SUGAR-TOLERANT YEASTS IN FERMENTED EXTRACTS OF HORTICULTURAL CROPS

（園芸生産物の発酵液から分離した耐糖性酵母の研究）

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1997

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A dissertation submitted to
The United Graduate School of Agricultural Sciences, Kagoshima University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in Agricultural Science

United Graduate School for Agricultural Sciences
Kagoshima University
Kagoshima, Japan

1997

Dedicated to my beloved wife, **MOE**

who has been always near me

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Acknowledgements

I am greatly indebted to my supervisor, Professor Dr. Fumio Hashinaga for his invaluable guidance and encouragement in implementing this research work, as well as for all his kind help during my stay in Japan.

I am forever grateful to VEL Co.for its finicial support to my study, as well as for being my guarantor in Japan.

I would like to thank Professor Dr. Ikunosuke Tanabe of Laboratory of Applied Microbiology, Faculty of Agriculture, Kagoshima University, Professor Dr. Yasushi Uchida, Faculty of Agriculture, Saga University for their precious guidances; to Professor Dr. Taizo Sakata of Laboratory of Microbiology, Faculty of Fisheries, Kagoshima University, for his valuable advices and for kind permission of use of laboratory facilities; to Dr. Yuka Watanabe for her kind suggestions and for her help in preparation of photographs and slides.

I also extend my gratitude to my Japanese colleagues, Miss Mariko Kimura, and Mr. Naoki Onizuka, for their cooperation in conducting some experiments; to Mr. Tetsuro Minato for his assistance in GC-MS analyses.

I. GENERAL INTRODUCTION

Sugar-tolerant yeasts can grow in the presence of 40-70 % (w/w) sugar (Lodder, 1970) and are frequently isolated from such high-sugar foods as fruit jam, sugar syrup, juice concentrate, dried fruit, molasses, and honey (Munitis, et al., 1976; Tokuoka et al., 1985; Jermini et al., 1987). It has been regarded that these yeasts cause the spoilage of foods from which they are isolated (Tokuoka et al., 1985; Jermini et al., 1987, Golden and Beuchat, 1992). On the other hand, however, sugar-tolerant yeasts are not merely the undesirable microorganisms.They are osmophillic yeasts that produce polyalcohols and there is a potential of industrial application (Tokuoka et al., 1992; Groleau et al., 1995). This implies that there need to investigate and exploit more of them. Moreover, as suggested by Tokuoka et al. (1985), mechanism of sugar-tolerance of yeasts is less extensively studied than that of salt-tolerance.

Recently in Japan, there are some food products that employ sugar-tolerant yeasts during processing. These foods are variously called "functional foods," "health drinks," "enzyme drinks" or "non-alcoholic fermented vegetable extracts" etc. Exact manufacturing procedures of these foods are not disclosed. However, it is generally assumed that one important step is a slow fermentation of vegetable and/or fruit extracts under high-sugar concentration employing wild sugar-tolerant yeasts, the origin of which are unknown. One major difference of these novel foods from other high-sugar foods used by many researchers as source of isolation of sugar-tolerant yeasts is that yeasts are not contaminants in the finished products but deliberately inoculated during processing. The manufacturers claim that their products possess various health regulating functions such as antimicrobial action.

These food products contain 40-60 % sugar, and some are thought to be unpasteurized. It is considered that sugar-tolerant yeasts might contribute

in some way or other to the unique functions of these novel foods. So far, no work has been reported on such sugar-tolerant yeasts.

The scope of the present study has been laid down as follows:

(1) Isolation and identification of sugar-tolerant yeasts from commercial products of fermented extract of horticultural crops as well as from related products.

(2) Investigation of sugar-tolerance and salt-tolerance of isolated strains and determination of growth characteristics of some selected strains.

(3) Study on the antibacterial activity of isolated yeasts followed by identification of active substance(s).

(4) Study on the production of active metabolite compound(s) by selected strain.

(5) Study on proteolytic enzyme activity of isolated strains followed by isolation and partial characterization of enzyme.

II. ISOLATION, IDENTIFICATION AND CHARACTERIZATION

1. ISOLATION

1 - 1. Source materials

Nine samples of high-sugar fermented vegetable extract from five manufacturers were used for isolation of sugar-tolerant yeasts; five were obtained from the manufacturer and the others were bought at local health-food shops. To keep the confidentiality, the names of the product or the manufacturer were not mentioned; instead, sample codes were used throughout this paper. Sample V-93-1, V-93-4, O-95, C-95, M-95 and S-95 were finished products. Sample V-93-2 was an intermediate product; sample V-93-4 was the inoculum used in fermentation; sample V-93-5 consisted of sugar crystals precipitated at the bottom of fermentation tank. For comparison, homemade fermented ume (Japanese apricot) extract was also used. It was selected as a source of isolation because its processing method is thought to be similar to that of commercial high-sugar fermented extracts of horticulture crops. Honey was also chosen as a source since its sugar content and pH were comparable to those of commercial products. Moreover, slow fermentation of commercial products is considered to be similar to the natural process of honey from nectar by bees. Fresh honey was obtained by pressing a young beehive that was just under construction at the roof-corner of a house in Oita Prefecture, Japan. Under a microscope fresh honey from young beehive showed no sign of microorganisms. It was diluted with a little water and let stand for a few days after which it was used as a source sample for isolation of yeasts.

Sugar content of the samples were determined by a hand refractometer (Atago Co. Ltd., Japan) and pH was measured by Horiba pH meter F-12. Since sample V-93-5 was a cluster of crystals, the sugar content and pH were not determined.

1- 2. Isolation and maintenance

Yeasts were isolated by direct streaking 0.1 mL of source on YM30, YM50 or YM60 agar media (0.5% polypeptone, 0.3% malt extract, 0.3% yeast extract, 2.5% agar; all w/v, with glucose concentration of 30%, 50% or 60% w/w respectively); pH adjusted to 4.0. Plates were incubated at 25°C for five days and the colonies were picked up. Enrichment cultures were also accomplished for one week at 25°C in YM30, YM50 or YM60 broths with the same glucose concentration as in direct streaking. With sample C95, for which no growth of yeast was observed in YM30 enrichment culture, we accomplished enrichment culture again using YM medium containing 2% w/w glucose. Each of the enrichment culture was followed by streaking on the corresponding agar medium. Colonies were plated twice on agar medium of the same glucose composition and pure cultures were maintained on YM agar slants containing 2% w/v glucose at 4°C, recultured every month and subcultured at six-month intervals.

2. IDENTIFICATION

2 - 1. Morphological, biochemical and physiological properties

Morphological, biochemical and physiological tests were carried out as described in "The Yeasts, A Taxonomic study," 3rd ed. (Kreger-van Rij, 1984). Cell sizes were measured after 3-day incubation in malt extract broth at 25°C. Ascospore formation was accomplished on various sporulation media described in the above-mentioned text as well as on YPG-50 agar medium (yeast extract, 0.5%; polypeptone, 1.0%; agar, 2%; all w/v; with 50% w/w glucose; pH unadjusted). Incubation was carried out at 25°C as well as at 20°C. Formation of pseudohyphae was observed on slide culture of potato agar medium. Fermentation tests were done using 2% and 10% sugars.

Assimilation tests were carried out by inoculating 0.1 mL suspension of freshly grown yeast cells (24-30 h incubation at 25°C on YM agar with 2% glucose; pH unadjusted) to each of the assimilation medium, as described by Kreger-van Rij (1984). With organic acids, the stock solutions were adjusted to pH 5.6 by addition of saturated sodium hydroxide solution. All solutions were filter-sterilized. DBB test was carried out after incubating on Sabouraud's 4% glucose-0.5% yeast extract agar plates for three weeks at 25°C. Assimilation and fermentation tests with supplementary compounds were done as described in "Yeasts: Characteristics and identification," 2nd ed. (Barnett et al., 1990a). Keys of both references were used for identification.

2 - 2. Mean molar percentage of guanine plus cytosine of nDNA

Molar composition of guanine plus cytosine of nDNA (mol % G+C) was determined for seven strains. Yeast cells were harvested from YPG broth culture (yeast extract, 0.5%; polypeptone, 1%; glucose, 2%; all in w/v) at the logarithmic phase and the DNA was isolated by the phenol-chloroform method (Takagi, 1994) with slight modifications. The DNA was spooled around a glass rod to eliminate mitochondrial DNA (Tewari, et al., 1966). The DNA base composition was determined by reverse-phase high-performance liquid chromatography (Tamaoka and Komagata, 1984).

