition method (3~8), yeast (*Saccharomyces rouxii*) addition method (9, 10) and other methods (11~14) have been used in the production of low salt-containing *miso*. The NaCl concentration used in these methods is 4~5 %.

Proteases have been reported to be inhibited by high concentrations of NaCl (15~20). Therefore, production of fermented foods in the presence of high concentrations of NaCl takes a long time. On the other hand, ethanol has an inhibitory effect on the growth of microorganisms including putrefactive bacteria (21, 22). A method for producing unsalted *miso* has been

developed by the direct addition of ethanol to the fermentation process (23, 24).

During this study on the production of fermented foods, the author designed a method of producing unsalted fermented protein foods. This sugar-yeast (SY) addition method utilizes the proteolytic and amylolytic enzyme activities of *koji* molds and alcohol fermentative activity of yeasts on various raw protein sources and glucose or starch. In this study, the author compared the effect of this method on the growth of putrefactive bacteria and enzyme activities during the fermentation process with those of the ethanol addition method and the NaCl addition method.

Materials and Methods

Microorganisms.

Asahi No. 1 seed *koji* mold (*Aspergillus oryzae*) purchased from Hishiroku Co. Ltd. and *Saccharomyces cerevisiae* (sake yeast Kyokai No. 7) stocked in our laboratory were used in this study.

Preparation of soy bean koji.

A solid *koji* medium composed of 50 g broken soy beans and 50 g wheat flour moistened with 80 ml water was prepared in a 15 cm-diameter petri dish. After autoclaving at 120 °C for 20 min, 0.5 g Asahi No. 1 seed *koji* was inoculated and mixed well. Cultivation was performed at 28 °C for 72 hr.

Culture of Saccharomyces cerevisiae.

S. cerevisiae was cultured in a liquid medium consisting of

3 % glucose, 0.5 % peptone, 0.3 % yeast extract, 0.03 % KH_2PO_4 , 0.03 % K_2HPO_4 and 0.01 % MgSO_4 , pH 7.0. Cultivation was carried out at 28 °C for 24 hr with shaking. The cells were harvested by centrifugation and suspended in distilled water to a concentration of 10^8 cells/ml.

Preparation of enzyme extract of koji.

Asahi No. 1 was cultivated with solid medium composed of 30 g wheat bran and 30 ml water, under the same conditions mentioned above. The culture was extracted with distilled water (2:1, v/w) at 4 °C for 12 hr, and then filtered through a cotton cloth and centrifuged. The supernatant solution was used as the enzyme solution.

Fermentation procedure.

Soy bean *koji* (180 g) was ground with distilled water (150 ml) using a mill for 1 hr (referred to as ground *koji* material), and a suspension of yeast cells (10^8 cells/ml water) and 9 % glucose or starch were added and mixed well (the SY addition method). The water content of this fermentation mixture was about 73 %. The final concentration of yeast cells was adjusted to be 10^5 cells per 1 g of ground *koji* material. The fermentation (in solid or liquid state) was allowed to proceed in a container with a lid at 22 °C.

In the NaCl addition method, NaCl was added at the concentration of 9 % (w/w) in place of 9 % glucose and in the ethanol addition method, 9 % ethanol was used.

Analytical methods.

Proteinase activity was determined by the method of Hagihara

(25) using Hammersten casein as a substrate, at 37 °C. TCA soluble protein was assayed by Lowry's method (26). Leucine aminopeptidase (LAP) (27) and glutaminase (28) activities were assayed by measuring *p*-nitroaniline released from leucine-*p*-nitroanilide and γ -glutamyl-*p*-nitroanilide, respectively, at 37 °C. Amylase activity was determined by the method of Noelting (29) using soluble starch as a substrate, at 37 °C.

The components of the fermented mixture were assayed using the supernatant solution mentioned below and were expressed as the concentration in the fermentation mixture. The supernatant solution was prepared by extraction of the fermented mixture with two-fold weight of distilled water followed by centrifugation (3500 rpm, 15 min). The total amounts of amino acids was determined colorimetrically with ninhydrin (30) with glutamic acid as the standard. Individual amino acids were determined by an amino acid analyzer (Kyowa Seimitsu K-101AS). Ethanol was assayed by the method of Bernt (31) using alcohol dehydrogenase or by gas chromatography (32). Glucose was determined by the method of Somogyi (33) and Nelson (34).

Determination of viable cells.

Fermenting mixtures were appropriately diluted and spread on a nutrient agar medium. After incubation at 30 °C for 2 days colonies were enumerated. Nutrient agar medium for yeast consisted of 1 % glucose, 0.2 % peptone, 0.15 % yeast extract, 0.01 % KH_2PO_4 , 0.004 % MgSO_4 , 50 units/L penicillin G and 0.2 % Na-propionate, at pH 5.5, and that for bacteria 1 % meat extract, 1 % peptone, 0.5 % NaCl and 0.1 mg/ml kabicidin, at pH 7.2. The viable cell number of yeast and bacteria was expressed as the number in the fermentation mixture.

Enzymes and chemicals.

Leucine-*p*-nitroanilide and γ -glutamyl-*p*-nitroanilide were purchased from Nacalai Tesque Co. Ltd., and Hammersten casein from Merck. Alcohol dehydrogenase from beef liver was from Oriental Yeast Co. Kabicidin was from Wako Pure Chemical Industries.

Results and Discussion

Effect of addition of glucose, ethanol and NaCl on bacterial growth and enzyme activity.

The author compared the antibacterial effects of ethanol with those of NaCl, and found that at the same concentration ethanol showed the same bacteriostatic activity as those of NaCl.

The inhibition of enzymes including proteinases by NaCl has been well investigated (15~20), but few studies have been done on the inhibition by glucose and ethanol (35). Therefore, the effect of addition of glucose, ethanol and NaCl on the enzyme activities which occur during the fermentation process were investigated.

As shown in Fig. 1, proteolytic enzymes were scarcely inhibited by glucose. Although proteinase, LAP and amylase were inhibited by ethanol, the activities of proteinase and amylase were less inhibited by ethanol than by NaCl, but LAP was more inhibited by ethanol than by NaCl. Glutaminase was slightly activated by ethanol and was strongly inhibited by NaCl.

These enzymes were more stable in an ethanol-containing solution than in an NaCl-containing solution (data not shown). Moreover, most of these enzyme activities, and especially the

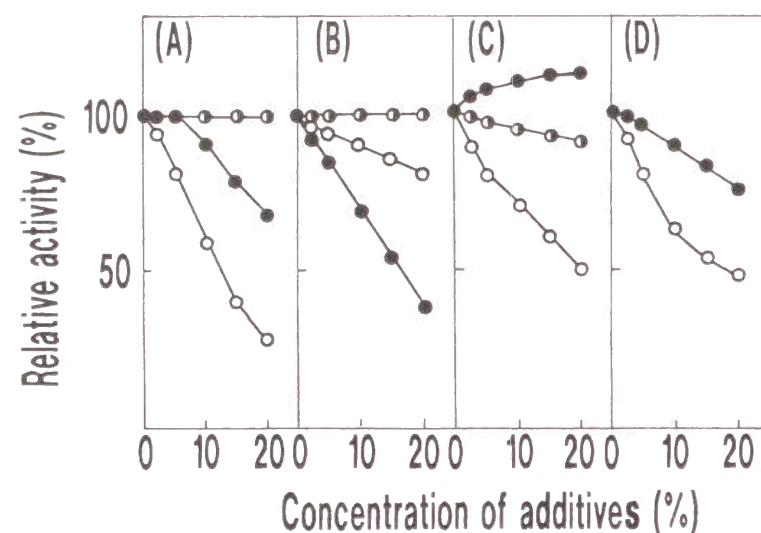


Fig. 1. Effect of addition of glucose, ethanol and NaCl on enzyme activities.

Each enzymatic activity was measured as described in Materials and Methods. The amylase activity in the presence of glucose could not be determined, because of the high blank value produced by glucose. The activities of (A) proteinase, (B) leucine aminopeptidase (LAP), (C) glutaminase and (D) amylase were measured.

Symbols: ○, glucose; ●, ethanol; ○, NaCl.

proteinase activity, from other sources (*A. oryzae*, *A. saitoi*, etc.) and commercial products (actinase E, papaya, pepsin) were inhibited less by ethanol than by NaCl (data not shown).

Effect of addition of ethanol and NaCl on proteolysis and amylolysis.

Although the effect of addition of ethanol and NaCl on each enzyme activity was as indicated above, the action of these reagents to liberate amino acids from protein is not clear. To clarify the effects of addition of these reagents on the digestion of protein, the proteolysis and amylolysis activities of the *koji* enzyme extract was investigated in the presence of these reagents. Heated soy bean powder was used as a substrate. The amounts of proteolysis products (water soluble and 5 % trichloroacetic acid soluble proteins), and products of amylolysis (reducing sugars) in the reaction mixture containing ethanol were greater than in that containing NaCl (Fig. 2).

Fermentation by the SY addition method.

Fermentation by the SY addition method was examined using various concentrations of additives and the products were evaluated by measuring the amounts of glutamic acid and total amino acids and by their color tone (Table I).

An increase in the concentration of the additive decreased the amounts of glutamic acid and total amino acids liberated by each fermentation method. The SY addition method gave the highest amount of total amino acids of the three methods and similar amount of glutamic acid to the ethanol addition method.

The amounts of glutamic acid and total amino acids liberated by the NaCl addition method were smaller than those liberated by

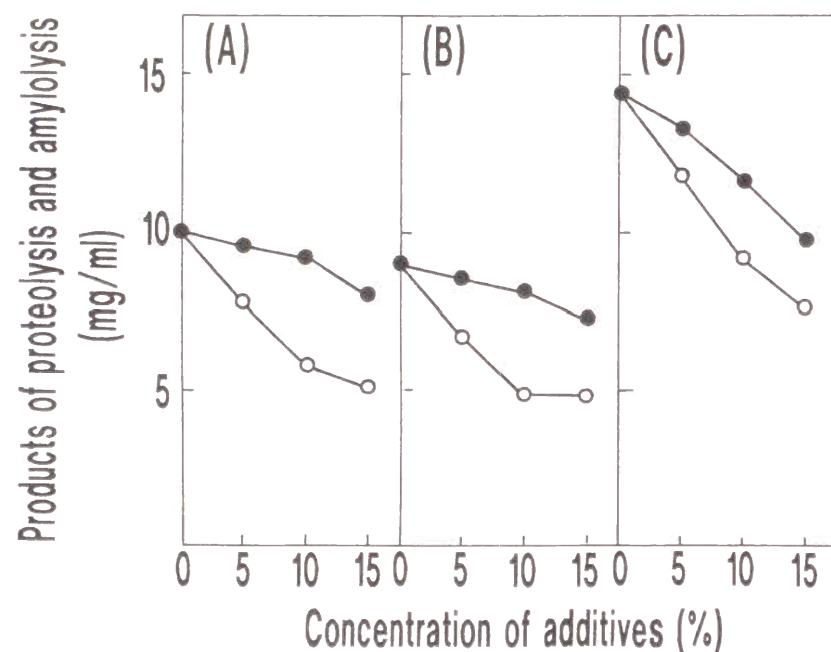


Fig. 2. Effect of addition of ethanol and NaCl on proteolysis and amylolysis.

The reaction mixture contained: 2 g soy bean powder, 16 ml of enzyme extract of *koji* and the indicated concentration of ethanol or NaCl in a final volume of 20 ml, and was incubated at 30 °C for 2 days with shaking. Water soluble protein (A), 5 % TCA soluble protein (B) and reducing sugar (glucose) (C) were measured.

Symbols: ●, ethanol; ○, NaCl.

Table I. Comparison of products by each fermentation method.

The control experiment contained ground *koji* material and yeast as described in Materials and Methods. The concentration of each additive (ethanol, NaCl and glucose) was weight percent per weight of ground *koji* material. Each experiment system contained 10^5 yeast (*S. cerevisiae*) cells/g fermentation mixture. The fermentation was carried out at 22 °C for 30 days.

Additives	Product found					
	Amino acids (mg/g)	Glutamic acid (mg/g)	Y% ^a (%)	ΔA ^b	pH	Ethanol (%)
Control experiment	33.4	8.5	61	0.53	4.4	0.1
Ethanol 5 %	36.1	11.0	57	0.53	5.6	4.6
10 %	33.6	9.9	54	0.58	5.8	8.9
15 %	30.6	9.3	53	0.53	5.8	13.6
NaCl 5 %	28.0	8.9	56	0.49	5.6	0.5
10 %	26.7	5.0	56	0.51	5.8	0.4
15 %	18.7	3.4	57	0.48	5.8	0.3
Glucose 5 %	43.0	10.2	60	0.57	5.6	2.1
10 %	38.7	8.7	64	0.60	5.8	4.3
15 %	34.1	7.6	62	0.54	5.8	6.8

^a The increase of Y% indicate the increase of light of color.

^b The increase of ΔA indicate good quantity of color.

the ethanol addition method was also indicated in Figs. 1 and 2.

The color tone, the values of $Y\%$ (36) and ΔA (37) in the supernatant of each product were similar for all methods, but slightly higher values were observed by the SY addition method. Therefore, the color obtained by this method was not different from that obtained by other methods. The final products were brown-yellowish.

In the fermentation mixture, 0.3~0.5 % ethanol was formed by the NaCl addition method, but 1~2 % ethanol decreased by the ethanol addition method. By the SY addition method, alcoholic fermentation was rapidly developed, the concentration of ethanol formed increased in proportion to the amount of glucose added, and the amount of ethanol formed was about 80 % of the theoretical yield of ethanol from glucose. Yoshida (24) described that in the case of *miso* production, putrefaction did not occur in the presence of 2~4 % ethanol. Therefore, the addition of the about 10 % glucose might be sufficient to prevent bacterial contamination.

Figure 3 shows the time course of fermentation under conditions containing 9 % of each additive.

In the control experiment (Fig. 3(A)), the amounts of glutamic acid and total amino acids liberated from soy bean protein were increased, but glucose which is contained in and can be liberated from *koji* (ground *koji* materials) was decreased throughout the fermentation period. Yeast and bacteria grew to 6.8×10^6 and 1.2×10^8 cells/g after 5 days of fermentation and gradually decreased to 1.0×10^3 and 1.0×10^4 cells/g at 30 days of fermentation, respectively. The pH gradually fell and that of the 30 day fermentation product was 4.4. A putrefactive odor was

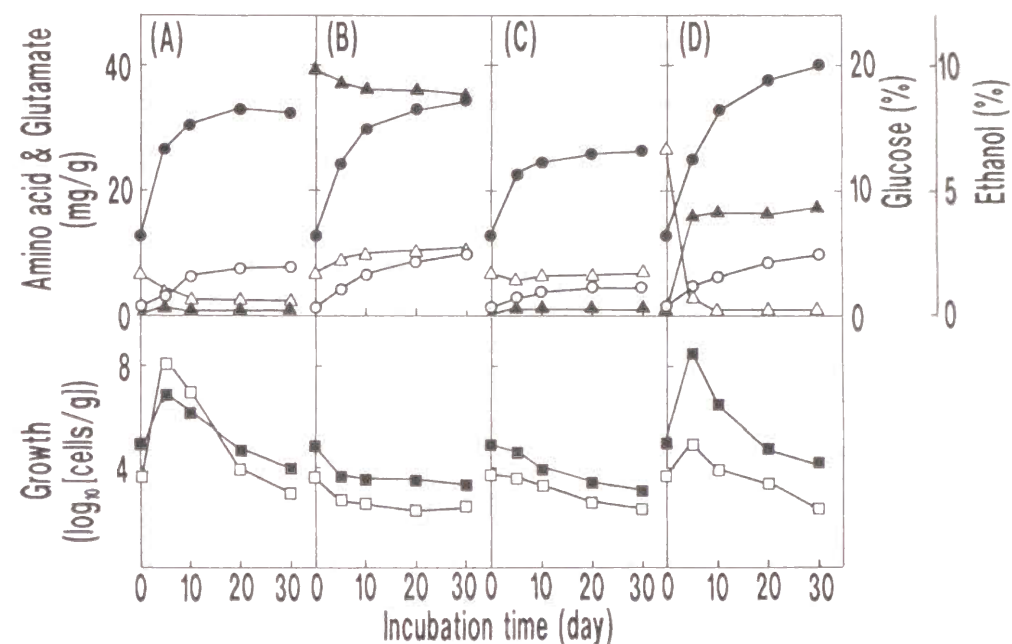


Fig. 3. Comparison of fermentation methods.