3. CHARACTERIZATION

3 - 1. Sugar-tolerance and salt-tolerance tests

For testing sugar-tolerance and salt-tolerance of yeasts, YM agar (yeast extract, 0.5%; malt extract, 0.3%; glucose, 1%; agar, 2%; all in w/v) was used as basal medium. To this, 10 to 60% w/w of glucose or zero to 2.5 M of NaCl

were added. Actively grown cultures on YM agar with 2% w/v glucose were stab-inoculated onto the agar plates with different concentrations of glucose or NaCl. Plates were incubated at 25°C for five days and the size of each colony was measured. Average of three was taken. An osmophilic strain of *Zygosaccharomyces rouxii* N28, that was obtained from the Laboratory of Applied Microbiology, Kagoshima University was employed in sugar- and salt-tolerance tests for comparison. This strain was isolated from miso and it was salt-tolerant (Onishi, 1957).

3 - 2. Growth characteristics of strain V19

Since strain V19 showed strong glucose-tolerance it was selected and determined its growth curve at different glucose concentrations and different pH. Five mL each of YPG medium (yeast extract, 0.5%; polypeptone, 1.0%; both w/v; glucose 5% to 80%, w/w) in 15-mm test tube was inoculated with 0.1 mL of inoculum. The inoculum was a cell suspension in YPG (20% w/v glucose) medium at the logarithmic phase of growth and approximately contained 1.1×10^8 cells per mL. The test tubes were put on a reciprocal shaker and were incubated at 25°C. Growth of yeast was measured by determining the turbidity at 650 nm with Klett-Summerson meter and the results were expressed in arbitrary Klett unit.

3 - 3. Characterization of alcohol dehydrogenase (ADH)

Strain V19 was cultivated in YPG medium containing 20% (w/v) glucose at 25°C in shake culture for 3 days and in still cultures for 7 days. After that, cell- free extracts of yeast were prepared and submitted to polyacrylamide gel electrophoresis as described by Tanabe et al. (1994).

4. RESULTS AND DISCUSSION

4 - 1. Isolation of yeasts

Isolated yeast strains and their corresponding sources are shown in Table 4-1. All source samples were high in sugar content and low in pH.

Thirty-four isolates were obtained from eight sources. Yeasts were isolated from samples V-93-1 to O-95 employing 50% and 60% (w/w) glucose media. With other samples, since total counts were low (data not shown), 30% and 50% (w/w) glucose media were employed; with sample S-95 and H-95 direct streaking was not carried out. Since there was no growth in enrichment culture of sample C-95 containing 30 or 50%(w/w) glucose, isolation medium containing 2% glucose was used. Yeasts were not isolated from samples V-93-3, O-95, and M-95 even though isolation was carried out using 2% glucose medium with some sources. The samples were considered to be sterilized.

Table 4-1. Sources and isolated yeast strains [a]

Source sample	Properties of sample		Direct streak method			Enrichment culture method				No. of isolates
			% glucose, w/w			% glucose, w/w				
	%sugar	pH	30%	50%	60%	2%	30%	50%	60%	
(a) Commercial high-sugar fermented vegetable extract										
V-93-1	61	4.23	NT	NT	V14	NT	NT	V1,V2,V3	V13	5
V-93-2	49	4.25	NT	V16,V22	V15	NT	NT	V4,V5	NT	5
V-93-3	52	4.31	NT	ng	ng	NT	ng	ng	ng	0
V-93-4	61	4.47	NT	V24,V25	V17,V18	NT	NT	V6,V7	ng	6
V-93-5	NT	NT	NT	V9,V10	NT	NT	NT	NT	V19,V20	4
O-95	57	3.28	ng	ng	NT	NT	ng	ng	ng	0
C-95	67	3.08	ng	ng	NT	C1	ng	ng	NT	1
M-95	33	3.83	ng	ng	NT	ng	ng	ng	NT	0
S-95	63.2	4.31	NT	NT	NT	S1	NT	S2	NT	2
(b) Local fermented ume (Japanese apricot) extract										
P-95	48.4	2.79	P2, P3, P4	P1, P1-A	NT	NT	P5, P6	P7	NT	8
(c) Local honey, diluted										
H-95	70	2.82	NT	NT	NT	NT	H1, H2	H3	NT	3
Total			3	8	4	2	4	10	3	34

a) Numeral alphabets are strain numbers of isolated yeasts; ng, not grown; NT, not tested.

4 - 2. Identification of yeasts

Table 4-2 shows the morphological, physiological and biochemical properties of the isolates. In the table the identified species is mentioned.

Zygosaccharomyces rouxii (Boutroux) Yarrow

Thirty isolates including 23 strains were identified as *Z. rouxii*. There were some identical isolates. On the other hand, most of the strains differed each other in some way or other. However, almost all of the strains fit the properties of *Z. rouxii* , which is one of the species that includes versatile range of fermentation and assimilation characteristics. There are discrepancies between the authors Yarrow (1984) and Barnett et al. (1990c). While the former allowed slow assimilation of D-ribose for *Z. rouxii* , the latter did not. Similarly, while the former described that some strains of *Z. rouxii* seldom assimilated succinic acid, the latter did not. Using both references in identification, it was possible to include a wide range of strains of *Z. rouxii.*

In view of fermentation of maltose and sucrose, there are variations among standard type strains of *Z. rouxii* (Barnett et al., 1990c; Yarrow 1984). Some strains ferment maltose or sucrose but the others may not. Such variations among the isolated *Z. rouxii* strains were also observed and accordingly they were divided nominally into four groups based on their fermentation properties so that the variations between each other can be seen easily.

(a) Strains that fermented maltose: Six isolates including five strains belonged to this group. Strains in this group fermented maltose; delayed with most of them. The strains did not ferment sucrose. Cells were oval to ellipsoidal except with strain C1, with which they were globose. Strains P2, P5 and C1 produced well developed, branched pseudohyphae. With all strains, two ascospores per ascus were observed on malt extract agar and

vegetable juice agar medium. Conjugation between separate cells preceeded spore formation in all but strain C1, with which no conjugation was observed. Variations of the strains lay in the differences in assimilation of maltose, trehalose, ethanol, ribitol, D-mannitol, D-glucono-1,5-lactone and D-gluconate and growth in vitamin-free medium. Although strain C1 was isolated on 2% glucose medium, it showed sugar-tolerance up to 60% (w/w) glucose and was included in this group. Strain C1 fermented maltose quickly while the other strains did slowly. It was the only strain in this group which not only grew at 37°C but also did slowly at 40°C (after two weeks). Strain H3 was the only strain that assimilated trehalose; a thin film was observed on the surface of the assimilation medium after one week. Kreger-van Rij (1984) did not include ethanol in the assimilation tests, on the probable presumption that most yeasts assimilate it; however, it was found out that some of the isolated strains did not. This was true also for *Z. rouxii* strains in other groups. Nevertheless, the results were in agreement with those described by Barnett et al. (1990c). All strains in this group grew well on 50% and 60% (w/w) glucose agar media. Representative strain P1-A (MAFF 237552) was registered for future reference at Culture Collection Center of Ministry of Agriculture, Forestry and Fisheries.

(b) Strains that fermented maltose weakly: Eight isolates including six strains belonged to this group. Most strains fit the standard species description. Appreciable amount of gas did not evolve during fermentation of maltose. Frequently, fermentation was not only weak but also delayed. The strains did not ferment sucrose. Cells were ovoidal. With strain S1 and S2, branched, Mycocandida-type pseudohyphae were observed under the cover slip of Dalmau plate. All strains in this group were sporogenous. Ascospores were observed only on vegetable juice agar either incubated at 25°C or at 20°C. Low temperature incubation led to slow formation of ascospores. In all

strains conjugation between the two cells preceeded spore formation. All strains matched the standard species description. In this group variations of the strains lay in the differences in assimilation of sucrose, ethanol, ribitol, xylitol, D-glucono-1,5-lactone and D-gluconate and in growth at 37°C. Strain S1 had weak and delayed urease activity. Dry part of the inoculated slant agar became pink after one week of incubation and the whole surface of the slant culture turned red within two weeks. All strains in this group grew well on 50% and 60% (w/w) glucose agar media. Representative strain S1 (MAFF 237555) was registered for future reference at Culture Collection Center of Ministry of Agriculture, Forestry and Fisheries.