The experiment (A), control experiment, contained 330 g ground *koji* material (soy bean *koji* 180 g and water 150 g, see Materials and Methods) and 10^5 yeast cells/g. The experiment (B), ethanol addition method, contained 330 g ground *koji* material, 33 g ethanol (final concentration, 9 %) and 10^5 yeast cells/g. The experiment (C), NaCl addition method, contained 330 g ground *koji* material, 33 g NaCl (final concentration, 9 %) and 10^5 yeast cells/g. The experiment (D), SY addition method, contained 330 g ground *koji* material, 33 g glucose (final concentration, 9 %) and 10^5 yeast cells/g. The fermentation was carried out at 22 °C.

Symbols: ○, glutamic acid; ●, total amino acids; △, glucose; ▲, ethanol; ■, yeast; □, bacteria.

noticed, and the product was putrefied.

No putrefaction occurred during the fermentation by the ethanol addition and the NaCl addition methods (Figs. 3(B), 3(C)). Much glutamic acid and total amino acids were liberated, but the liberation rate of these compounds obtained by the ethanol addition method was 1.3 times as fast as those obtained by the NaCl addition method. A gradual increase in the amount of glucose was obtained by the ethanol addition method, but a decrease by the NaCl addition method. A gradual decrease in the cell counts of yeast and bacteria was obtained by both methods, but the rate obtained by the ethanol addition method was faster than that obtained by the NaCl addition method.

By the SY addition method (Fig. 3(D)), glutamic acid and total amino acids were also liberated. At the beginning of fermentation, these compounds were liberated at the same rate as in the ethanol addition method, and in the late fermentation period, total amino acids were continuously liberated in this method but not in the ethanol addition method.

The glucose added was consumed quickly; about 4.0 % ethanol was formed after 5 days of fermentation, and it increased gradually.

The cell counts of yeast and bacteria increased to 1.8×10^8 and 7.9×10^4 cells/g after 5 days of fermentation and gradually decreased thereafter, and after 30 days of fermentation the cell count was 1.2×10^4 and 2.6×10^2 cells/g, respectively. This number of bacterial cells was the same as that of the ethanol and NaCl addition methods at 30 days. No putrefactive odor was observed and the pH of product was not decreased (pH 5.6).

These results indicate that the SY addition method can be utilized as a method of fermentation just as well as the ethanol

and NaCl addition methods. Therefore, the fundamental conditions of this method were investigated in the following experiments.

Effect of yeast cell concentration on fermentation.

Figure 4 shows the effect of the initial yeast cell concentration on fermentation. The yeast cell concentration did not influence the liberation of glutamic acid and total amino acids but the ethanol formation. The initial rates of glucose consumption and ethanol formation increased with an increase in the number of yeast cells. The maximum concentration of ethanol (4.9 %) was observed after 10 days of fermentation, in a fermentation mixture initially containing 10^7 yeast cells/g.

The number of yeast cells was about 10^8 cells/g after 5 days of fermentation in all fermentation mixtures, and decreased to 10^4 cells/g after 30 days of fermentation. On the other hand, the bacterial cells increased with a decrease of the initial yeast cell concentration; 4.1×10^7 cells/g of bacteria were found in the mixture containing 10^4 cells/g yeast cells which might have been due to insufficient ethanol production. In mixtures with 10^5 yeast cells/g or more, there were less than 10^5 bacterial cells/g every time.

For satisfactory fermentation, 10^5 yeast cells/g was used in the following experiments.

Effect of water content on fermentation.

The liberation of glutamic acid and total amino acids was observed in all the fermentation mixtures containing from 50 to 90 % water content (Fig. 5). The maximum count of yeast cells was similar for all mixtures and that of bacteria increased with the decrease of water content, and the number of viable cells of

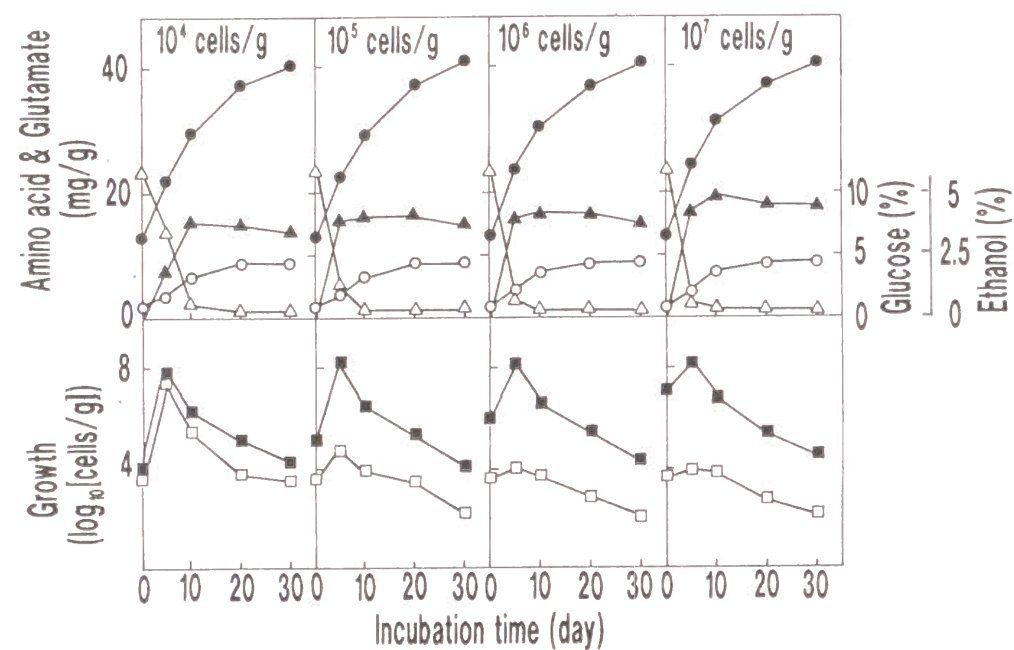


Fig. 4. Effect of initial yeast-cell concentration on fermentation.

Each fermentation mixture contained 330 g ground *koji* material and 33 g glucose, and yeast cells as shown in the Figure. The fermentation was carried out at 22 °C.

Symbols: ○, glutamic acid; ●, total amino acids; △, glucose; ▲, ethanol; ■, yeast; □, bacteria.

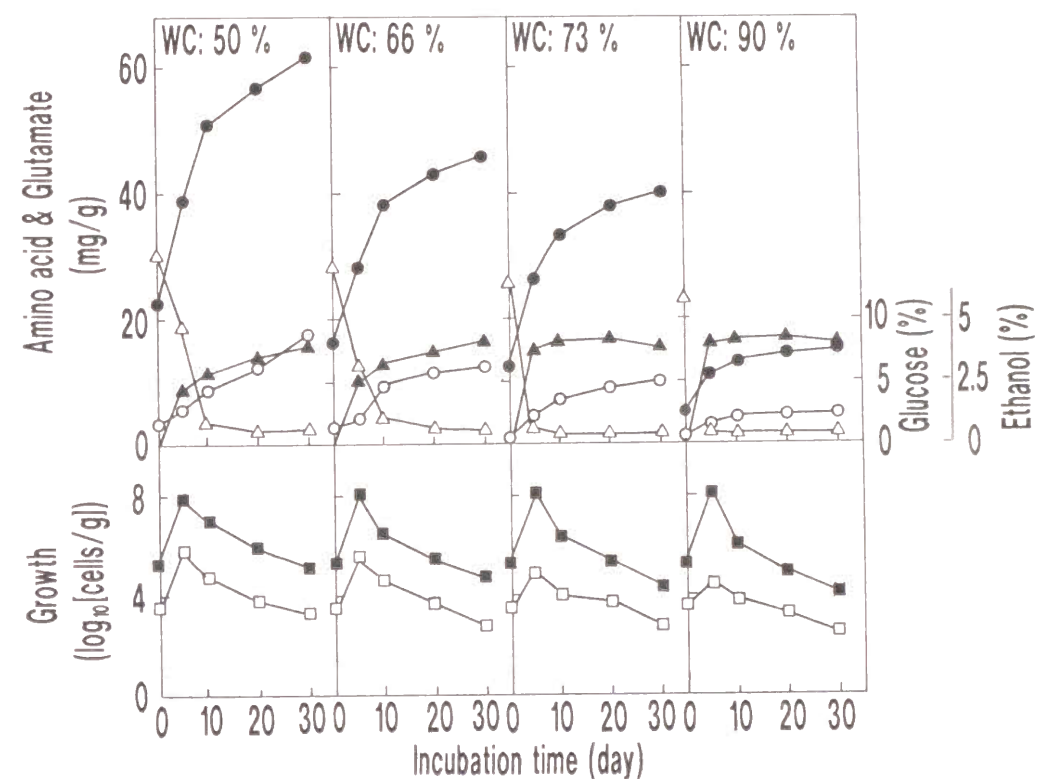


Fig. 5. Effect of water content on fermentation.

The water content (WC) was controlled at the time of ground treatment by changing added amount of water. Each fermentation mixture contained 330 g ground *koji* material different in water content and 33 g glucose and 10^5 yeast cells/g. The fermentation was carried out at 22 °C.

Symbols: ○, glutamic acid; ●, total amino acids; △, glucose; ▲, ethanol; ■, yeast; □, bacteria.

bacteria at 30 days fermentation also increased with the decrease of water content.

This phenomenon may be dependent on the concentration and rate of formation of ethanol (in cases of water content 50 % and 90 %, 2.5 % and 3.8 % ethanol formed in 5 days preservation, respectively). Although the maximum cell count of bacteria increased with the decrease of water content, it did not increase beyond 10^6 cells/g in any mixture. Accordingly, the SY addition method can be utilized even in cases of low water content.

Fermentation using starch as a sugar source.

Fermentation by the SY addition method was examined using 9 % starch in place of glucose. The amounts of glutamic acid and total amino acids liberated were not influenced, but the formation rate and the amount of ethanol in the mixture were less than when glucose was used (Fig. 6). On the other hand, the count of yeast cells did not vary. However the bacterial cells increased in the starch mixture and the maximum count of bacterial cells was 2.0×10^5 cells/g, putrefactive fermentation did not occur. Therefore, starch can be utilized as an carbon source in place of glucose in the SY addition method.

Removal of alcohol from fermented product.

The fermented product described above was a paste or liquid from which alcohol was removed by distillation under reduced pressure. The final product can be shipped not only as manufactured foods, but also as intermediate materials for food processing.

Fermentation of sardine koji using the SY addition method.

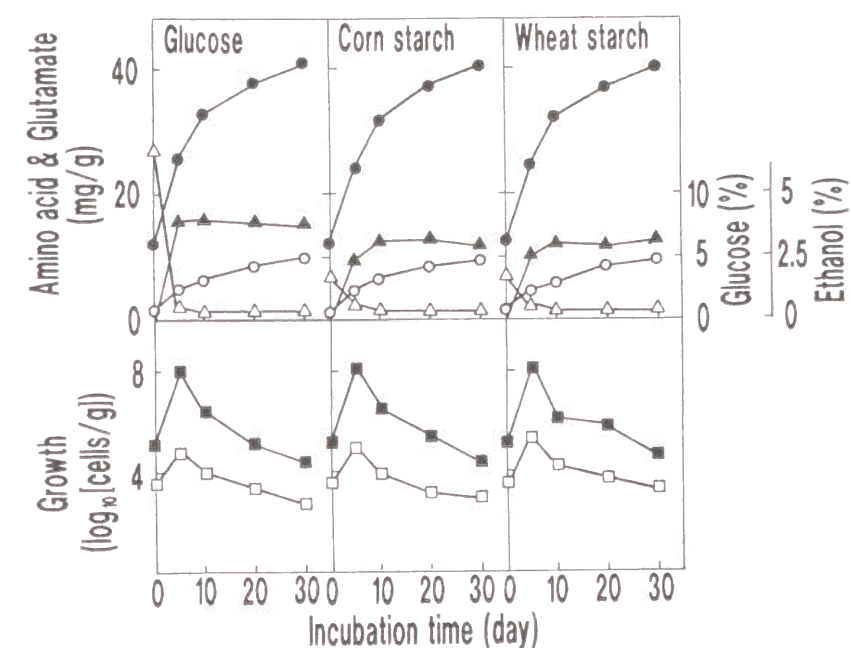


Fig. 6. Fermentation using starch as sugar source.

Each fermentation mixture contained 330 g ground *koji* material and 33 g sugar source ((A): glucose, (B): corn starch or (C): wheat starch) and 10^5 yeast cells/g. The fermentation was carried out at 22 °C.

Symbols: ○, glutamic acid; ●, total amino acids; △, glucose; ▲, ethanol; ■, yeast; □, bacteria.

To modify the SY addition method for the production of fermented fish, the author used sardines *koji* in place of soy bean *koji*, and carried out fermentation as above. As shown in Fig. 7, the amounts of glutamic acid and total amino acids liberated differed from the case using soy beans (Fig. 3(D)). This difference might be due to the difference in the contents of amino acids and in the raw material.

On the other hand, the cell counts of yeast and bacteria obtained using sardines were similar to those obtained with soy bean.

The products obtained by the SY addition method had a slightly bitter taste, and the author is currently trying to modify the method to improve the taste.

These results show that putrefactive fermentation does not occur in the SY addition method, and this method can utilize solid and liquid raw materials, and is useful in the commercial production of marine products and livestock. On the other hand, this method differs from the ethanol addition method since the ethanol used was made from glucose (starch) via alcoholic fermentation of yeast (and *koji* amylolytic enzymes). It has already been indicated that ethanol in *miso* not only has a preservation effect but also improves the flavour (37, 38). Therefore, a new kind of fermented food with or without alcohol might be developed using this method. Moreover, utilization of other yeasts, such as *S. ellipsoides*, *S. carlsbergensis* and so on may produce fermented foods which have different flavour.

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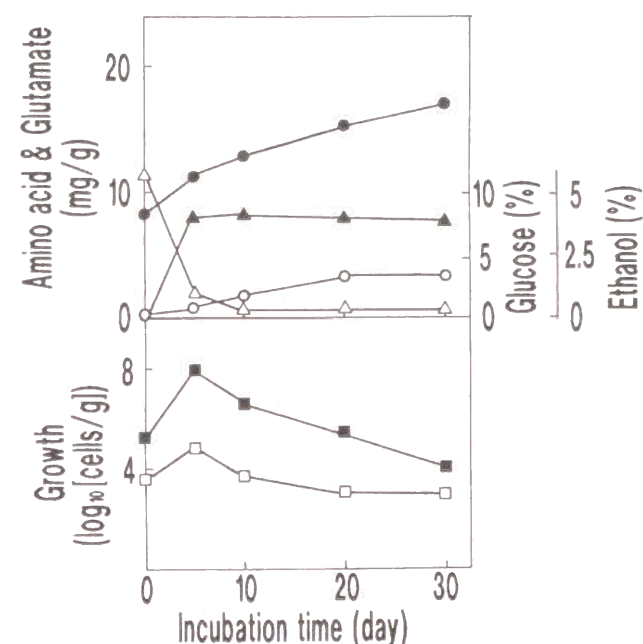


Fig. 7. Fermentation of sardine *koji* by the SY addition method.

The sardine *koji* (in a 15 cm-diameter petri dish) composed of 100 g minced sardine (whole body) and 50 g wheat flour was treated in the same way as treatment of soy bean *koji*. The fermentation mixture contained 330 g ground sardine *koji* material and 33 g glucose and 10^5 yeast cells/g. The fermentation was carried out at 22 °C.

Symbols: ○, glutamic acid; ●, total amino acids; △, glucose; ▲, ethanol; ■, yeast; □, bacteria.

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Summary

The author developed a sugar-yeast (SY) addition method for the production of peptide foods without salt. In the preliminary experiments, ethanol showed the same bacteriostatic activity with and less inhibitory effect on the enzymes from *koji* culture than NaCl.

The fermentation mixture consisting of ground *Aspergillus koji* made of soy beans and wheat flour, 9 % glucose and 10^5 yeast (*Saccharomyces cerevisiae*) cells/g, was incubated at 22 °C. Glucose was consumed quickly and about 4.0 % ethanol was produced after 5 days of fermentation. At the beginning of fermentation, the number of contaminated bacteria increased to 4.8×10^5 cells/g, but decreased to about 10^3 cells/g after 30 days of fermentation. The fermentation mixture did not putrefy. The liberation rates and yields of total amino acids and glutamic acid from raw materials obtained by the SY addition method were greater than or the same as those of the ethanol addition method, and were higher than those obtained by the NaCl addition method. This method can be utilized by replacing starch for glucose as an ethanol source and by replacing soy beans for sardines.

Section 2. Preservation of raw fish and meat

Introduction

These days, low salt foods, especially fermented foods, are favored for health reasons such as the prevention of hypertension. Therefore, several kinds of production methods, such as the ethanol addition method (1~6) and yeast mass addition method (7), were developed to produce low salt fermented foods.

In section 1 of chapter IV, the author reported a new method, the sugar-yeast (SY) addition method, which did not utilize a high concentration of NaCl for the production of fermented protein foods. This method utilized the bacteriostatic effect of ethanol produced from sugar sources by alcoholic fermentation of added yeast (*Saccharomyces cerevisiae*), and also amylolysis of *koji* mold.

The fermentation mixture, consisting of ground soybean *koji* of *Aspergillus*, a sugar source (glucose was mainly used), and yeast cells, was incubated at 22 °C. Under this condition, yeast grew well, ethanol was quickly produced, and glutamic acid and amino acids were also quickly liberated. The number of contaminated bacteria increased to 10^6 cells/g at the beginning of fermentation, then gradually decreased. The fermentation mixture did not putrefy.

From this result, the author thought that the SY addition method could be utilized as a preservation and processing method for raw foods in place of the NaCl addition method (salting method). Therefore, the author investigated the application of the SY addition method in preserving raw marine products and

livestock meat. This section deals with variations of the component content, such as eicosapentaenoic acid, and also biochemical properties such as freshness and the water holding capacity of raw meat during preservation.

Materials and Methods

Microorganism and materials.

Saccharomyces cerevisiae (sake yeast, Kyokai No. 7) was used to preserve raw meat, and was cultivated as described in section 1 of chapter IV.

Sardine, pork and beef meats minced with a blender were mainly used as a starting material.

Preservation procedure.

To determine microbial growth, the amount of eicosapentaenoic acid (EPA), and the freshness of the meat, raw sardine meat (100 g) without head and internal organs, or raw pork meat (100 g), was minced with a blender, and 10 % glucose (powder, 11 g) and a suspension of yeast cells (10^8 cells/ml water, 1 ml) were added and mixed well. The final concentration of yeast cells was adjusted to 10^6 cells per g of raw material. The preservation was allowed to proceed in a container with a lid at 12 °C.

To determine the water holding capacity (WHC), a block (500 mg) of white sardine flesh or of pork meat was soaked for 3 days in a 5 ml solution containing glucose and yeast cells. In the NaCl addition method, NaCl was added at a concentration of 10 % (w/w) in place of the yeast cells and 10 % glucose, and in the ethanol addition method, 10 % ethanol was used.

Analytical method.

Viable cells, glucose and ethanol were determined as described in section 1 of chapter IV.

Determination of EPA: The fermentation mixture (5 g) was suspended in 5 ml water. Then 25 ml of chloroform was added and mixed well. As an internal standard, nonadecanoic acid (5 mg) was usually added to the chloroform mixture. After extraction, a 0.2 ml chloroform layer was taken and mixed with 0.2 ml phenyl trimethyl ammonium hydroxide as a methylation reagent, and 2 μ l mixture was subjected to gas chromatography. The analytical conditions were as follows: Apparatus-Shimadzu GC-7A equipped with a flame ionization detector; column-glass column (3 mm x 3 m) packed with 5 % Shinchrom E-71 on 80/100 mesh Shimalite (AW) (Shimadzu); column temperature-200 °C; injection port temperature-270 °C; N₂ (carrier gas) flow rate-50 ml/min; and integrator-Shimadzu C-R1A.

Determination of freshness: The freshness of the sardines was determined by measuring the K value (8). A sample for determination of K value was prepared as follows. Five grams of preserved meat was ground with 25 ml of 5 % cold perchloric acid solution by hand in a mortar. The extract was centrifuged, and the supernatant was neutralized to pH 6.5 with 1 M KHC0₃, then centrifuged again. The supernatant was used for analysis. The amount of hypoxanthine, inosine and nucleotides (adenine- and hypoxanthine-) was determined by high performance liquid chromatography (Hitachi Co., Ltd.) on a Shim-pack CLC-ODS column (6 x 150 mm). Elution was carried out with 24 mM 2-dimethylamino-ethanol and 16 mM citric acid solution at 40 °C. The elution was monitored at 250 nm.

Determination of water holding capacity (WHC): The amount

of water of 500 mg white flesh (M) was determined by dryness at 105 °C. White flesh (500 mg) soaked in a solution of each additive was held between 10 sheets of filter paper (Toyo filter paper No. 4) previously weighted (A_1) and pressed with a presser (Riken Co., Ltd.) at 10 kg/cm² for 1 min. Residual white flesh was then rapidly peeled off and the filter paper, including water, was weighted (A_2). WHC was calculated according to the following formula (9):

$$\text{WHC (\%)} = 1 - \frac{A_2 - A_1}{M} \times 100$$

Results and Discussion

Time course of the SY addition method.

Using sardine, pork and beef meat, the time course of alcoholic fermentation and the cell numbers of yeast and contaminated bacteria in the SY addition method were compared to those in the control experiment without additions (Table I).

In the control experiment using each meat, the cell number of contaminated bacteria increased to about 10⁹ cells/g after 5 days of preservation, and putrefactive odor, methylmercaptan and H₂S were detected.

By contrast, in the SY addition method using each meat, the glucose was consumed quickly and 3~4 % ethanol was formed after 5 days of preservation. The cell numbers of yeast and bacteria increased to about 10⁸ and 10⁶ cells/g, respectively; but putrefactive odor was scarcely detected.

Effect of additives on the preservation of EPA.

Table I. Growth of microorganisms and formation of ethanol in a mixture of the control experiment and SY addition method.

Each control experiment contained only minced meat (100 g). The SY addition mixture consisted of minced meat (100 g), 10 % glucose and 10⁶ yeast cells/g of preservation mixture. Preservation was carried out at 12 °C for 5 days. Other conditions were the same as those given under Materials and Methods.

Material and preservation method	Incubation time (days)	Cell number (cells/g)		Glucose	Ethanol
		Bacteria	Yeast	(%)	(%)
Sardine					
Control experiment	0	6.8 x 10 ⁴	0	0.3	0
	1	5.2 x 10 ⁶	0	0.2	0
	3	1.1 x 10 ⁸	0	0.2	0.1
	5	8.8 x 10 ⁸	0	0.2	0
SY addition method	0	5.3 x 10 ⁴	2.3 x 10 ⁶	10.7	0
	1	6.8 x 10 ⁵	1.7 x 10 ⁷	7.6	0.7
	3	1.3 x 10 ⁶	9.8 x 10 ⁷	3.4	2.6
	5	7.4 x 10 ⁵	9.6 x 10 ⁷	0.5	3.8
Pork meat					
Control experiment	0	9.3 x 10 ⁴	0	0.3	0
	1	7.5 x 10 ⁶	0	0.3	0.1
	3	2.1 x 10 ⁸	0	0.2	0.1
	5	8.4 x 10 ⁸	0	0.2	0
SY addition method	0	8.4 x 10 ⁴	2.3 x 10 ⁶	10.7	0
	1	9.2 x 10 ⁵	2.4 x 10 ⁷	7.3	0.6
	3	2.5 x 10 ⁶	7.4 x 10 ⁷	3.8	2.4
	5	8.4 x 10 ⁵	9.6 x 10 ⁷	0.8	3.5
Beef meat					
Control experiment	0	4.8 x 10 ⁴	0	0.3	0
	1	4.4 x 10 ⁶	0	0.2	0
	3	1.5 x 10 ⁸	0	0.2	0.1
	5	7.6 x 10 ⁸	0	0.3	0.1
SY addition method	0	5.3 x 10 ⁴	2.3 x 10 ⁶	10.7	0
	1	6.3 x 10 ⁵	2.6 x 10 ⁷	7.4	0.6
	3	1.6 x 10 ⁶	6.9 x 10 ⁷	3.5	2.6
	5	7.9 x 10 ⁵	9.6 x 10 ⁷	0.7	3.6

Meat of sardine contains a large amount of EPA and higher unsaturated fatty acids (10~12). EPA is known as a precursor of prostaglandins and has preventing function related to thrombosis (13) and other disorders (14~19). Therefore, the preservation of EPA was investigated under conditions containing each additive (Fig. 1).

EPA was quickly decomposed in the control experiment (Fig. 1(A)). In the NaCl addition method (Fig. 1(C)), the viable cell numbers of contaminated bacteria decreased gradually, and putrefaction was not observed. But, EPA was quickly decomposed in the same way as the control experiment. On the other hand, the ethanol addition method (Fig. 1(B)) did not cause bacterial growth or putrefactive preservation, and it repressed the decomposition of EPA.

Moreover, in the SY addition method (Fig. 1(B)), alcoholic fermentation proceeded, bacterial growth was repressed as described above, and the decomposition of EPA was repressed in the same way as the ethanol addition method.

Effect of components of the SY addition method on the preservation of EPA.

The effect of the glucose and yeast components in the SY addition method on the preservation of EPA was investigated (Fig. 2).

Omission of either glucose or yeast caused a decrease in EPA content and an increase in the number of bacterial cells; and preservation mixtures putrefied. Therefore, both glucose and yeast were required to preserve of EPA by the SY addition method.

It is generally known as phytochemical reduction that at the stage of alcoholic fermentation by yeast, the inside of

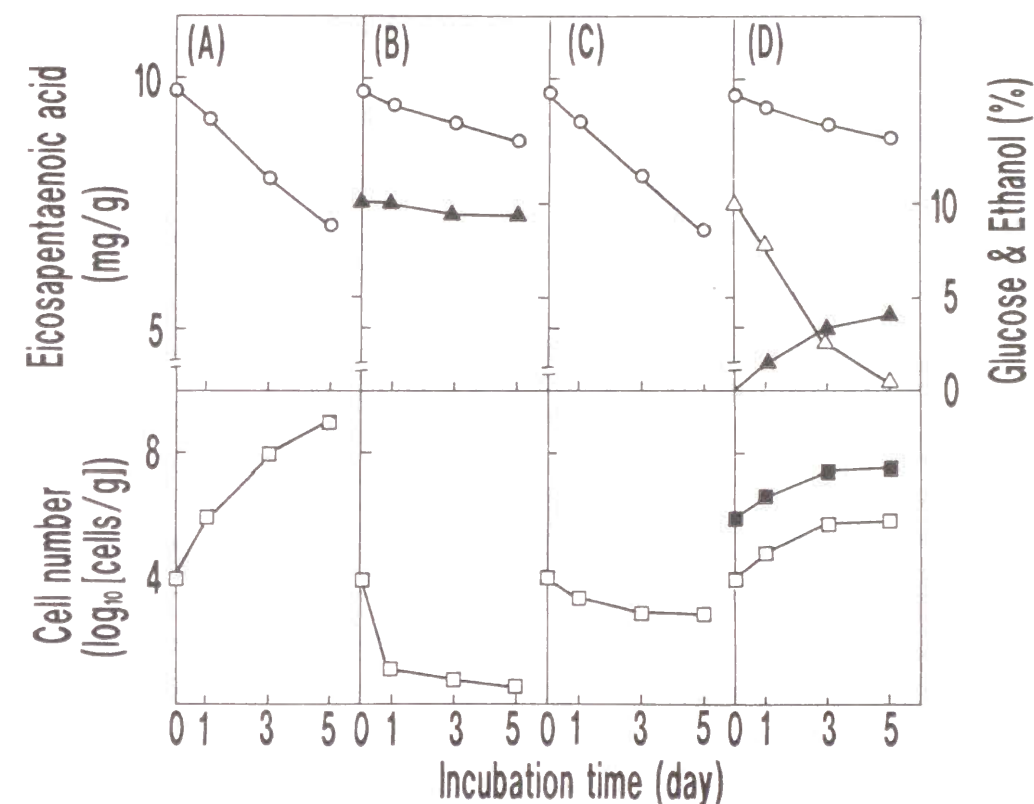


Fig. 1. Effect of additives on the preservation of EPA.

The control experiment (A) contained only 100 g of sardine meat. Experiment (B), the ethanol addition method, contained 100 g sardine meat and 11 g ethanol. Experiment (C), the NaCl addition method, contained 100 g sardine meat and 11 g NaCl. Experiment (D), the SY addition method, contained 100 g sardine meat, 11 g glucose and 10^6 yeast cells/g. Preservation was carried out at 12 °C.

Symbols: ○, EPA; △, glucose; ▲, ethanol; ■, yeast; □, bacteria.

fermentation mixture could be kept in remarkably reduced state (20~22). Therefore, it was thought that EPA was not decomposed in the SY addition method.