(c) Strains that did not ferment maltose: Fourteen isolates including 10 strains belonged to this group. Strains in this group did not ferment maltose, nor did sucrose either. Most strains fit the standard species description. Cells of strain V1, V24, and V25 were globose; others were ovoidal. Developed pseudohyphae were observed with strain V1, V6, V7, V24, and V25. Strain V2 produced hyphae that resembled true septate hyphae. All but four isolates produced two ascospores per ascus. Like the strains in group (b), ascospore formation was observed only on vegetable juice agar. With strains V6, V7, V17 and V18 spore formation was did not observed. Barnett et al. (1990d) mentioned in "Tables for identifying individual species" that *Z. rouxii* is distinguishable from *Candida bombicola* by presence of ascospores. However, as can be seen from their standard descriptions, there are other differences; while *C. bombicola* ferments raffinose and grows in 0.01% cycloheximide slowly (Barnett et al., 1990 b), *Z. rouxii* does not (Barnett et al., 1990c). On the other hand, Yarrow (1984) stated that imperfect state of *Z. rouxii* is *C. mogii* although the latter does assimilate citric acid (Meyer et al., 1984c) while the former does not (Yarrow, 1984). The results of above-mentioned four strains did not agree with the standard species description

of either *C. bombicola* or *C. mogii* but fit that of *Z. rouxii*. Reported values of G+C content for *C. bombicola* and *C. morgii* were 49.8 mol% (Meyer et al., 1984a) and 46.1 mol% (Meyer et al., 1984c) respectively while that for *Z. rouxii* lay between 39.0 to 41.2 mol% (Yarrow, 1984). G+C content of strains V7 and V18 were 38.6 mol% and 39.8 mol% respectively (Table 4-2). Jermini et al. (1987) reported that out of 41 strains of *Z. rouxii* isolated from high sugar foods only seven strains were sporogenous. Wickerham & Burton (1960) have described that taxonomy of *Z. rouxii* was complicated by the rapidity with which they might lose the ability to sporulate and have reported on heterothallism in *Z. rouxii*. Barnett et al. (1990a) also warned that picking up a single colony in isolation might lead to the selection of asporogenous strain which otherwise may be sporogenous.

In this group, variations of the strains lay in the differences in assimilation of galactose, L-sorbose, sucrose, maltose, ethanol, ribitol, D-glucitol, D-gluconate and succinic acid, growth at 37°C and growth in vitamin-free medium. Strain P7 was the only strain that assimilated succinic acid. Strains V2 and V25 did not assimilate D-glucitol, which did not agree with what described by Barnett et al. (Barnett et al., 1990c); Yarrow (1984) did not mention the assimilation of D-glucitol. All strains in this group grew well on 50% and 60% (w/w) glucose agar media. Representative strain V1 (MAFF 237554) was registered for future reference at Culture Collection Center of Ministry of Agriculture, Forestry and Fisheries.

(d) Strains that fermented sucrose: Two strains belonged to this group. Both matched the standard species description. Strains in this group fermented sucrose but they did not ferment maltose. Cells were ovoidal to ellipsoidal. Pseudohyphae was absent in both strains. Both produced two round ascospores per ascus on YPG agar medium. Characteristics of the two were the same except that strain P1 assimilated ribitol and D-mannitol while

strain P3 did not. Strain P3 grew well on both 50% and 60% (w/w) glucose media, whereas strain P1 grew well only on 50% glucose medium but weakly on 60% glucose medium. Representative strain P1 (MAFF 237553) was registered for future reference at Culture Collection Center of Ministry of Agriculture, Forestry and Fisheries.

***Zygosaccharomyces bailii* (Linder) Guilliermond**

Strain P6 almost fit the standard species description of *Z. bailii* except that it did not assimilate ethylamine·HCl. Rudimentary pseudohyphae was observed. Two oval ascopores per ascus were formed on vegetable juice agar. Strain P6 assimilated raffinose while the strains of *Z. rouxii* did not. It grew well on 1% acetic acid medium agar, which differentiated it from *Z. rouxii*. Growth on 50% (w/w) glucose was delayed and weak; that on 60% (w/w) was absent.

***Torulaspora delbrueckii* (Linder) Linder**

Strain V16 almost matched the standard species description. Cells were ovoidal. Two round ascospores per ascus was observed on vegetable juice agar. Strain V16 fermented glucose and sucrose and it assimilated sucrose, raffinose, ethanol, ribitol and lysine only.

***Candida bombicola* (Spencer, Gorin et Tulloch) Meyer et Yarrow**

Strain H1 almost fit the standard species description. Assimilation of D-ribose and growth at 37°C of strain H1 did not match what described by Meyer et al. (1984a), but agreed with species description of Barnett et al. (1990b). However, strain H1 assimilated succinic acid only weakly while both authors described slow assimilation of it by standard strains.

Table 4-2. Characteristics of isolated yeast strains

Strain	Cell size, μm	Ascospore	Pseudohyphae	Fermentation							
				Glucose	Galactose	Sucrose	Maltose	Lactose	Raffinose	Cellobiose	Inulin
Zygosaccharomyces rouxii											
(a) Maltose-fermenting strains											
P1-A	(2.5-4.8) x (3.8-6)	Oval, 2	R	+	–	–	D	–	–	–	–
P2, P5	(3.8-5) x (5.3-8.8)	Oval, 2	L	+	–	–	D	–	–	–	–
P4	(2.5-5.5) x (4.8-8)	Oval, 2	A	+	–	–	D	–	–	–	–
C1	(5.3-7) x (5.3-7.5)	Round, 2	L	+	–	–	+	–	–	–	–
H3	(3-5.5) x (4.5-7)	Oval, 2	R	+	–	–	D	–	–	–	–
(b) Strains that fermented maltose weakly											
V3	(2.8-8) x (4.5-10)	Oval, 2	A	+	–	–	D,w	–	–	–	–
V9, V19	(2.5-6) x (4.8-6.8)	Oval, 2	R	+	–	–	w	–	–	–	–
V10, V13	(4.5-7) x (5-7.8)	Oval, 2	A	+	–	–	w	–	–	–	–
V20	(2.5-6) x (4.8-6.8)	Oval, 2	R	+	–	–	w	–	–	–	–
S1	(2.5-8) x (3-10.3)	Oval, 2	L	+	–	–	D,w	–	–	–	–
S2	(3-5.5) x (3.5-6)	Oval, 2	L	+	–	–	D,w	–	–	–	–
(c) Strains that did not ferment maltose											
V1	(3.8-7.3) x (3.8-7.3)	Oval, 2	L	+	–	–	–	–	–	–	–
V2	(3.5-6.5) x (5-6.5)	Oval, 2	S	+	–	–	–	–	–	–	–
V4, V5	(3.5-6.3) x (5-7.8)	Oval, 2	A	+	–	–	–	–	–	–	–
V6, V7	(4-7.5) x (4.5-8)	Absent	L	+	–	–	–	–	–	–	–
V14, V15	(3.3-6.5) x (5.5-7)	Oval, 2	A	+	–	–	–	–	–	–	–
V17, V18	(3.8-6) x (4-7.5)	Absent	R	+	–	–	–	–	–	–	–
V22	(3.8-5) x (4.5-7)	Oval, 2	A	+	–	–	–	–	–	–	–
V24	(3.5-5.3) x (3.5-5.3)	Oval, 2	L	+	–	–	–	–	–	–	–
V25	(3-5.5) x (3-5.5)	Oval, 2	L	+	–	–	–	–	–	–	–
P7	(3-7.5) x (3-8.5)	Round, 2	A	+	–	–	–	–	–	–	–
(d) Sucrose-fermenting strains											
P1	(2.5-7.5) x (5-10)	Round, 2	A	+	–	+	–	–	–	–	–
P3	(2-6.5) x (2.5-8.3)	Round, 2	A	+	–	+	–	–	–	–	–
Zygosaccharomyces bailii											
P6	(2.5-4.5) x (4.3-6)	Oval, 2	R	+	–	+	–	–	–	–	–
Torulaspora delbrueckii											
V16	(1.8-3.3) x (3-5)	Round, 2	A	+	–	+	–	–	–	–	–
Candida bombicola											
H1	(2-3.8) x (2.5-5)	Absent	A	+	–	+	–	–	–	–	–
Sporogenous sp.											
H2	(2.5-5.5) x (4.5-7.5)	Round, 2-	L	D	–	–	D	–	–	–	–

+, positive; -, negative; w, weak; D, delayed.

A, absent; R, rudimentary; L, developed; S, septate.

Table 4-2. (Continued).

Strain	Assimilation																			
	Galactose	L-Sorbose	Sucrose	Maltose	Cellobiose	Trehalose	Lactose	Melibiose	Raffinose	Melezitose	Inulin	Soluble starch	D-Xylose	L-Arabinose	D-Arabinose	D-Ribose	L-Rhamnose	Me- -D-glucoside	Salicin	Arbutin
Zygosaccharomyces rouxii																				
(a) Maltose-fermenting strains																				
P1- A	+	–	–	–	–	–	–	–	–	–	w	–	–	–	–	–	–	–	–	–
P2, P5	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P4	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
C1	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
H3	+	–	–	+	–	+	–	–	–	–	w	–	–	–	–	–	–	–	–	–
(b) Strains that fermented maltose weakly																				
V3	w	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
V9, V19	+	–	+	+	–	–	–	–	–	–	–	w	w	w	–	–	–	–	–	–
V10, V13	+	–	+	+	–	–	–	–	–	–	–	w	w	–	–	–	–	–	–	–
V20	+	–	+	+	–	–	–	–	–	–	–	w	w	–	–	–	–	–	–	–
S1	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
S2	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
(c) Strains that did not ferment maltose																				
V1	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
V2	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
V4, V5	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
V6, V7	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
V14, V15	+	–	+	+	–	–	–	–	–	–	–	w	w	w	–	–	–	–	–	–
V17, V18	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
V22	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
V24	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
V25	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
(d) Sucrose-fermenting strains																				
P1	+	–	–	w	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P3	w	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Zygosaccharomyces bailii																				
P6	–	–	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
Torulaspora delbrueckii																				
V16	–	–	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
Candida bombicola																				
H1	+	+	+	–	–	–	–	–	+	–	–	–	–	–	–	+	–	–	–	–
Sporogenous sp.																				
H2	+	–	–	+	–	–	–	–	–	–	w	–	–	–	–	–	–	–	–	–

+, positive; -, negative; w, weak; D, delayed.

Table 4-2. (Continued).

Strain	Assimilation																			
	Ethanol	Glycerol	Erythritol	Ribitol	Xylitol	D-Glucitol	D-Mannitol	Galactitol	myo-Inositol	D-Glucono-1,5-lactone	2-Keto-D-gluconate	D-Gluconate	DL-Lactic acid	Succinic acid	Citric acid	Nitrate	Ethylamine.HCl	Cadaverine.2HCl	Lysine	D-Glucosamine.HCl
Zygosaccharomyces rouxii																				
(a) Maltose-fermenting strains																				
P1-A	–	+	–	–	–	w	–	–	–	–	–	–	–	–	–	–	w	+	+	–
P2, P5	w	+	–	–	–	w	+	–	–	–	–	+	–	–	–	–	w	+	+	–
P4	–	+	–	–	–	w	–	–	–	–	–	+	–	–	–	–	+	+	+	–
C1	+	+	–	+	–	+	+	–	–	+	–	+	–	–	–	–	+	+	+	–
H3	+	+	–	+	–	+	D	–	–	–	–	D	–	–	–	–	+	+	+	–
(b) Strains that fermented maltose weakly																				
V3	–	–	–	–	–	w	–	–	–	–	–	w	–	–	–	–	+	+	+	–
V9, V19	w	+	–	–	–	+	+	–	–	–	–	+	–	–	–	–	+	+	+	–
V10, V13	w	+	–	–	–	+	+	–	–	–	–	+	–	–	–	–	+	+	+	–
V20	+	+	–	–	–	+	+	–	–	–	–	–	–	–	–	–	+	+	+	–
S1	+	+	–	+	–	+	D	–	–	+	–	D	–	–	–	–	+	+	+	–
S2	+	+	–	+	+	+	+	–	–	w	–	+	–	–	–	–	+	+	+	–
(c) Strains that did not ferment maltose																				
V1	–	+	–	+	–	+	+	–	–	–	–	–	–	–	–	–	+	+	+	–
V2	–	+	–	–	–	–	w	–	–	–	–	+	–	–	–	–	w	+	+	–
V4, V5	–	w	–	–	–	+	+	–	–	–	–	+	–	–	–	–	+	+	+	–
V6, V7	+	+	–	–	–	+	+	–	–	–	–	+	–	–	–	–	+	+	+	–
V14, V15	–	+	–	–	–	+	+	–	–	–	–	–	–	–	–	–	+	+	+	–
V17, V18	+	+	–	–	–	+	+	–	–	–	–	+	–	–	–	–	+	+	+	–
V22	–	+	–	–	–	+	+	–	–	–	–	+	–	–	–	–	+	+	+	–
V24	–	+	–	–	–	+	+	–	–	–	–	+	–	–	–	–	+	+	+	–
V25	+	+	–	–	–	–	+	–	–	–	–	D	–	–	–	–	+	+	+	–
P7	–	+	–	–	–	+	D	–	–	–	–	w	–	+	–	–	+	+	+	–
(d) Sucrose-fermenting strains																				
P1	–	+	–	+	–	+	+	–	–	–	–	+	–	–	–	–	w	+	+	–
P3	–	+	–	–	–	w	–	–	–	–	–	+	–	–	–	–	+	+	+	–
Zygosaccharomyces bailii																				
P6	–	+	–	–	–	+	–	–	–	+	–	w	–	–	–	–	–	+	+	–
Torulaspora delbrueckii																				
V16	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Candida bombicola																				
H1	+	+	–	–	–	+	+	–	–	+	–	–	–	w	–	–	w	+	+	–
Sporogenous sp.																				
H2	–	+	–	–	w	+	+	–	–	–	–	+	–	–	–	+	+	+	+	–

Table 2. (Continued).

Strain	Growth at/in																			
	37°C	0.01% Cycloheximide	0.1% Cycloheximide	50% Glucose	60% Glucose	Vitamin free	Biotin free	Inositol free	Niacin free	Pyridoxine free	Thiamin free	1% Acetic acid	DBB test	Urease test	Starch formation	G+C content (mol%)	Sugar-tolerance type	Salt-tolerance type	MAFF Number
Zygosaccharomyces rouxii																			
(a) Maltose-fermenting strains																			
P1A	–	–	–	+	+	–	w	+	+	+	+	–	–	–	–		1	3	237552
P2, P5	–	–	–	+	+	–	w	+	+	+	+	–	–	–	–		1	3	
P4	–	–	–	+	+	–	+	+	+	+	+	–	–	–	–		1	3	
C1	+	–	–	+	+	–	w	+	+	+	+	–	–	–	–	39.5	3	3	
H3	–	–	–	+	+	+	+	+	+	+	+	–	–	–	–		2	3	
(b) Strains that fermented maltose weakly																			
V3	+	–	–	+	+	–	w	+	+	+	+	–	–	–	–		1	2	
V9, V19	w	–	–	+	+	–	w	+	+	+	+	–	–	–	–	40.1 (V19)	1	3	
V10, V13	+	–	–	+	+	–	–	+	+	+	+	–	–	–	–		1	2	
V20	+	–	–	+	+	–	w	+	+	+	+	–	–	–	–		1	3	
S1	–	w	–	+	+	–	w	+	+	+	+	–	–	D	–		1	3	237555
S2	–	–	–	+	+	–	w	+	+	+	+	–	–	–	–		1	3	
(c) Strains that did not ferment maltose																			
V1	+	–	–	+	+	+	w	+	+	+	+	–	–	–	–		2	3	237554
V2	+	–	–	+	+	–	+	+	+	+	+	–	–	–	–		2	3	
V4, V5	+	–	–	+	+	–	+	+	+	+	+	–	–	–	–		2	3	
V6, V7	–	–	–	+	+	–	w	+	+	+	+	–	–	–	–	38.6 (V7)	3	3	
V14, V15	+	w	–	+	+	–	w	+	+	+	+	–	–	–	–		1	3	
V17, V18	–	–	–	+	+	+	w	+	+	+	+	–	–	–	–	39.8 (V18)	1	3	
V22	+	–	–	+	+	–	w	+	+	+	+	–	–	–	–		1	3	
V24	–	–	–	+	+	+	w	+	+	+	+	–	–	–	–		3	3	
V25	–	–	–	+	+	+	w	+	+	+	+	–	–	–	–		3	3	
P7	–	–	–	+	+	–	w	+	+	+	+	–	–	–	–		1	3	
(d) Sucrose-fermenting strains																			
P1	–	–	–	+	w	–	+	+	+	+	+	–	–	–	–	39.2	1	3	237553
P3	–	–	–	+	+	–	+	+	+	+	+	–	–	–	–		1	3	
Zygosaccharomyces bailii																			
P6	–	–	–	D,w	–	–	w	–	+	+	–	+	–	–	–	42.5	3	3	
Torulaspora delbrueckii																			
V16	–	–	–	w	–	–	+	+	+	w	+	–	–	–	–		3	3	
Candida bombicola																			
H1	–	–	–	+	+	–	+	+	+	+	+	–	–	–	–	49.1	3	3	
Sporogenous sp.																			
H2	+	w	–	+	+	+	w	+	+	+	+	–	–	–	–		1	3	

+, positive; -, negative; w, weak; D, delayed.