Moreover, a substrate glucose and the product ethanol in the SY addition method also function as reductants. Therefore, it is thought that the amount of decomposed EPA in this method is less than that in the NaCl addition method (Fig 1(C)). So, this method might effectively function not only to prevent the oxidative degradation of higher unsaturated fatty acids, but also of lipids and vitamins in meat.

Effect of component concentration in the SY addition method on the preservation of EPA.

Figure 3 shows the effect of the initial concentration of yeast cells on the preservation of EPA. The yeast cell concentration influenced both the formation of ethanol and the preservation of EPA. The initial rates of glucose consumption and ethanol formation increased with an increase in the yeast cell concentration.

In mixtures with 10^5 cells/g or less, EPA was quickly decomposed, and the number of bacterial cells increased with a decrease in the initial yeast cell concentrations. About 10^8 cells/g of bacteria were found in the mixture containing 10^5 cells/g yeast cells. This result might be due to insufficient alcoholic fermentation to produce ethanol, which works as a reductant and as a bacteriostatic reagent. On the other hand, in a mixture with 10^6 cells/g yeast and more, alcoholic fermentation quickly proceeded, so that both the decomposition of EPA and the growth of bacteria were repressed.

Furthermore, the effect of glucose concentration was

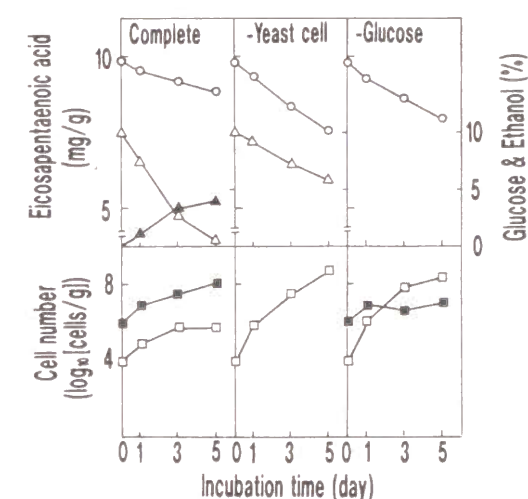


Fig. 2. Effect of components of the preservation mixture on the preservation of EPA.

The complete system contained 100 g sardine meat, 11 g glucose and 10^6 yeast cells/g. Preservation was carried out at 12 °C.

Symbols: ○, EPA; △, glucose; ▲, ethanol; ■, yeast; □, bacteria.

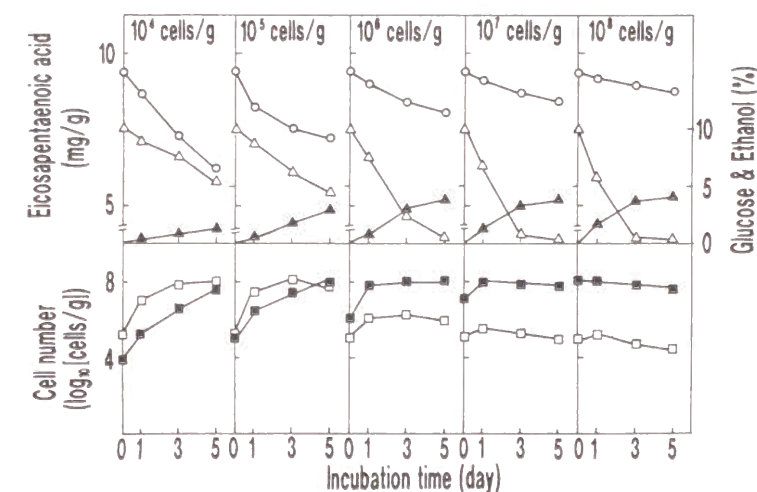


Fig. 3. Effect of initial yeast cell concentration on preservation of EPA.

Each preservation mixture contained 100 g sardine meat, 11 g glucose, and yeast cells as shown in Figure. Preservation was carried out at 12 °C.

Symbols: ○, EPA; △, glucose; ▲, ethanol; ■, yeast; □, bacteria.

investigated (data not shown). Under the condition containing 10^6 cells/g yeast and using less than 10 % glucose, alcoholic fermentation proceeded quickly; however, decomposition of EPA and bacterial growth occurred, because alcoholic fermentation did not continue and ethanol concentration in the mixture was insufficient. This could have been due to a lack of glucose as a substrate. Using 10~15 % glucose, alcoholic fermentation proceeded quickly so that both EPA decomposition and bacterial growth were repressed. Yet, with the addition of 20 % glucose or more, alcoholic fermentation was retarded at the beginning of preservation, where the decomposition of some EPA and a slight growth of yeast and bacteria was observed. However, since an increase in the initial concentration of yeast cells brought about quick alcoholic fermentation, the decomposition of EPA and the growth of contaminated bacteria were repressed greatly.

The effect of preservation temperature on the SY addition method was also investigated (data not shown). Preservation at above 20 °C caused bacterial growth (about 10^8 cells/ml) at the beginning of preservation, and a decrease of EPA content was observed. Below 12 °C, neither decomposition of EPA or bacterial growth was significantly observed.

Consequently, for the satisfactory preservation of EPA, the addition of more than 10^6 cells/g yeast and 10~15 % glucose was required, and the optimum temperature for incubation was less than 12 °C. However, a shift of incubation temperature from 12 °C to 22 °C after accumulation of 3~4 % alcohol did not stimulate further decomposition of EPA or growth of contaminated bacteria.

Removal of ethanol from the preserved-processed product.

The conditions for removing ethanol from fermented meat mixtures was investigated, because removal of ethanol was needed in order to use the preserved-processed meat not only as manufacturing foods, but also as intermediate materials for food processing and for shipping.

As shown in Fig. 4, ethanol in the product was almost completely removed by heating at 40~60 °C for 30~40 min under reduced pressure. The product from which ethanol was removed can easily be shipped and utilized for food processing.

Preservation of the freshness of meat.

Regarding the preservation of fish, the freshness is extremely important. Figure 5 shows the effect of the preservation method on the freshness of sardines. In both the control experiment and the ethanol addition method, freshness deteriorated remarkably. However, in the SY and NaCl addition methods, freshness was lost only gradually, and the deterioration of freshness in the SY addition method was slightly less than that in the NaCl addition method.

Effect of additive concentration on freshness.

Table II shows the effect of additive concentration on freshness with or without yeast. Without yeast, the increase of concentration of each additive (glucose, NaCl and ethanol) was only slightly effective for the preservation of freshness, and the addition of NaCl being most effective. By adding 10^6 cells/g yeast cells to each preservation mixture described above, preservation of freshness was observed only in the glucose-added mixture, i. e., in the SY addition method.

In the SY addition method, the effect of the initial

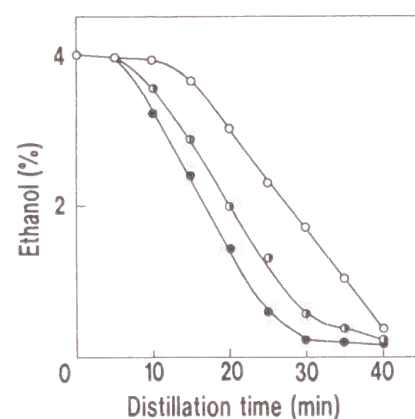


Fig. 4. Removal of ethanol with distillation.

Preservation mixture (100 g) containing about 4 % ethanol was distilled under the conditions of reduced pressure (about 15 mmHg) and heating at 40 (○), 50 (◐) and 60 °C (●).

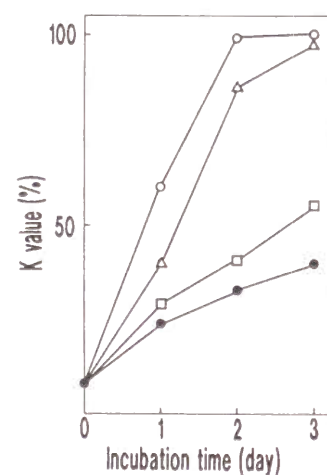


Fig. 5. Effect of additives on freshness.

The control experiment contained only 100 g sardine meat. The ethanol addition method contained 100 g sardine meat and 11 g ethanol. The NaCl addition method contained 100 g sardine meat and 11 g NaCl. The SY addition method contained 100 g sardine meat, 11 g glucose and 10^6 yeast cells/g. Preservation was carried out at 12 °C.

Symbols: ○, control experiment; △, ethanol addition method; □, NaCl addition method, ●, SY addition method.

Table II. Effect of concentration of additives and yeast cells on freshness.

The concentration of added yeast cells was 10^6 cells/g of preservation mixture. The concentration of each additive (glucose, NaCl and ethanol) was weight percent per weight of sardine meat. Preservation was carried out at 12 °C for 2 days. Other conditions were the same as those given under Materials and Methods.

Additive	Concentration (%)	K value (%)	
		Without yeast	With yeast
Control experiment		100	100
Glucose	2.5	89	64
	5	86	60
	10	84	52
	15	83	46
	20	84	44
NaCl	2.5	76	77
	5	69	68
	10	59	60
	15	59	58
	20	58	56
Ethanol	2.5	98	99
	5	96	95
	10	86	87
	15	88	88
	20	88	86

concentration of yeast cells on freshness was investigated under the condition containing 10 % glucose (Table III). An increase in yeast cell concentration maintained the preservation of freshness. But the addition of only yeast did not affect the preservation of freshness.

Therefore, these findings indicate that alcoholic fermentation contributed to the preservation of freshness, although the reason was not evident. The decomposition of IMP to inosine might be inhibited by alcoholic fermentation, because a small amount of AMP, ADP and ATP found in sardines decomposed and disappeared, but the hypoxanthine, inosine or IMP content scarcely changed during the preservation period.

The effect of temperature on the preservation of freshness by the SY addition method was investigated as well (Fig. 6). Low temperatures repressed the deterioration of freshness while high temperatures (above 22 °C) deteriorated freshness. Therefore, alcoholic fermentation was effective in preserving freshness at low temperature, although at high temperatures alcoholic fermentation proceeded actively, and freshness deteriorated. Accordingly, it was thought that at high temperatures, other factors, such as stimulation of nucleotidase activity, contributed to the deterioration of freshness.

Effect of preservation method on WHC.

The texture of the foods was strongly influenced by components within the foods. Water, protein, lipid content and so on provide good examples of this. Water content was remarkably influenced by dehydration which could occur with the addition of additives. Therefore, WHC, a factor influencing food texture, was investigated (Fig. 7).

Table III. Effect of yeast cell concentration on freshness.

The amount of added glucose was 10 % of the preservation mixture. Preservation was carried out at 12 °C for 2 days. Other conditions were the same as those given under Materials and Methods.

Yeast concentration (cells/g)	K value (%)
10^3	81
10^4	76
10^5	67
10^6	52
10^7	49
10^8	48

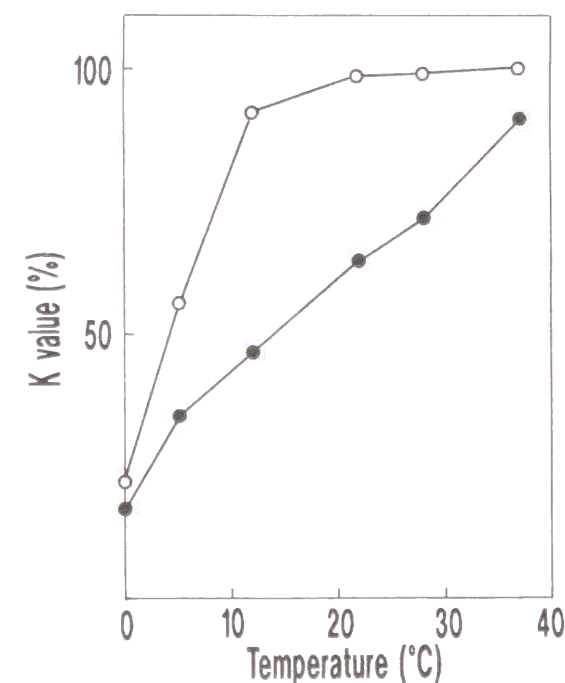


Fig. 6. Effect of temperature and addition of yeast on freshness.

The experiment systems without yeast cells (○) and with yeast cells (10^6 cells/g) (●) contained 100 g sardine meat and 11 g glucose. The temperature of preservation was changed as described in the Figure. Preservation was carried out at 12 °C for 2 days.

The addition of more than 5 % NaCl caused expansion of meat, and a decrease in WHC, whereas the addition of only glucose without yeast caused a slight increase in WHC. Little putrefaction was observed when only glucose was added. On the other hand, in the SY addition method, WHC scarcely varied, similar to the findings of the ethanol addition method. This method is effective for preserving WHC.

WHC was also influenced by yeast cell concentration and preservation temperature (Table IV). A decrease in initial yeast cell concentration combined with preservation at a high temperature brought about increase in WHC. The same result was obtained using livestock meat.

These results indicate that variations of EPA content and physical properties scarcely occurred in the SY addition method. Thus, this method was superior to the commonly used NaCl addition (salting) method for preserving raw foods.

Since it has already been reported that the cultivation of several kinds of yeast in fish extracts caused a deodorization of fish odor (23), the flavor of products preserved by the SY addition method might be improved.

Recently, using ethanol, the production of "marine beef" (24), a processing method for fish meat containing protein as a main component, was developed. Marine beef is now experimentally being used as a material to be cooked by itself or by mixing it with other meats or foods. Products obtained by the SY addition method might also be utilized in the same way as marine beef, because the meat is processed without NaCl, and the product is easily seasoned.

Moreover, pathogenic germs, food poisoning germs and parasitic worms might be sterilized by ethanol, which is produced

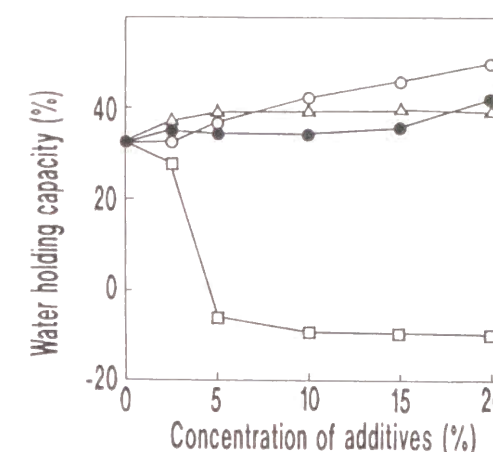


Fig. 7. Effect of additives on water holding capacity.

A 500 mg block of white sardine fish was soaked in a 5 ml solution containing each of the additives at 12 °C for 3 days. Each additive (glucose, ethanol or NaCl) concentration in the glucose (only glucose) addition method (○), ethanol addition method (△), NaCl addition method (□) and the SY addition method (●) was changed as shown in the Figure. In the SY addition method, the concentration of yeast was 10^6 cells/g and the glucose concentration was changed.

Table IV. Effect of yeast cell concentration and temperature on water holding capacity.

Exp. 1: The amount of added yeast cells was varied as indicated in the Table. Preservation was carried out at 12 °C for 3 days. Exp. 2: The preservation temperature was changed as indicated in the Table. The amount of added yeast cells was 10^6 cells/g. Preservation was carried out for 3 days. Other conditions were the same as those given under Materials and Methods.