Strain H2

Strain H2 produced two to four round ascospores on one-month old YM agar. Spores were persistent. Among the standard sporogenous species, strain H2 resembled *Z. rouxii* . However, there were major differences. Strain H2 assimilated potassium nitrate while the standard strains of *Z. rouxii* do not. Moreover, strain H2 formed elaborate pseudohyphae abundantly. The pseudohyphae consisted of long branches and it was bare of blastospores. With *Z. rouxii* , it was reported that pseudohyphae was either absent or rudimentary (Yarrow, 1984). On the other hand, the properties of strain H2 were similar to the standard description of *C. etchellsii,* although there were some differences. It grew well at 37°C and weakly grew at 40°C on YM agar while the standard *C. etchellsii* strains do not (Meyer et al., 1984b). The properties of strain H2 did not resemble the unidentified strains described by Tokuoka et al. (1985), or the four new *Candida* species reported by the same authors (1987). This strain was not identified as belonging to any species reported to date.

4 - 3. Sugar- and salt-tolerance of isolated yeast strains

Types of sugar- and salt- tolerance for each strain are described at the end of Table 4-2. Typical growth patterns of isolates with regard to glucose and NaCl concentrations are shown in Fig. 4-1 and 4-2, respectively. Strains that did not grow well on YM agar but showed their maximum growth at 30 or 40% glucose or at 1.5M NaCl are said to have strong tolerance toward those solutes and are designated to type 1. Strains that grew well on YM agar as well as on 10% or 20% glucose or at 1.0 M NaCl are said to have moderate tolerance and are designated type 2. Strains that showed their maximum growth only at glucose concentration of less than 10% or YM agar (without NaCl) and the growth successively declined with concentration are said to

have weak tolerance and are designated type 3. In Table 4-2, types of sugar- and salt-tolerance of isolated strains are described. Sixteen strains belonged to type 1 (strong); four strains to type 2 (moderate) and the remaining four to type 3 (weak) sugar-tolerance. However, in view of salt-tolerance, of all the isolated strains none belonged to type 1 (strong); only two strains belonged to type 2 (moderate) and remaining all to type 3 (weak). Some of the strains even did not grow at 2M NaCl concentration. Lodder (1970) described that the yeasts capable of developing in high sugar concentrations may be suppressed by high concentrations of sodium chloride. This and present results suggest that sugar-tolerant yeasts are not so tolerant toward salt and that sugar-tolerance and salt-tolerance of yeasts are not necessarily the same aspect as has been discussed by some authors (Jermini et al., 1987; Munitis et al., 1976). On the other hand, *Z. rouxii* N28, which we used for comparison, belonged to type 2 sugar-tolerance and type 1 salt-tolerance. Being isolated from high-salt food miso, it had strong salt-tolerance while it possessed moderate sugar-tolerance.

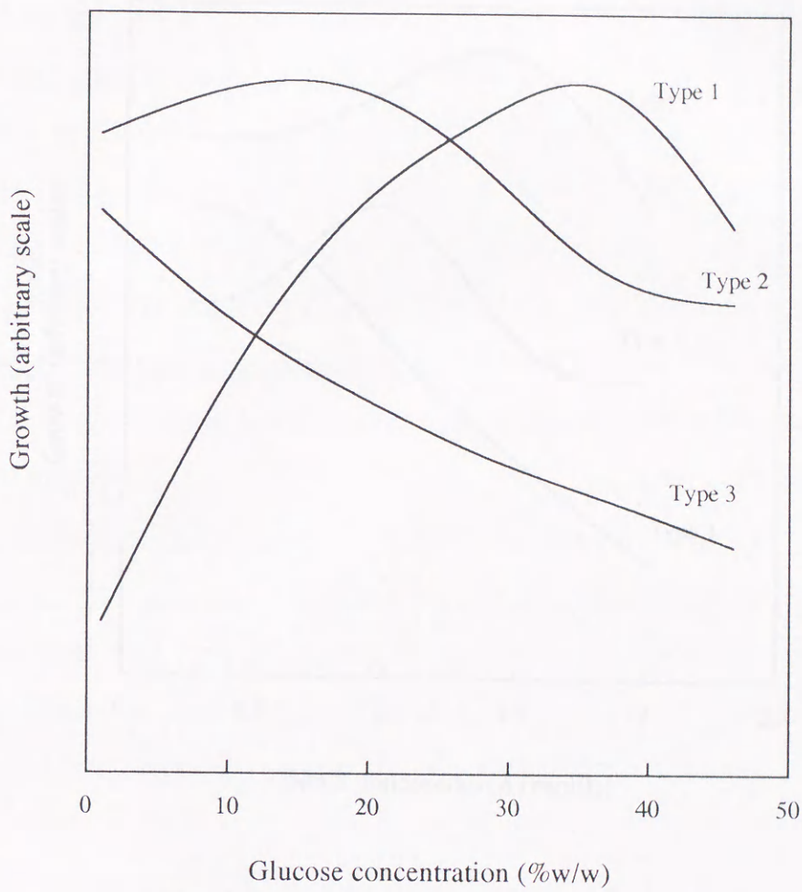


Fig. 4-1. Type of sugar-tolerance

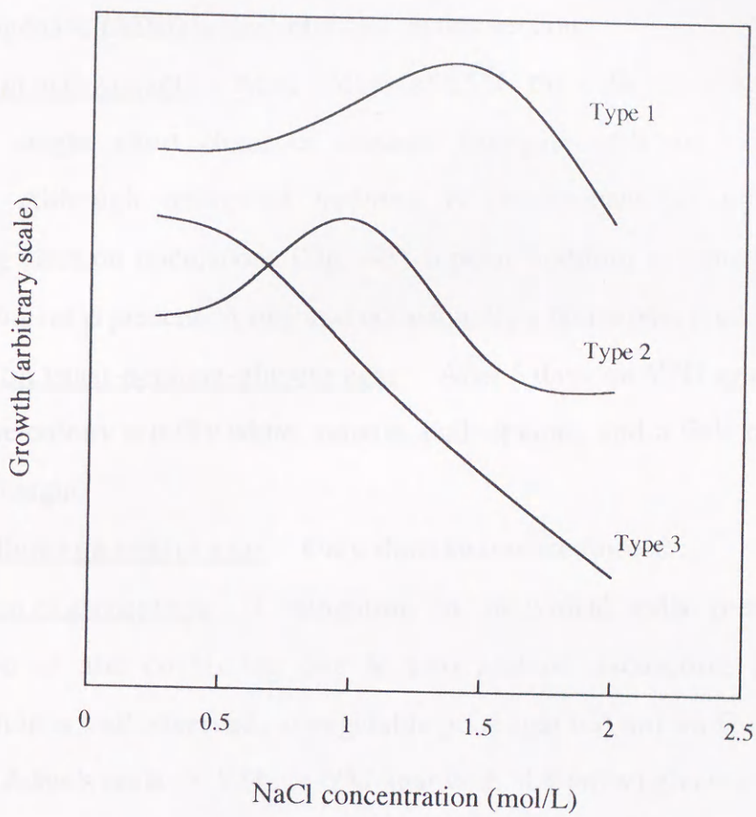


Fig. 4-2. Type of salt-tolerance

4 - 4. Description and characterization of *Z. rouxii* V19

As strain V19 showed the strongest sugar-tolerance and it was the strain that was used for the following studies, which are described in coming sections, its growth as well as sexual behavior are documented and its alcohol dehydrogenase (ADH) is characterized in this section.

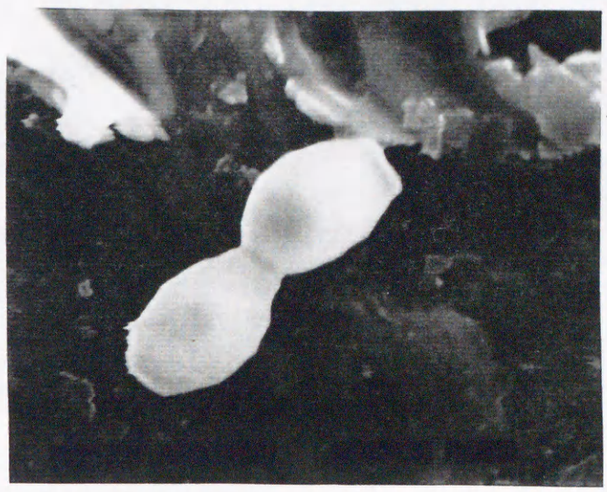
Growth in malt extract: After 3 days at 25°C the cells are subglobose or ovoidal; single, short chain or clusters. Elongate cells are occasionally present. Although multipolar budding is predominant, as observed in scanning electron microscope (Fig. 4-3), bipolar budding is sometimes seen. Thin sediment is present. A ring and occasionally a film is observed.

Growth on yeast-peptone-glucose agar: After 5 days on YPG agar plates at 25°C, the colony is milky white, smooth, dull, opaque, and a little raised with ragged margin.

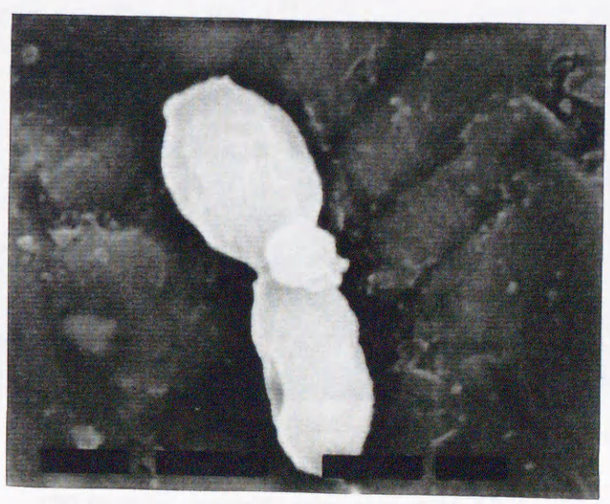
Slide cultures on potato agar: Only short chains are formed.

Formation of ascospores: Conjugation of individual cells preceeds the formation of asci containing one to two globose ascospores (Fig. 4-4). Sporulation is well observed on vegetable juice agar but not on Gorodkowa-, potato-, Adam's acetate-, YM-, or YM-agar with 50%(w/w) glucose.

Yeast ADH: Electrophoretic polyacrylamide gels of ADH of *Z. rouxii* V19 are shown in Fig. 4-5. Both shake culture and still culture result in the same ADH isozyme patterns.

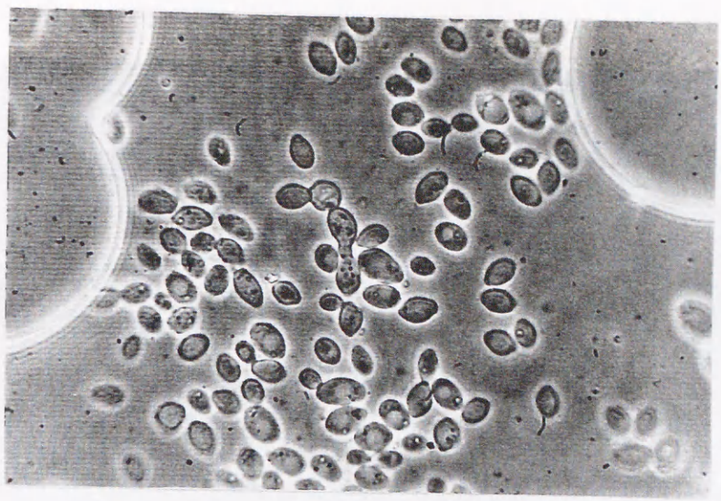


(a) 8,000 X

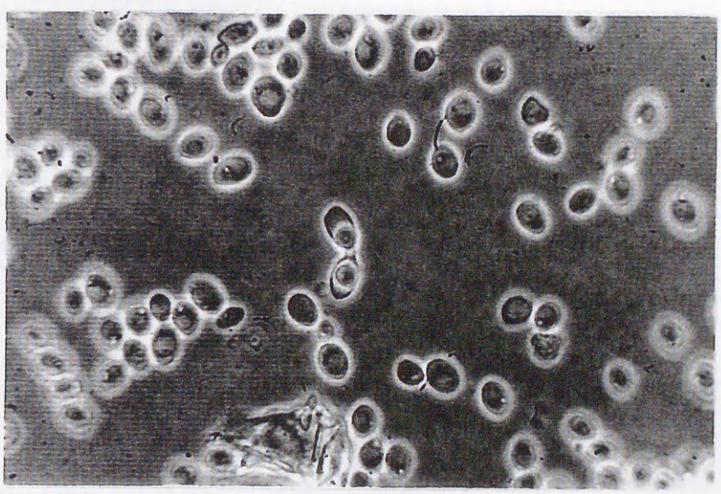


(b) 10,000 X

Fig. 4-3. Scanning electron miscropic photographs of vegetative cells of *Zygosaccharomyces rouxii* strain V19



(a) Conjugating cells
(13 days on vegetable juice agar at 15°C)



(b) Spore-forming cells
(20 days on vegetable juice agar at 15°C)

Fig. 4-4. Conjugation and formation of ascospores in *Z. rouxii* V19 under phase-contrast miscrope (1,500 X)

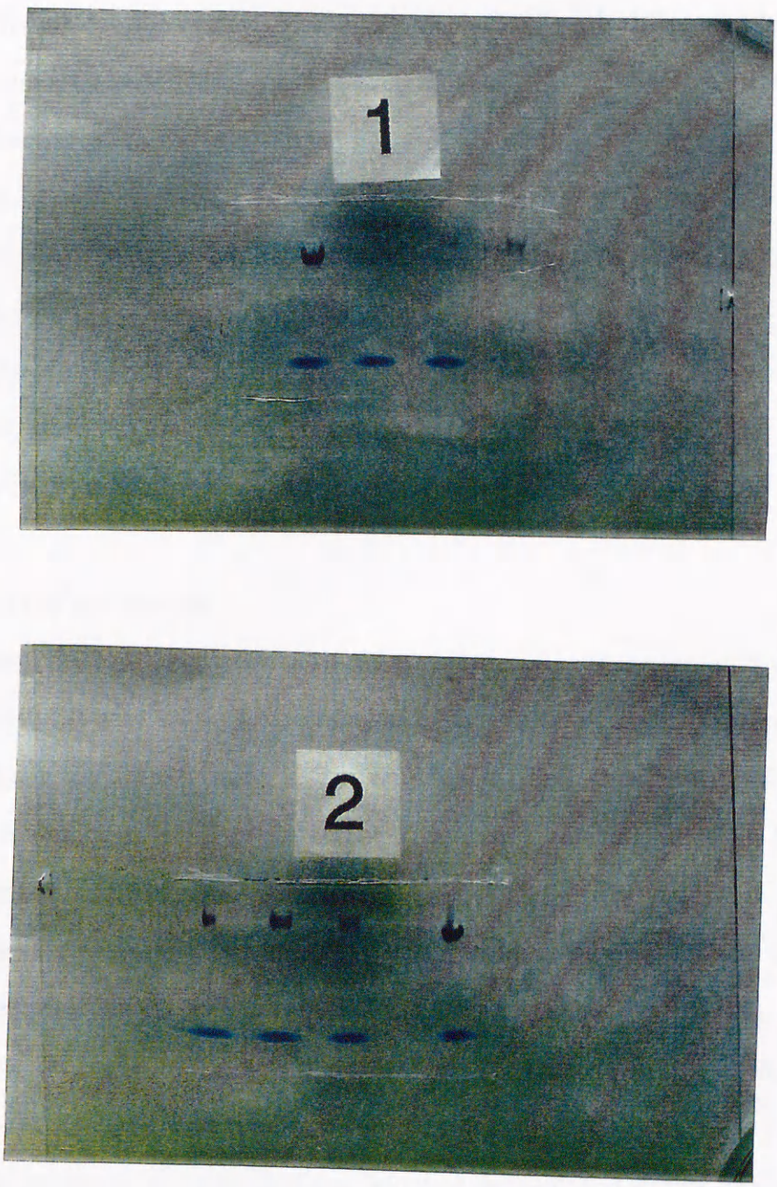


Fig. 4-5. Electrophoretic polyacrylamide gels of the alcohol dehydrogenase of *Z. rouxii* V19

(1): Still culture; (The left most lane, standard ADH; others, sample)

(2): Shake culture; (The right most lane, standard ADH; others, sample)

4 - 5. Growth characteristics of *Z. rouxii* V19 at different conditions

Growth curves of *Z. rouxii* V19 in liquid medium with different glucose concentrations are shown in Fig. 4-6. Maximum growth was observed at 10-20% w/w glucose. It can be seen from Fig.4-3 that V19 grew well until 80% w/w glucose. As discussed by Lodder (1970), there are very few of yeasts that can grow in concentrations between 65-70% glucose. In this sense, strain V19 is one of the rare strains. The fact that this strain was isolated from sugar crystals of fermentation tank may explain its strong sugar-tolerance.