Condition	WHC (%)
Exp. 1	
10 ³ yeast cells/g	52
10 ⁴	51
10 ⁵	47
10 ⁶	42
10 ⁷	41
10 ⁸	38
Exp. 2	
5 °C	38
12	42
22	49
28	57
37	69

by the alcoholic fermentation of yeast.

In this section, the author discusses the preservation of raw fish and meat by the SY addition method from the standpoint of application of the method, though in these days, the freezing method seems to be best for preservation of these raw foods. The author envisions materials preserved by this method being used as materials for new types of foods.

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Summary

The sugar-yeast (SY) addition method, which was developed for the production of fermented protein foods, was applied to the preservation of raw fish and meat and compared with the NaCl and ethanol addition methods. The preservation mixture, consisting of minced raw meat of fish or livestock, 10 % glucose and 10^6 yeast cells/g, was incubated at 12 °C. Glucose was consumed quickly and about 2~4 % ethanol was produced after 3~5 days of preservation. About 10^5 cells/g of contaminating bacteria present in the preservation mixture increased to about 10^6 cells/g after 1 day of preservation, but decreased to about 10^4 cells/g after 5 days of preservation. The preservation mixture did not putrefy. The amount of eicosapentaenoic acid contained in sardines was preserved effectively by the SY addition method and by the ethanol addition method, but not well by the NaCl addition method. The freshness (K value) of sardines preserved by the SY addition method was maintained at the same level as that using the NaCl method. However, deterioration of freshness was observed in the ethanol addition method. The water holding capacity (WHC) of the NaCl addition method decreased, but WHC was well maintained by the SY and ethanol addition methods.

Chapter IV. Production of useful materials using yeast alcoholic fermentation

Section 1. Production of deoxyribonucleoside triphosphates through coupled fermentation with energy transfer

Introduction

Tochikura *et al.* have already succeeded in the production of various ribonucleoside triphosphates by using dried baker's yeast cells as an enzyme source (1, 2). In addition, they established a process in which yeast fermentation of sugar is combined with endergonic reaction(s) catalized by enzyme(s) from other microorganisms (coupled fermentation with energy transfer), and demonstrated the possibility of producing many substances even though the biosynthetic activity of the yeast is insufficient (3, 4).

It has already been shown that dried baker's yeast phosphorylates dAMP and dGMP, but the amounts of dATP and dGTP formed are low (5). However, a method for producing deoxyribonucleoside triphosphates (dNTPs) in high yields has not been investigated in detail. Therefore, the author tried to produce dNTP via coupled fermentation with energy transfer, in order to maintain a sufficient supply of this compound, for which demand has been increasing with recent progress in the field of applied genetics.

Materials and Methods

Enzyme preparation.

Baker's yeast supplied from Oriental Yeast Co. Ltd. was dried with an electric fan at room temperature and stored at -20°C .

dAMP, dGMP, dCMP and dTMP kinases were partially purified from cell-free extracts of *Escherichia coli* B by DEAE-cellulose column chromatography according to the method of Okazaki and Kornberg (6). The addition of enzyme(s) catalyzing the phosphorylation of dNDP was not necessary: the dNMP kinase preparations and/or baker's yeast might contain such enzymes.

Reaction condition.

The standard reaction mixture for dNTP formation contained 100 mM deoxyribonucleoside monophosphate (dNMP), 400 mM glucose, 400 mM potassium phosphate buffer (KPB, pH 7.0), 10 mM MgSO_4 , 0 or 1 unit/ml deoxyribonucleoside monophosphate kinase corresponding to the substrate dNMP and 100 mg/ml dried baker's yeast. Incubation was carried out at 28°C with shaking and terminated by immersing the mixture in boiling water for 3 min. The mixture was centrifuged for 15 min at 3000 rpm and the resultant supernatant was used for the assay.

Preparation of hydrolyzed DNA.

Hydrolysis of DNA was carried out by the method of Fujimoto *et al.* (7, 8) with slight modification. Commercial DNA (100 mg) dissolved in 10 ml of water was heated in boiling water for 10 min and then cooled quickly with ice. To the solution were added 1 ml of 0.02 M acetate buffer (pH 5.5) and 0.001 mg of Nuclease

P_1 . The mixture was incubated at 37°C for 15 hr. The lyophilized mixture was used as a mixture of dNMPs.

Assay.

Protein was measured by Lowry's method (9). FDP and glucose were measured by the methods of Roe *et al.* (10) and Somogyi (11), respectively.

dNMP kinase activity was assayed using the method of Miech *et al.* (12) with NADH, pyruvate kinase and lactate dehydrogenase. Oneunit of each kinase activity was defined as the amount producing 1 μmole of deoxyribonucleoside diphosphate (dNDP), for 1 min at 25°C . dNMP, dNDP and dNTP were determined by measuring the optical densities at 260 nm, after the extraction of their spots with 0.01 N HCl after paper chromatography on Toyo filter paper No. 51A, developed with *iso*-butyric acid and 0.5 N ammonia water (5:3, by volume). They were also determined by high performance liquid chromatography (Waters Co. Ltd) with a Cosmosil 5NH₂ column (4.6 x 15 cm). Elution was carried out with a convex gradient of $\text{NH}_4\text{H}_2\text{PO}_4$ (0.01 M, pH 3.0 to 0.4 M, pH 5.0). Eluates were monitored at 254 nm. The concentration of deoxyribonucleotides was determined by comparison with the absorbance of standard solutions.

dNDP and dNTP were isolated and identified by paper chromatography with various solvent systems (13~15). Their phosphate content was determined by the method of Fiske and Subbarow (16) after hydrolysis by the wet-ashing method (17). Deoxyribose in the products was identified by the Diphenylamine-acetic acid method (18).

Enzymes and chemicals.

Pyruvate kinase and lactate dehydrogenase were purchased from Sigma Chemical Co. dAMP, dGMP, dCMP, dTMP (sodium salts) and Nuclease P₁ were obtained from Yamasa Co. Ltd., and DNAs (salmon testes and herring sperm) were from Sigma Chemicals Co.

Results and Discussion

Production of dATP by baker's yeast cells.

Figure 1 shows the effect of yeast cell concentration on dATP formation. dATP formation was dependent on the addition of yeast cells and glucose (data not shown), and reached a maximum concentration (70~80 mM) in 10 hr or 6 hr of incubation, respectively in which the mixture had 50 mg/ml or 100 mg/ml of dried yeast cells, respectively. The amount of dADP formed was 17 mM or 22 mM in each mixture. The initial rate of dATP formation increased with an increase in the yeast cell concentration, but the maximum amount decreased. The dATP formed degraded to dADP and dAMP when the accumulated FDP decreased to a certain level (10~20 mM).

The maximum amount of dATP increased with an increase in the glucose concentration (Fig. 2). About 70~80 mM dATP was formed in the reaction mixture containing 500~600 mM glucose, but the phosphorylation was retarded with an increase in the glucose concentration. In mixtures with 300 mM glucose or less, phosphorylation of dAMP to dATP did not occur sufficiently and large amounts of dADP were formed, which might be due to a lack of an energy source.

For satisfactory dATP formation with a small amount of dADP, 100 mg/ml dried baker's yeast cells and 400 mM glucose were

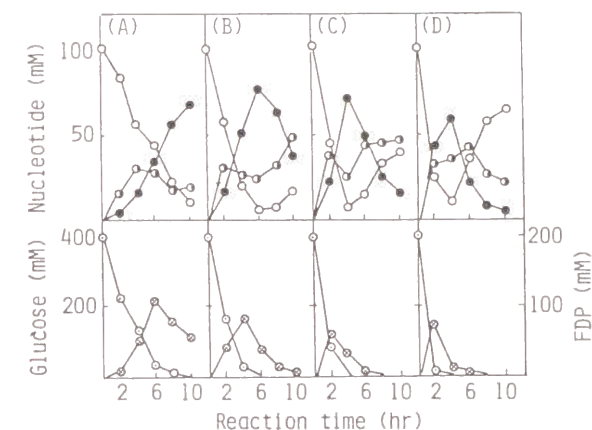


Fig. 1. Effect of the Concentration of Yeast Cells on dATP Production.

The experimental conditions are given under **Materials and Methods**. The yeast cell concentrations were 50 mg/ml (A), 100 mg/ml (B), 150 mg/ml (C) and 200 mg/ml (D). Symbols: ○, dAMP; ●, dADP; ●, dATP; ○, glucose; ⊗, FDP.

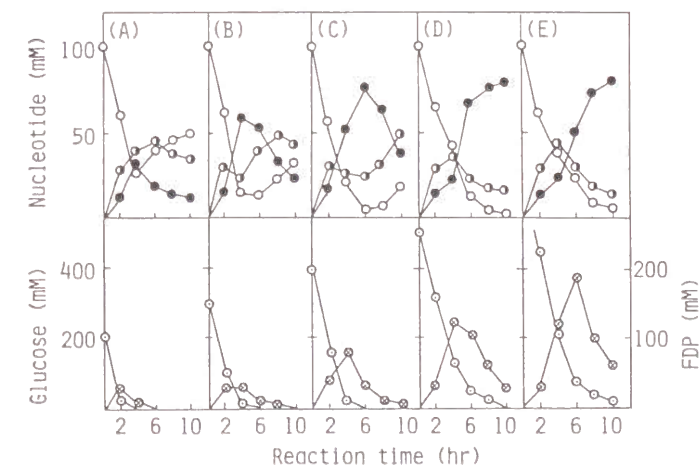


Fig. 2. Effect of the Glucose Concentration of dATP Production.

The experimental conditions are given under **Materials and Methods**. The glucose concentrations were 200 mM (A), 300 mM (B), 400 mM (C), 500 mM (D) and 600 mM (E). Symbols: ○, dAMP; ●, dADP; ●, dATP; ○, glucose; ⊗, FDP.

chosen for the following experiments.

Figure 3 shows dATP formation with different concentrations of dAMP as a substrate. In the mixture containing 50 mM dAMP, about 40 mM dATP and 10 mM dADP were formed, indicating that glucose was sufficient as an energy source for the phosphorylation. The products were stable for 4~6 hr until sugar components were consumed. With 100 mM dAMP, 76 mM dATP and 30 mM dADP were formed in 6 hr of incubation. When the concentration of dAMP was increased to 150 mM, the maximum amount of dATP decreased (70 mM) and considerable amounts of dAMP remained, which indicated consumption of the energy source prior to phosphorylation. In these experiments, the conversion ratios of dAMP to dATP were 80, 70 and 40 %, respectively, and the yields based on glucose as an energy source were 10, 19 and 17 %, respectively.

The findings that retardation of sugar fermentation occurs with an increase in dAMP concentration and that only 5 mM of dIMP was formed in the mixture with 100 mM of dAMP (not shown in Fig. 3) were characteristics of dATP production considering the previous results in ATP production (3): the increase in AMP as a substrate promoted sugar fermentation of the yeast and that AMP was remarkably deaminated to form 30 mM of IMP under similar conditions. Yoshino *et al.* (19) reported that yeast AMP deaminase showed low reactivity to dAMP, and a deaminating enzyme specific to dADP and/or dATP was not reported.

Figure 4 shows dATP production in the mixture with various concentrations of another substrate, inorganic phosphate, which controls the rate of ATP regeneration through its repressive effect on the sugar fermentation. The formation of dATP increased in response to the phosphate concentration, and most of

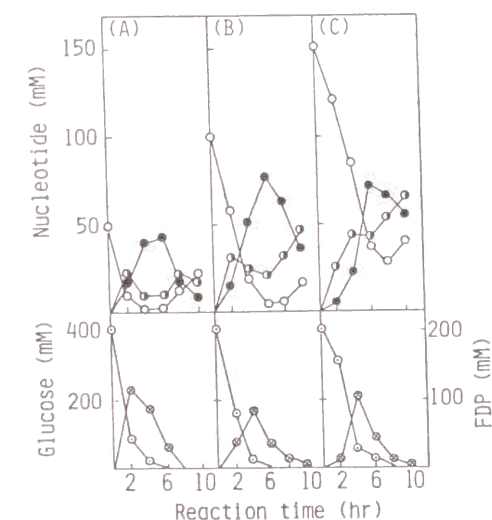


Fig. 3. Effect of the dAMP Concentration on dATP Production.

The experimental conditions are given under **Materials and Methods**. The dAMP concentrations were 50 mM (A), 100 mM (B) and 150 mM (C). Symbols: ○, dAMP; ◐, dADP; ●, dATP; ○, glucose; ⊗, FDP.

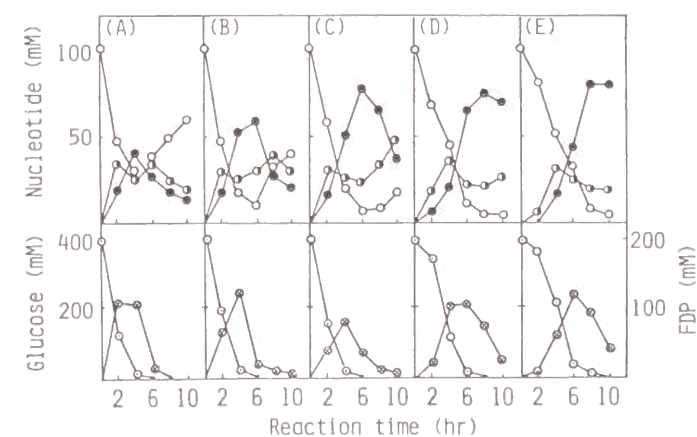


Fig. 4. Effect of the Phosphate Concentration on dATP Production.

The conditions are given under **Materials and Methods**. The phosphate concentrations were 200 mM (A), 300 mM (B), 400 mM (C), 500 mM (D) and 600 mM (E). Symbols: ○, dAMP; ◐, dADP; ●, dATP; ○, glucose; ⊗, FDP.

the dAMP was phosphorylated to dATP and dADP in mixtures containing more than 400 mM of phosphate. However, retardation of sugar fermentation and phosphorylation was observed with 500 mM of phosphate or more. Sufficient phosphorylation of dAMP did not occur in the mixture with 300 mM of phosphate or less. The enzyme for dAMP phosphorylation could not utilize the fermentative energy, because of the rapid fermentation of sugar.

The enzymes for glycolysis and the phosphorylation of dAMP require Mg^{2+} as a cofactor. Figure 5 indicates the effect of Mg^{2+} concentrations. Phosphorylation of dAMP and sugar fermentation proceeded gradually without the addition of Mg^{2+} , and were remarkably enhanced by the addition of 10 mM Mg^{2+} . On the other hand, when Mg^{2+} was added at a concentration of 20 mM or more, yeast fermentation proceeded too fast, and a decrease in dATP formation and the rapid degradation of dATP to dAMP were observed in the early period of incubation.

Table I summarizes dATP production under optimum conditions, which were defined on the basis of the results of the above experiments, namely the yields of the substrates and the reaction period.

Isolation and identification of dADP and dATP.