In Fig. 4-7, growth curves of V19 at different pH are shown. It can be seen that strain V19 grew well at pH 2.5 and still grew at pH 2.0.

4 - 6. General discussion

It is noteworthy that a considerable number of strains were isolated using the media with 50 and 60% w/w glucose. The results of identification and characterization indicated that *Z. rouxii* constituted nearly 90% of the isolates. It is clear that various strains of *Z. rouxii* are distributed in high-sugar fermented extracts of horticultural crops. All 23 isolated strains of *Z. rouxii* fermented glucose; some of them fermented maltose or sucrose. There were some strains which did not fit the standard species description. All isolates grew on the media containing 50% (w/w) glucose and all but two grew on 60% (w/w) glucose. At least one strain still grew up to 80% (w/w) glucose concentration in liquid medium. Most of the isolated strains belonged to the strong sugar-tolerance type while they were included in the weak salt-tolerance type. Strong sugar-tolerance of yeasts may be an annoying problem in view of food protection, but this indicates that further study is required to understand the mechanism and to elucidate their role in foods.

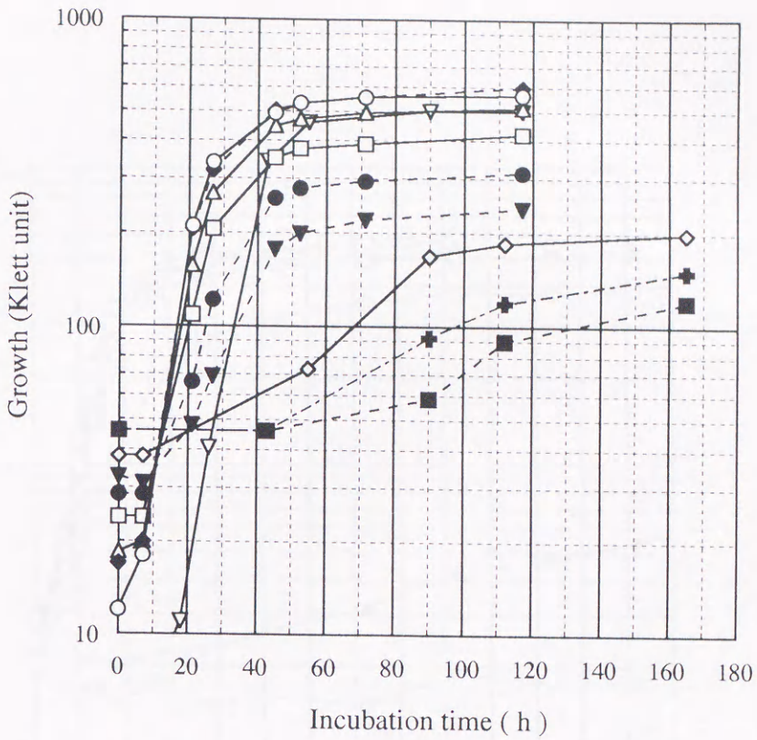


Fig.4-6. Growth curve of strain V19 at different glucose concentrations in YPG medium

5%w/w; 10% ; 20% ; 30% ;
40% ; 50% ; 60% ; 70% ;
75% ; 80%.

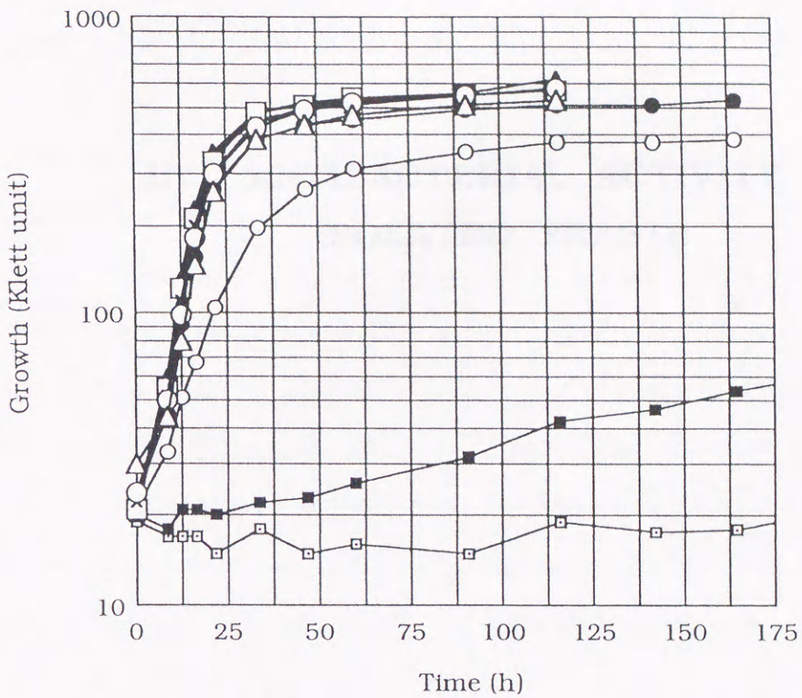


Fig.4-7. Growth curve of V19 at different pH in YPG medium

pH 1.5 pH 2 pH 2.5 pH 3
pH 3.5 pH 4 pH 5 pH 6
pH 7 pH 8

III. ANTIBACTERIAL ACTIVITY OF ISOLATED YEASTS

5. INTRODUCTION

Although there have been many works on the anti-microbial activity of lactic acid bacteria against yeasts, little has been known about the antibacterial activity of yeasts. Cook et al. in 1941 reported that growth of *Escherichia coli* and *Staphylococcus aureus* were greatly depressed by bakers' yeast. However, the authors did not identify the active substanse(s). Sasaki et al. (1984) reported on a strain of *Saccharomyces cerevisiae* that killed beer-spoilage bacteria. In this cace, again, the antibacterial substance produced by the yeast was not revealed. The manufacturers of high-sugar fermented extract of horticulture crops claim that one function of their food products is antibacterial activity. This is partly due to the high concentration of sugar but there may be some factors associated with sugar-tolerant yeasts.

In this section, the antibacterial activity of some isolated strains were detected. The work dealt also with isolation, purification and identification of the substances that contribute to the antibacterial activity.

6. DETECTION OF ANTIBACTERIAL ACTIVITY

6- 1. Bacterial Strains

List of Bacteria used in the antibacterial assay are listed below. Bacteria were maintained on nutrient agar at 4^{o}C except for *Enterococcus faecalis* , which was maintained on one-third concentration of brain heart infusion agar (Difco).

Strains of bacteria used and their sources

Bacterial strain	Source
Escherichia coli B	IFO 13168
Escherichia coli C	IFO 13892
Salmonella enteritidis	IFO 3313
Micrococcus luteus	IFO 12708
Bacillus subtilis	IFO 3009
Staphylococcus aureus	IFO 3761
Enterococcus faecalis	IFO 12580

6 - 2. Detection of antibacterial activity

YM hard agar (2% w/v) plates were overlaid with 3.5 mL of soft YM agar (0.5% w/v) inoculated with 0.1 mL of an overnight culture of the indicator bacteria strain. The agar plates were left for 30 min to solidify. After that the yeast samples were taken from the freshly prepared slant by a platinum wire and inoculated by plunging vertically into the soft agar medium. The agar plates were incubated at 27^{o}C for three days and were subsequently examined for zones of inhibition.