The reaction mixture (10 ml) obtained after 6 hr of incubation, under the conditions described in the Materials and Methods with 1 mmole of dAMP as a substrate, was treated with activated carbon and separated by column chromatography on Dowex 1 x 2 (Cl^- form). The R_f values of the substances in eluate were developed on and identified by paper chromatograms with various solvent systems, the contents of phosphorus and deoxyribose (Table II), and absorption spectra (pH 2.0, 7.0 and 12.0) were

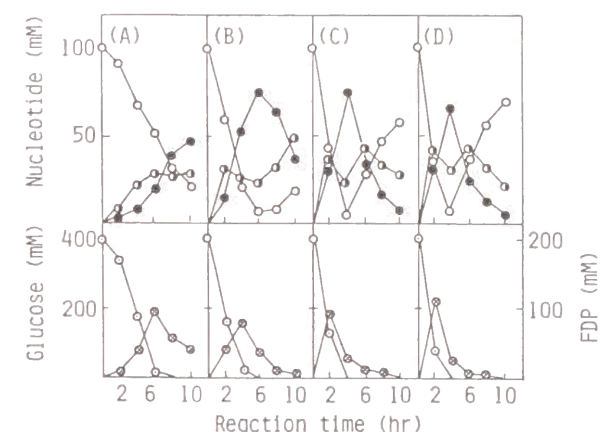


Fig. 5. Effect of the $MgSO_4$ Concentration on dATP Production.

The conditions are given under **Materials and Methods**. The $MgSO_4$ concentrations were 0 mM (A), 10 mM (B), 20 mM (C) and 30 mM (D).

Symbols: ○, dAMP; ●, dADP; ◐, dATP; □, glucose; ◑, FDP.

Table I. dATP production under the optimum conditions.

The components of the reaction mixture and their concentrations are indicated in the Table. The concentration of $MgSO_4$ was 10 mM.

Conditions				Products (mM)	
				dADP	dATP
				time	
A)	dAMP	100 mM			
	Glucose	100 mM	6 hr	21	76
	KPB	400 mM			
	Yeast	100 mg/ml			
B)	dAMP	100 mM			
	Glucose	600 mM	9 hr	19	82
	KPB	600 mM			
	Yeast	150 mg/ml			
C)	dAMP	150 mM			
	Glucose	800 mM	18 hr	23	127
	KPB	800 mM			
	Yeast	150 mg/ml			

Table II. Identification of dADP and dATP.

Sample	Phosphorus [#]	Deoxyribose [#]
Isolated dADP	2.01	1.10
Authentic dADP	2.03	1.03
Isolated dATP	2.98	0.96
Authentic dATP	3.05	1.02

[#] Ratio to 1 mole of deoxyriboadenosine.

Table III. Phosphorylation of deoxyribonucleoside monophosphates and effects of the addition of dNMP kinase.

Deoxyribonucleoside monophosphate kinase preparation corresponding to the substrate added. Other conditions are the same as those described in Materials and Methods.

Substrate	Kinase (unit/ml)	Reaction time	Products (mM)	
			dNDP	dNTP
dAMP	0	6 hr	28	70
	1	6 hr	24	72
dGMP	0	8 hr	9	34
	1	8 hr	15	48
dCMP	0	8 hr	19	26
	1	8 hr	38	44
dTMP	0		0	0
	1	8 hr	18	32

the same as those of authentic dADP and dATP.

The results indicated that the deoxyriboadenosine derivatives formed were dADP and dATP, respectively.

Phosphorylation of deoxyribonucleoside monophosphates.

Table III indicates phosphorylation of dAMP, dGMP, dCMP and dTMP in the mixture with or without the addition of the corresponding deoxyribonucleoside monophosphate kinase preparations.

In the case of dAMP as a substrate, the addition of dAMP kinase preparation showed no effect on the phosphorylation suggesting sufficient amounts of dAMP kinase were present in the yeast for the reaction. Phosphorylation of dGMP or dCMP was increased 1.5~2 times by the addition of the kinase preparation, and 48 mM dGTP or 44 mM dCTP was obtained with considerable amounts of dGDP or dCDP in an 8 hr incubation. The formation of a certain amounts of dGTP or dCTP without the addition of the kinase preparation indicated that dGMP or dCMP kinase was present in the yeast cells even though in insufficient quantities. The finding that dTMP was phosphorylated only in the mixture with dTMP kinase preparation indicated that dTMP kinase activity was extremely low in the yeast cells. Sugar fermentation in the mixture with dGMP, dCMP and dTMP was repressed in comparison to that in the mixture with dAMP.

The production of dATP was achieved with a high yield of dAMP by the yeast cells alone, but other dNTP production was not. The formation of the latter was improved to some extent by the addition of the bacterial kinase preparation. Tachiki *et al.* (20) have already indicated that the ratio of the activities of sugar fermentation by the yeast and the energy utilizing system was

Table IV. Production of deoxyribonucleoside triphosphate from DNA.

DNA was hydrolyzed by Nuclease P_1 as shown in Materials and Methods. One hundred mg/ml of the hydrolyzate were used as the substrate and dNMP kinase preparations (1 unit/ml, each) were added to the reaction mixture. Other conditions are the same as those described in Materials and Methods.

Substrate (mM)		Reaction time	Products (mM)			
DNA-Na (Salmon testes)	dAMP 40	6 hr	dADP 4	dATP 29		
	dGMP 32		dGDP 11	dGTP 20		
	dCMP 36		dCDP 12	dCTP 17		
	dTMP 29		dTDP 8	dTTP 10		
DNA-Na (Herring sperm)	dAMP 32	10 hr	dADP 7	dATP 22		
	dGMP 28		dGDP 5	dGTP 18		
	dCMP 37		dCDP 6	dCTP 11		
	dTMP 24		dTDP 6	dTTP 5		

important for satisfactory production with a limited energy source. As the activity of dNMP kinase, especially dTMP kinase, was low with the *E. coli* B used in this study, it is necessary to use other microorganisms with higher dTMP kinase activities for dTTP production.

Production of deoxyribonucleoside triphosphates from DNA.

Production of dNTPs was examined using hydrolyzed DNA in place of dNMP (Table IV). When purified DNA from salmon testes was used, 76 mM dNTPs were formed in 6 hr of incubation. On the other hand, 56 mM NTPs were formed in 10 hr with crude herring sperm. The rate of sugar fermentation by the yeast in the latter mixture was lower than that in the former mixture.

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Summary

Production of deoxyribonucleoside triphosphate (dNTP) from deoxyribonucleoside monophosphate (dNMP) was investigated by using dried baker's yeast in the presence of a high concentration of inorganic phosphate. The amounts of dATP formed by 150 mg/ml of the yeast cells alone was 127 mM with a 84 % yield of the substrate, dAMP, in 18 hr at 28 °C, under the conditions of 150

mM dAMP, 800 mM glucose, 800 mM potassium phosphate buffer (pH 7.0), and 10 mM MgSO₄. The amounts of dGTP or dCTP formed by baker's yeast was about 26~34 mM from 100 mM of dGMP or dCMP in an 8 hr incubation, and their formation increased remarkably with the addition of partially purified dGMP kinase or dCMP kinase from *Escherichia coli* B. dTTP was formed only when the dTMP kinase preparation was present in the reaction mixture.

In a reaction mixture containing dNMP-mixture, which was prepared by the hydrolysis of salmon testes DNA or herring sperm DNA with nuclease P₁, 56~76 mM of dNTP were obtained.

Section 2. Production of ATP from adenine by a combination of bacterial and baker's yeast cells

Introduction

In 1967, Tochikura *et al.* reported the production of ATP from AMP using various cell preparation of baker's yeast as enzyme sources (1), which was done by energy released fermentatively from sugar by the yeast. That was the first of studies on the production of various substances by yeast cells (2 ~7). These methods are characteristic in using high concentrations of substrates and giving products in good yields. However, as to ATP production, increasing the concentration of the substrate AMP was not effective because AMP at a high concentration was easily deaminated to IMP before its phosphorylation to ATP (1). But in 1974, the authors produced ATP from adenosine, adenosine was scarcely deaminated and at the same time the level of AMP remained rather low throughout the reaction (8). To use adenine as a substrate has been unsuccessful because of the low ribosylation activity for adenine in the yeast cells.

On the other hand, the authors have indicated the possibility of coupling of yeast fermentation with endergonic reactions catalyzed by enzymes prepared from different microorganisms (8, T. Tochikura, Proc. First General Meet. Yeast Symp., Japan, p. 71, 1974; T. Tochikura *et al.*, Abstr. Fifth Intn. Ferment. Symp. Berlin, p. 411, 1976). In this section, the author indicates the production of ATP from adenine and uridine, inosine, or cytidine using bacterial nucleoside phosphorylases (9, 10) and the

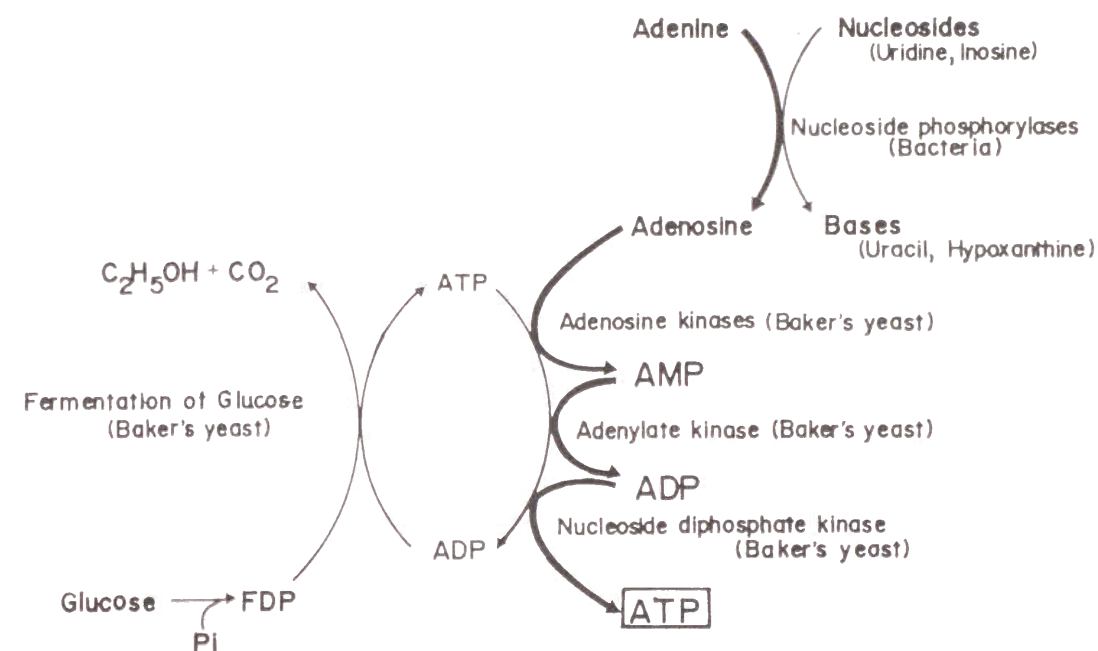


Fig. 1. ATP production through the coupled reaction of transribosylation of adenine and fermentation of sugar.

alcoholic fermentation system of glucose by yeast cells (Fig. 1): adenine is transribosylated to adenosine with a nucleoside as a ribosyl donor through the coupled reactions of adenosine phosphorylase and the corresponding nucleoside phosphorylase, and the adenosine thus formed is preferentially phosphorylated to ATP by the yeast system.

Materials and Methods

Reaction mixture for ATP production.

The mixture contained 50~150 mM (7~20 mg/ml) adenine, 75~200 mM uridine, inosine, or cytidine, 300~600 mM potassium phosphate buffer (pH 7.0), 10 mM MgSO₄, 100 mM fructose-1,6-diphosphate (FDP) or 400~800 mM glucose, 0.01~0.5 units/ml bacterial phosphorylase preparation (as adenosine phosphorylase), and 25~100 mg/ml dried baker's yeast cells. The enzyme reaction was done at 28 °C with shaking and stopped by immersing the reaction tubes in boiling water for 3 min. Adenine was added as a powder, since its actual concentration was low owing to its poor solubility in water. The powder dissolved with the progress of the reaction, and finally disappeared from the mixture.

Enzyme preparation.

Dried baker's yeast cells.

Pressed baker's yeast supplied by Oriental Yeast Co. Ltd. was dried by method of Tochikura *et al* (1~4).

Nucleoside phosphorylases.

Four preparations (i)~(iv) were used in this study. They contained all of the adenosine-, uridine-, and inosine-

phosphorylases. (i) Intact cells: *Erwinia carotovora* IFO 3057, *Escherichia coli* IFO 3806, or *Aerobacter aerogenes* IFO 3319 was cultivated for 24 hr at 28 °C on a reciprocal shaker in 2-L Sakaguchi flasks containing 500 ml of a medium (2.0 % peptone, 0.5 % yeast extract, and 0.3 % NaCl, pH 7.0). Cells were collected, washed, and suspended in 0.01 M potassium phosphate buffer (pH 7.0). (ii) Cell-free extracts: Cell suspensions were sonicated at 0~5 °C for 10 min. After centrifugation for 30 min at 10,000 x g, the supernatants were dialyzed against 0.01 M potassium phosphate buffer at 4 °C for 15 hr. Nucleoside phosphorylase activities in cell-free extracts of *Er. carotovora*, *E. coli*, and *A. aerogenes* were 33, 29, and 26 munits/mg protein for adenosine, and 40, 32, and 33 munits/mg protein for inosine, respectively. (iii) Partially purified enzymes: A cell-free extract of *Er. carotovora* (50,800 mg protein) was fractionated with ammonium sulfate (35~75 % saturation). The precipitate obtained by centrifugation was dissolved in 0.01 M potassium phosphate buffer (pH 7.0) and then dialyzed against the same buffer. After insoluble materials were removed, the enzyme solution (8,770 mg protein) was put on a DEAE-cellulose column (3.2 x 45 cm) previously equilibrated with 0.01 M potassium phosphate buffer (pH 7.0), and then eluted with an increasing NaCl concentration in the buffer. Adenosine-, inosine-, and uridine-phosphorylase activities were all found in the same fractions, *i. e.*, in the buffer containing 0.3 M NaCl. Cytidine phosphorylase activity was not found in any fraction, as was reported for other bacteria (11~13). The active fractions were pooled, followed by precipitation with ammonium sulfate (80 % saturation). The precipitate was dissolved in 0.01 M potassium phosphate buffer (pH 7.0) and then dialyzed against the same

buffer. The specific activities (units/mg protein) of adenosine-, inosine-, and uridine-phosphorylases were, respectively, 26, 28, and 17-fold higher than those in the cell-free extract. Adenosine- and cytidine-deaminating enzymes that occurred in the cell-free extracts could not be detected in the final preparation. The enzyme preparation (1,020 mg protein) was stored at -20 °C.

The phosphorylases in the preparation were stable at high temperatures, as were the enzymes of other organisms (13~15): adenosine-, inosine-, and uridine-phosphorylases showed 80 %, 50 %, and 80 % of their initial activities, respectively, after incubation at 60 °C for 30 min in the presence of 10 mM each corresponding nucleoside. The optimum pH for the adenosine-, inosine-, and uridine-phosphorylase reaction were 6.5, 9.0, and 7.0, respectively. The transribosylation between adenine and other nucleoside by the enzyme preparation proceeded smoothly at around pH 7.0. (iv) Immobilized enzyme : In 4 ml of the partially purified enzyme solution (12 mg protein, 10 units as adenosine phosphorylase), 750 mg of acrylamide, 40 mg of *NN'*-methylenebisacrylamide, 0.5 ml of 5 % β -dimethylaminopropionitrile, 0.5 ml of 1 % $K_2S_2O_3$, and 100 mg of bovine serum albumin were dissolved and mixed. This mixture was immediately poured into 4~5 glass tubes (5 mm x 6 cm) and left at room temperature for 1 hr (16). The resulting gel rods were sliced (1 mm thickness). The gel pieces were washed with 0.01 M potassium phosphate buffer (pH 7.0) and then stored in the same buffer at 4 °C. The adenosine-, inosine-, and uridine-phosphorylase activities were 0.16, 0.22, and 0.45 units/g of gel (23 %, 24 %, and 32 % recoveries), respectively.

Enzyme assay.

Nucleoside phosphorylase activity was measured by the amount of nucleic base released in the mixture containing 10 mM nucleoside and 50 mM potassium phosphate buffer (pH 7.0). After incubation at 30 °C, the reaction was stopped by the addition of trichloroacetic acid, and the deproteinized supernatant was used for the measurement of the base that appeared. One unit of nucleoside phosphorylase is defined as the amount which releases 1 μ mol of each nucleic base per min.

Analytical methods.

Nucleic acid-related compounds in the mixture for ATP production were measured spectrophotometrically after paper chromatography with a solvent system of *iso*-butyric acid-0.5 N NH_4OH (5:3, pH 3.6) or 95 % ethanol-1 M ammonium acetate (7.5:3, pH 7.4) (17, 18).

Nucleic bases in the assay mixture for phosphorylase activity were measured by high performance liquid chromatography, which was done on a μ Bondapak C_{18} column (4 mm x 30 cm) with a solvent system of 0.007 M K_2HPO_4 (pH 7.8)-80 % (v/v) methanol (9:1) at a flow rate of 1.5 ml/min (19) (M6000A pump; U6K injector; M440 UV detector, fixed wavelength at 254 nm; Waters Assoc., Milford, MA, USA).

Protein was measured by Lowry's method (20). FDP and glucose were measured by the methods of Roe *et al.* (21) and Somogyi (22), respectively.

Results and Discussion

Production of ATP with FDP as an energy source.

The production of ATP from adenine and uridine was examined using the cell-free extract of *Er. carotovora* and baker's yeast cells. Figures 2A, 2B, and 2C show that a combination of the transribosylation and the alcoholic fermentation was indispensable for ATP formation. During 8 hr of incubation, 25 mM ATP was produced concomitantly with the decrease in FDP (Fig. 2A). Further incubation did not cause any increase in ATP production due to depletion of the energy source. Preincubation for transribosylation without the sugar fermentation system was more effective for increasing the ATP yield (Fig 2D, about 35 mM), but preincubation for more than 4 hr was worse because adenosine produced from adenine by transribosylation was deaminated to inosine before phosphorylation to adenine nucleotides (data not shown).

The total adenine nucleotides (ATP + ADP + AMP) produced in these experiments (Figs. 2A and 2D) amounted to 65 mM in 28 hr, which indicated that 87 % of the added adenine was transribosylated during the fermentation. This was much more than that in the mixture without yeast cells (in Fig. 2B, adenosine plus inosine that was formed from adenosine by deamination amounting to 25 mM, in 33 % yield on adenine in 24 hr; another product being 17 mM hypoxanthine), suggesting that the coupled reaction of nucleoside phosphorylases with yeast fermentation led to more effective ribosylation of adenine due to the rapid extraction of adenosine by conversion to adenine nucleotides through the fermentation.

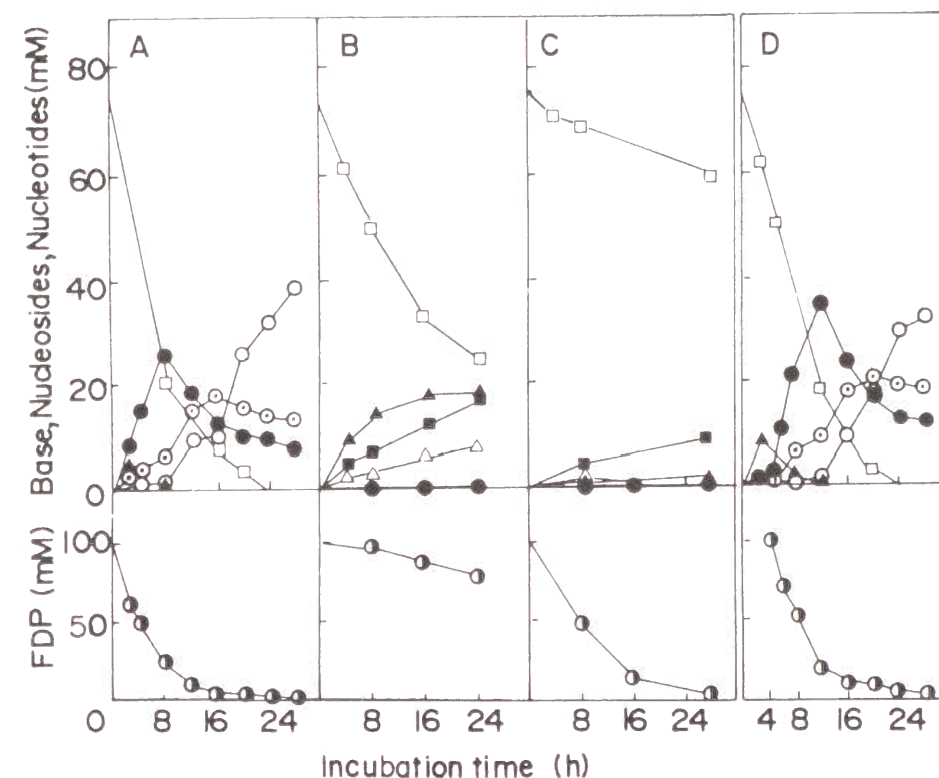


Fig. 2. Production of ATP with FDP as an energy source.

(A) The reaction mixture contained 75 mM adenine, 100 mM uridine, 600 mM potassium phosphate (pH 7.0), 100 mM FDP, 10 mM $MgSO_4$, 10 mg protein/ml cell-free extract of *Er. carotovora*, and 25 mg/ml baker's yeast cells. (B) Baker's yeast cells were omitted from the mixture in (A). (C) Cell-free extract of *Er. carotovora* was omitted from the mixture in (A). (D) The mixture in (A) without baker's yeast cells and FDP was incubated for 4 hr, then the reaction was started by their addition. FDP consumption in the reaction mixture is shown underneath each Figure.

Symbols: ATP (●), ADP (⊙), AMP (○), adenosine (▲), inosine (△), adenine (□), hypoxanthine (■), FDP (◐).

Production of ATP with glucose as an energy source.

As shown in Fig. 3, the production of ATP with glucose as an energy source occurred after temporary accumulation of FDP. ATP production and FDP accumulation were greatly influenced by the concentration of inorganic phosphate. In the mixture with 300 mM phosphate, the accumulated FDP disappeared rapidly, but only gradually disappeared in the mixture with 600 mM phosphate.

The changes in glucose consumption, FDP metabolism (rise and fall), and ATP accumulation suggested that the sugar fermentation in the mixture with 300 mM phosphate proceeded too much faster than the ribosylation of adenine, and eventually the energy released from glucose was not used effectively for the phosphorylation of adenosine. Presumably repression of the sugar fermentation by 600 mM phosphate might be advantageous for the transribosylation and phosphorylation. Table I summarizes the ATP production in mixtures containing various concentrations of phosphate and glucose. The data indicate the importance of activity balance of sugar fermentation and transribosylation. The results also indicated that too great an increase in the concentration of glucose as an energy source delayed ATP formation. The total amounts of adenine nucleotides produced in 24 hr in the mixture with 800 mM glucose were less than those in the mixtures with 400~600 mM glucose. Further incubation of these mixtures with 800 mM glucose resulted in increases in the nucleotides, where considerable amounts of FDP, which is more effective as an energy source (see, Fig. 2), still existed.

ATP production from various ribosyl donors using cell-free extracts of bacteria.

As shown in Table II, ATP production was compared in

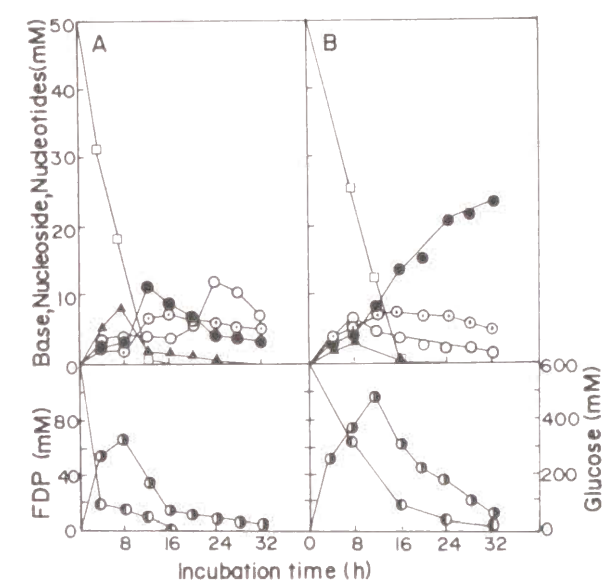


Fig. 3. Production of ATP with glucose as an energy source.

The reaction mixture contained 50 mM adenine, 75 mM uridine, 600 mM glucose, 10 mM $MgSO_4$, 10 mg protein/ml cell-free extract of *Er. carotovora*, 25 mg/ml baker's yeast cells, and 300 (A) or 600 mM (B) potassium phosphate (pH 7.0). Consumption of glucose, formation of FDP, and its decrease in the reaction mixture are shown underneath each Figure.

Symbols: ATP (●), ADP (○), AMP (□), Adenosine (▲), adenine (□), glucose (●), FDP (●).

TABLE I. Effects of glucose and phosphate concentrations on ATP production

Glucose (mM)	Products (mM)	Potassium phosphate (mM)					
		400		500		600	
		24 h	30 h	24 h	30 h	24 h	30 h
400	AMP	14.7	28.9	4.2	30.8	1.6	21.6
	ADP	20.2	5.5	15.1	10.7	12.2	15.1
	ATP	16.8	9.4	28.6	7.5	31.6	10.7
	FDP	6.3	0	4.6	0	13.9	1.7
600	AMP	6.0	22.8	2.7	5.1	5.6	6.1
	ADP	15.2	12.1	11.7	13.6	14.4	15.8
	ATP	20.9	8.1	25.4	22.6	18.4	26.4
	FDP	13.9	2.9	18.5	6.1	38.8	6.1
800	AMP			7.4	5.8	6.5	5.3
	ADP			6.2	12.4	6.1	13.5
	ATP			12.5	17.8	7.8	21.4
	FDP			63.8	22.7	54.1	23.3

The reaction mixture contained 75 mM adenine, 100 mM uridine, 10 mM $MgSO_4$, 9.2 mg protein/ml cell-free extract of *Er. carotovora*, 25 mg/ml baker's yeast cells, and the indicated concentration of potassium phosphate (pH 7.0) and glucose.

mixtures with several bacterial cell-free extracts as nucleoside phosphorylase preparations, and with inosine, uridine, or cytidine as the ribosyl donor.

Inosine or cytidine could be substituted for uridine in the mixture with the *Er. carotovora* preparation. Cytidine was ascertained to be converted to uridine by a deaminating enzyme in the cell-free extract. Though cytidine phosphorylase is not present in *Er. carotovora* (see Materials and Methods), cytidine might be effective as a ribosyl donor due to the coupled reaction of cytidine deaminating enzyme and uridine phosphorylase in the crude extract. On 8~16 hr of incubation, 35 mM ATP was formed with inosine or uridine as a ribosyl donor, and 30 mM ATP with cytidine.

The cell-free extract of *A. aerogenes* or *E. coli* was less effective for production of ATP as well as total adenine nucleotides than that of *Er. carotovora*.

ATP production with various phosphorylase preparations.

The ATP production with various kinds of enzyme preparations derived from *Er. carotovora* is summarized in Table III. Increasing the amounts of phosphorylase preparations increased the ATP yields. Phosphorylases in immobilized preparations were stabilized with bovine serum albumin (see Materials and Methods) and the residual activities after 4 times of use in ATP production were about 80 % (data not shown), suggesting the feasibility of industrial use of the bacterial preparations.

Adenosine production by transribosylation with intact cells of *Er. carotovora* at high temperature.

Table IV shows adenosine production through trans-

TABLE II. Production of adenine nucleotides from various ribosyl donors with cell-free extracts of bacteria

Enzyme preparation	Incubation (h)	Ribosyl donor								
		Uridine			Inosine			Cytidine		
		Nucleotides formed (mM)			Nucleotides formed (mM)			Nucleotides formed (mM)		
		AMP	ADP	ATP	AMP	ADP	ATP	AMP	ADP	ATP
<i>Er. carotovora</i>	8	1.8	8.6	34.8	1.9	8.8	21.3	0.9	5.2	26.2
	16	18.1	21.2	17.2	4.3	21.8	35.2	1.3	12.7	30.2
	24	32.3	18.8	12.7	18.9	18.6	26.4	15.1	20.5	17.3
<i>E. coli</i>	8	1.7	9.3	20.6	1.1	4.2	22.7	1.2	5.8	15.4
	16	0.9	10.4	30.8	1.3	8.6	18.5	3.0	7.6	13.8
	24	14.3	15.7	15.2	5.8	11.3	14.6	1.4	3.7	22.4
<i>A. aerogenes</i>	8	0.8	4.3	13.9	1.7	7.9	23.6	0.8	2.3	14.7
	16	1.2	3.1	19.6	1.2	10.4	32.1	1.1	3.3	18.2
	24	10.7	7.9	6.2	1.9	9.8	31.2	6.5	18.3	6.0

The reaction mixture contained 75 mM adenine, 100 mM nucleoside, 600 mM potassium phosphate (pH 7.0), 100 mM FDP, 10 mM MgSO₄, 10 mg protein/ml bacterial cell-free extract, and 25 mg/ml baker's yeast cells. Preincubation without baker's yeast cells was done for 4 h at 28°C.

TABLE III. Production of ATP with various kinds of enzyme preparations as phosphorylases derived from *Er. carotovora*

Enzyme preparation (units/ml)	Nucleotides formed (mM)			Total
	AMP	ADP	ATP	
Expt. 1 Cell-free extract				
0.015	1.5	2.7	4.5	8.7
0.05	3.1	8.9	18.7	30.7
0.1	6.1	15.8	26.4	48.3
Expt. 2 Partially purified enzyme				
0.018	1.9	4.2	15.6	21.7
0.048	2.6	6.3	21.6	30.5
0.17	1.4	8.3	31.4	41.1
0.43	2.0	9.1	37.0	48.1
Expt. 3 Immobilized enzyme				
0.011	1.9	6.5	23.5	31.9
0.022	2.6	7.6	35.6	45.9
0.045	2.1	10.4	55.4	67.9

The reaction mixture contained 75 mM adenine, 100 mM uridine, 600 mM glucose, 600 mM potassium phosphate (pH 7.0), 10 mM MgSO₄, 25 mg/ml baker's yeast cells, and the indicated amounts of the enzyme preparations. Incubation at 28°C for: 24 h in Expt. 1; 48 h in Expt. 2; 48 h in Expt. 3.

ribosylation between adenine and inosine at a high temperature, 60 °C. Interesting points are (i) the phosphorylase was very stable at 60 °C (see Materials and Methods) and the reaction rate was accelerated greatly at the high temperature. Further, adenosine-deaminating and hypoxanthine-forming enzymes (see Fig. 2B) might be inactivated at rather high temperatures. (ii) Larger amounts of adenine could be used due to the increased solubility at the high temperature. According to preliminary experiment by the author, the solubility of adenine may be 8.2 mM at 28 °C, 28.3 mM at 45 °C, 33.6 mM at 50 °C, 40.0 mM at 55 °C, 45.5 mM at 60 °C, 56.5 mM at 65 °C. (iii) 112~198 mM (30~53 mg/ml) adenosine were produced from 150~300 mM (20~40 mg/ml) adenine at 60 °C, but only 18 mM (5 mg/ml) adenosine was produced from 75 mM (10 mg/ml) adenine at 28 °C (see Fig. 2B).

Production of ATP through a two-step reaction with Er. carotovora and baker's yeast cells with a temperature shift.

ATP production through a two-step reaction was examined as shown in Fig. 4. The reaction consisted of, first, trans-ribosylation between adenine and inosine by intact cells of *Er. carotovora* at 60 °C and, second, phosphorylation of adenosine by dried baker's yeast cells with glucose as an energy source at 28 °C.

On the addition of dried yeast cells, glucose, and MgSO_4 to the transribosylation mixture, 98 mM ATP (about 50 g/L) were produced at a yield of 65 % from 150 mM adenine in 7 hr of fermentation at 28 °C.

This method might be very useful for ATP production because of the simple procedure for preparing bacterial phosphorylases as intact cells without purification, the increased solubility of

TABLE IV. Production of adenosine by transribosylation with intact cells of *Er. carotovora* at 60°C

Substrate (mM)		Incubation time (h)	Adenosine formed (mM)	Yield from adenine (%)
Adenine	Inosine			
8	10	0.5	4.3	50
16	20	0.5-1	9.1	57
30	40	2	19.0	63
50	70	6	34.0	68
75	100	8	50.0	68
150	200	12-16	112.0	75
300	400	24-28	198.0	66

The reaction mixture contained 4 mg/ml *Er. carotovora* cells, 100 mM potassium phosphate (pH 7.0), and the amounts of adenine and inosine indicated in the Table. Incubation was at 60°C.

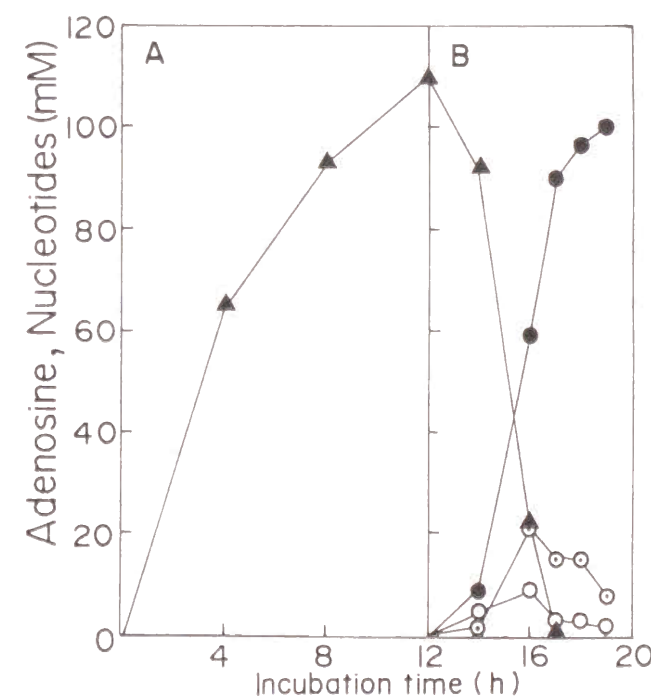


FIG. 4. Established ATP production system through a two-step reaction under temperature shift. (A) Transribosylation was done at 60°C for 12 h in a mixture containing 150 mM adenine, 200 mM inosine, 600 mM potassium phosphate (pH 7.0), and 4 mg/ml intact cells of *Er. carotovora*. (B) Phosphorylation was started at 28°C by adding 600 mM glucose, 10 mM MgSO_4 , and 100 mg/ml baker's yeast to the above mixture (A). Symbols: ATP (●), ADP (◐), AMP (○), adenosine (▲).

adenine, one of the initial substrates, the acceleration of transribosylation velocity, and the inactivation of enzymes deaminating adenosine produced by transribosylation at high temperatures.

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Summary

A new process for ATP production from adenine was designed involving the use of intact bacterial cells containing nucleoside phosphorylases and dried baker's yeast cells containing an energy generating system: it consists of (I) the formation of adenosine through trans-*N*-ribosylation between adenine and uridine,

cytidine, or inosine catalyzed by the nucleoside phosphorylases and (II) the phosphorylation of adenosine to ATP through alcoholic fermentation of the yeast cells. *Erwinia carotovora* was better than *Escherichia coli* or *Aerobacter aerogenes* as the source of nucleoside phosphorylases. Inosine and uridine were effective as ribosyl donors, and cytidine was also usable, if the reactions were done with cell-free extracts. ATP production was greatly influenced by the concentrations inorganic phosphate and glucose in the mixture. The immobilized phosphorylase preparation was better than the cell-free extract in the ATP production. The nucleoside phosphorylases were stable at 60 °C but adenosine deaminating enzymes were not, and large amounts of adenine could be used due to the increase in solubility at that high temperature. Through a two-step reaction, i. e., transribosylation with intact cells of *Er. carotovora* at 60 °C followed by fermentation with baker's yeast cells at 28 °C, 98 mM ATP (about 50 g/L) was obtained from 150 mM adenine.

CONCLUSIONS

Biomass can be roughly divided into two types: rich (high) quality biomass produced for utilization, and poor (low) quality biomass obtained as by-products (agricultural, stockbreeding and industrial) of biological origin during human activities. In this thesis, the author studied the utilization of the latter type (which had not been carefully studied before) as raw materials in food processing and medicine, as well as the development of new manufacturing techniques of food processing which chiefly use fermentation microorganisms, on the grounds that fermentation microorganisms fit for the production of various fermented foods are already known, that the safety of these microorganisms has been established, and that these poor quality biomasses are natural organic substances which are promising for utilization as foods and feeding-stuffs.

In Section 1 of Chapter I, the author studied the optimum setting for the decomposition and extraction of the protein and sugars which are contained in surplus sludge after wastewater treatment, as regarding their utilization as a microbial cultivation medium. This study revealed that most of the protein and sugars were hydrolyzed and extracted into amino acids and monosaccharide after being heat-treated at 120 °C in the presence of 0.3 N HCl. When these hydrolyzates were used as a cultivation medium after neutralization, they allowed good growth of many microorganisms. In addition, the above-mentioned decomposition and extraction reduced the sludge amount to less than 1/3.

In Section 2 of Chapter I, the author observed that sugars in active sludge take the form of complex carbohydrates and also succeeded in isolating a *Fusarium oxysporum* capable of producing

a new enzyme, α -L-fucosidase, from soil using sludge hydrolyzate. The author then purified this enzyme and examined its properties. This enzyme acted on complex carbohydrates in pork gastric mucin as well as on blood group specific substances in human saliva. Thus, the substrate specificity of this enzyme was different from that of α -L-fucosidase from various microorganisms found already. When cultivated in a glucose medium, this strain produced no α -L-fucosidase. These results indicate that sludge hydrolyzate can be utilized not only as a cultivation medium for microorganisms, but also for the isolation of microorganisms producing specific enzymes and for the induction of enzyme production.

In Section 1 of Chapter II, the author studied glutaminase which was obtained by solid *koji* cultivation using wheat bran (a low-cost by-product from the food processing industry), which strongly participates in the release of glutamic acid, thereby affecting the taste of fermented food. Using *Aspergillus oryzae* MA-27-IM isolated from the commercial seed *koji* for soy sauce production as a strain with a high capacity of producing glutaminase, the purification method of extracellular and intracellular glutaminase and their characterization of this strain was performed. From these results, it was observed that these glutaminases possessed almost the same properties, suggesting their similarity.

Because the above-mentioned *koji* extracts contained not only glutaminase but also some other enzymes useful for fermentation (e. g., proteinase, leucine aminopeptidase(LAP)), wheat bran can be used as a raw material for the production of these enzymes. The residue after extraction can also be used as feeding-stuffs.

In Section 2 of Chapter II, the author described a new

cultivation method called the "soft gel cultivation method". Although solid *koji* cultivation is effective for enzyme production with fungus, it is not suitable for the analysis of the relationship between fungus growth and enzyme production, because the separation of fungus mycelia and cultivation medium is difficult. For this reason, the author designed the "soft gel cultivation method" by which mycelia were grown on a soft agar medium containing bran (0.2~0.4 % agar and 10 % wheat bran). When the production and localization of various enzymes with this cultivation method were compared to those with solid *koji* cultivation method and the liquid cultivation method using *Asp. oryzae* MA-27-125, the activity of glutaminase, LAP, proteinase and α -amylase produced with soft gel cultivation were 61, 59, 29 and 24 % of that with *koji* cultivation method, respectively, and were about twice that with liquid cultivation except for α -amylase which was 70 %. The localization of enzymes varied among different cultivation methods. In the soft gel cultivation method (0.4 % agar), glutaminase and LAP were specifically found in a mycelial mat (whole-mycelial-mat fraction), although these enzymes were found in both extracellular and intracellular fractions with the *koji* and liquid cultivation methods. 55 % and 37 % of proteinase and α -amylase were found in the mycelial mat (whole-mycelial-mat fraction) and remaining portion of these enzyme were released in the medium (extra-mycelial-mat fraction).

In Section 3 of Chapter II, the mycelial mat and medium obtained by soft gel cultivation were subject to treatment with a blender or grinding with a mill, and the fractions obtained (whole-mycelial-mat, inter-mycelial, intramycelial fractions) were compared with the intracellular and extracellular fractions obtained with the solid *koji* cultivation or liquid cultivation

methods as to the type of glutaminase. This analysis revealed that the enzymes Glu-I and Glu-II, which were released out of mycelia by the *koji* cultivation and liquid cultivation methods, were preserved within the mycelial mat. Furthermore, the types of enzymes produced with soft gel cultivation differed from those produced with *koji* cultivation and resembled those produced with liquid cultivation. These results indicated that soft gel cultivation resembles *koji* cultivation in terms fungal growth on solid surfaces and enzyme productivity, and that they resembles liquid cultivation in terms of enzyme types produced.

Soft gel cultivation is useful for the cultivation of fungus for the following reasons: (I) Because mycelia contains many kinds and many quantities of enzymes, and because membrane-bound glutaminase also contributes to glutamic acid release during the manufacture of fermented foods, this method provides an effective means for the preparation of enzymes used in enzymatic manufacture or bioreactors; and (II) Because this method allows the easy separation of a mycelial mat consisting of secreted enzymes and pure mycelia after cultivation, it is useful for the analysis of the relationship between fungus growth and enzyme production, and for the purification of intracellular enzymes. The medium residues can be utilized as feeding-stuffs.

In Section 1 of Chapter III, the author proposed the "sugar-yeast addition method" as a new method for food manufacture. This method was developed to solve the inhibitory effect of NaCl which was added during the manufacture of proteinous fermented foods in conventional methods. With the new method, the sugar source (glucose and starch-amylase) and a strain of yeast with a high sugar fermenting ability (*Saccharomyces cerevisiae*) are added at the start of fermentation;

this method utilized the ethanol produced by the yeast to prevent microbial contamination, as well as the reducing ability of sugar fermentation. When this method was tested, rapid ethanol production occurred by means of the consumption of glucose, resulting in the suppressed growth of the contaminant bacteria. The amount and rate of release of glutamic acid and total amino acids from the raw material were comparable or higher than those for the ethanol addition method (addition of ethanol at the start of fermentation) and were higher than those for the NaCl addition method (conventional method). These results indicate that this method can replace the conventional NaCl addition method in the manufacture of proteinous fermented foods. This method was also applicable to various protein materials.

In Section 2 of Chapter III, the author applied the sugar-yeast addition method for processing and preserving fresh fish and meat and also examined changes in the components and physical properties of the preserved fish and meat between this method and the conventional NaCl addition method (salting method). The oxidative degradation of eicosapentaenoic acid was smaller in this method than in the NaCl addition method, and the rate of loss of fish freshness (represented by K value) was also lower in this method than in the NaCl addition method. In addition, the change in the water holding capacity (a factor associated with food texture) was lower with this new method. Thus, the sugar-yeast addition method proved to be applicable to food preservation and processing.

In Chapter IV, the author discussed the method of conversion method into valuable substances from fish sperm, and adenine and nucleosides (e. g., inosine, uridine, etc.) utilizing the ATP energy that is produced by the yeast alcoholic fermentation.

In Section 1 of Chapter IV, the author dealt with the production of deoxyribonucleoside triphosphates from fish sperm DNA. When nuclease P₁-decomposed sperm DNA was used as a substrate in the presence of yeast as an enzyme source, dATP, dGTP and dCTP were formed, while dTTP was not formed. However, when partially purified dTMP kinase from *Escherichia coli* was added to the reaction mixture, dTTP formation occurred. In this way, a mixture of deoxyribonucleoside triphosphates (76 mM in total after 8 hr incubation) could be obtained from partially purified salmon testes DNA (100mg).

In Section 2 of Chapter IV, the author proposed an ATP production method by which nucleosides and adenine are made into adenosine, utilizing the ribosyl group transfer reaction catalyzed by bacterial nucleoside phosphorylase and the subsequent phosphorylation catalyzed by dried baker's yeast cells. *Erwinia carotovora* was an excellent source of nucleoside phosphorylase. As a ribosyl donor, uridine and inosine were useful, although cytidine was also applicable to reactions where cell-free extracts were used as a nucleoside phosphorylase. This enzyme was stable at high temperatures (60 °C). Therefore, using intact cells of *Erwinia carotovora* (4 mg/ml) as a source of the enzyme, 98 mM ATP (about 50 g/L) was produced from 150 mM adenine by a two-step reaction, *i. e.*, 12 hr transribosylation reaction at 60 °C and subsequent 8 hr phosphorylation reaction at 28 °C in the presence of yeast (100 mg/ml) and glucose. If inosine is used as a ribosyl donor, the produced hypoxanthine can be converted into adenine by chemical synthesis for reutilization as a substrate. Ethanol and CO₂ by-products from this reaction can be also utilized after collection.

This thesis describes the effective utilization of various

kinds of poor quality biomass (*i. e.*, agricultural, stockbreeding and industrial by-products of biological origin), which chiefly use fermentation microorganisms. The biomass examined accounts for only a small percentage of all poor quality biomass available, and the majority of other biomass is wasted on reclaimed land and is burned. From the viewpoints of the environmental pollution by destroying activities of human in ecosystem, the scarcity of natural resources on the earth and the advanced effective utilization of resources, multi-dimensional approaches and studies for utilization of the poor quality biomass are required.

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List of Publications

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- 9) T. Yano, Y. Kusumi, K. Yamamoto, H. Kumagai and T. Tochikura, to be submitted to *Agric. Biol. Chem.*