6 - 3. Cultivation and isolation of metabolites

From the results of section 5 - 2, strain V19 that showed the strongest activity was selected and incubated for further investigations. The culture medium consisted of: yeast extract, 5 g; polypeptone, 10 g; glucose, 20 g; water, 1000 mL. One loop from freshly cultured slant was inoculated into 25

mL of the culture medium in a 100 mL-flask. The flask was incubated at 25°C for two days, with gentle shaking once a day. In the morning of third day, one mL of the culture broth was taken and inoculated to 75 mL culture medium of the same composition in Sakaguchi flask. The flask was incubated at 25°C for five days with gentle shaking once a day.

After 5-day incubation, the cultural broth was centrifuged at 7000 x *g* for 15 min at 4°C and the cells were discarded. The supernatant was equally divided into three portions. The pH of each portion was adjusted to 3, 9, and 13 respectively, using 0.1N NaOH and 0.1N HCl. Each portion was extracted three times with equal volume of ethyl acetate. The solvent layers were collected and evaporated under vacuum followed by washing with water. The solution was again evaporated to dryness under vacuum. The residue was dissolved into a minimum amount of water and was used in antibacterial assay.

Some portion of the acidic fraction was dialyzed against 400 fold of distilled water at 4°C, with two changes of water during dialysis. The dialyzed sample was used in antibacterial assay.

Some portion of the acidic fraction was heated at 100°C for 20 min and was used in assay. Another portion of the acidic fraction (original pH, 2.0) was taken, pH adjusted to 6.5, and was used in assay.

6- 4. Antibacterial assay procedure

Antibacterial activity of the various fractions was measured by disc assay method. Assay plates were prepared in the same way as stated in section 5 - 2: "Detection of antibacterial activity of the yeast." After the soft agar had solidified, assay discs (8 mm diameter, Toyo Filter) were dipped in the sample to be tested and left for 15 min to dry. Then the discs were placed

on the seeded lawn of soft agar. The plates were incubated at 37°C for 24 h. Diameter of the clear zones around each disc was measured.

7. IDENTIFICATION OF ACTIVE COMPOUNDS

7 - 1. Partial Purification

The active acidic fraction was ion-exchanged with amberlite IR-120 [H^+] and amberlite IR-45 [OH^-] resins in two connected columns successively. The latter column was eluted with 2N NH_3 solution and the eluate was concentrated under vacuum. After that the sample was partially purified by passing through an ODS column, which was eluted with water. Eluate was collected by a fraction collector. Presence of the required compounds was detected by a UV 160A spectrophotometer (Shimadzu Co., Japan) at 210 nm. Active fractions were combined and evaporated to dryness under vacuum and dissolved into a minimum amount of water.

7 - 2. HPLC analysis

Purified sample was diluted 20 times and filtered through a 0.45 μm cellulose nitrate membrane (Toyo Co., Japan). HPLC analysis was accomplished with Shimadzu LC-10AS liquid chromatograph (Shimadzu Co., Japan), equipped with Toyosoda UV-8 model II spectrophotometer (Toyosoda Co., Japan) and Shimadzu C-R4A Chromatopac integrator (Shimadzu Co., Japan). Analysis was carried out using a Shodex column (500cm x 4.6 mm i.d., packed with Shodex HC-125S resin). The mobile phase was 0.1% (w/w) phosphoric acid. The constant pressure analysis was accomplished with the following conditions: pressure, 38 kg/cm^2;

temperature, 55° C; flow rate, 0.3 mL/min; detector UV 210 nm; injection volume, 1 μL.

7 - 3. Methylation of sample

Purified sample was evaporated to dryness under vacuum and left inside a desiccator for a few days. Into a distillation tube, 50 mg of the dried sample and 1 mL of BF_3 methyl chloride were added and the methylation reaction was carried out at 80°C for 5 min under distillation with total reflux. After the reaction mass had been cooled down to room temperature, saturated NaCl solution (2 mL) and hexane (3 mL) were added. The tube was shaken thoroughly and the two layers were separated using a separating funnel. Some anhydrous sodium sulfate was added to the hexane layer. The dried organic layer was stored at 4°C and was analyzed by GC-MS.

7 - 4. Preparation of TMS esters

Purified and dried sample (10 mg) was taken into a screw-capped sample tube and one mL of pyridine was added. Then 0.1 mL of HMDS and 0.1 mL of TMCS were added subsequently. Esterified sample was stored at 4° C and was analyzed by GC-MS.

7 - 5. GC-MS analysis

GC-MS analyses were performed with a Hitachi M-80B gas chromatograph (Hitachi Co., Japan), equipped with a Chrompack capillary column (CP-Sil SCB, 25 m x 0.25 mm i.d.; 1.2 μm coating), and coupled to a Hitachi M-80B mass selective detector (Hitachi Co., Japan). Ionization potential was 70 eV, with a source temperature of 180°C. We applied the

following conditions: flow rate of the carrier gas (He), 30 mL/min; injector port temperature, 200°C; GC-MS interface temperature, 220°C. With methylated samples, oven temperature was held at 70°C for 20 min, then increased to 200°C at 10 °C/min. With TMS esterified samples, oven temperature was increased from 90°C to 250°C at 5°C/min.

8. RESULTS AND DISCUSSION

8 - 1. Antibacterial activity of isolated yeasts

Antibacterial activity of isolated yeast strains are shown in Table 8-1. In the table only strains that showed some degree of positiveness in the activity test are mentioned. Among these strains, strain V-19 showed the strongest activity against the wide spectrum of tested bacteria and it was selected for further study.

8 - 2. Antibacterial activity of different fractions

In Table 8-2, the activity of various fractions of metabolite extract of strain V-19 are shown. Acidic fraction showed the activity while neutral and basic fractions did not. This fraction was pH-sensitive, heat-resistant and non-dialyzable These results led to the deduction that the active substance(s) was organic acid in nature. The activity became more pronounced during its partial purification. The activity of purified sample with that of citrate buffer and acetate buffer, both at pH 4, was also compared.

Table 8-1. Antibacterial activity of selected isolated yeast strains [a]

Yeast strain	Bacterial strains						
	E.coli B	*E.coli C*	*S.enteritidis*	*M.luteus*	*B.subtilis*	*St.aureus*	*Ent.faecalis*
V1	–	–	–	++	+	+	–
V4	–	tr	–	++	+	–	–
V9	tr	+	tr	+	+	–	–
V13	–	tr	–	+	+	–	–
V15	–	+	–	–	+	–	tr
V19	tr	+	+	++	+	++	tr
V22	–	+	–	+	tr	–	tr
V25	–	+	tr	+	tr	–	–

a Antibacterial activity was detected by inoculating yeast directly to seeded lawn of YM soft agar

++ strongly active; + active; tr, weakly active; – inactive

Table 8-2. Antibacterial activity of various fractions and that of acidic fraction after various treatments [a]

Treatment	Bacterial strains						
	E.coli B	*E.coli C*	*S.enteritidis*	*M.luteus*	*B.subtilis*	*St.aureus*	*Ent.faecalis*
Neutral fraction	–	–	–	–	–	NT	NT
Alkaline fraction	–	–	–	–	–	NT	NT
Acidic fraction							
Before treatment	+	+	+	+	+	NT	NT
After dialysis	NT	–	–	–	–	NT	NT
After heating	NT	+	+	++	+	NT	NT
After adjusting pH to 6.5	NT	–	–	–	–	NT	NT
After ion-exchange	++	+	+	+	+	+	+
After ODS purification	++	++	+	++	++	++	++
Citrate buffer (0.1 M, pH4)	NT	+	+	+	++	NT	NT
Acetate buffer (0.1 M, pH4)	NT	+	tr	+	+	NT	NT

a Antibacterial activity was detected by disc assay method

++ strongly active; + active; tr, weakly active; – inactive; NT, not tested

8 - 3. Identification of organic acids

As can be seen from Fig. 8-1, malic acid, succinic acid, and fumaric acid were identified by HPLC analysis. The results were confirmed by GC-MS analyses, as shown in Fig. 8-2 and Fig. 8-3. Mass spectral data of authentic organic acids and those from attached computer library were used for confirmation. Methylated mixture of succinic acid and fumaric acid was resolved only when it was analyzed at an isothermal temperature of 80^{o}C or less. No peak of malic acid was observed with methylated sample. It was noted that there were still small peaks both in HPLC and GC-MS analyses which were yet to be identified.

8 - 4. Antibacterial activity of organic acids

Organic acids have been known to kill or suppress the growth of bacteria. Yamamoto et al. (1984) reported on the inhibitory activity of succinic acid and malic acid against a wide spectrum of bacteria. In their report, the authors placed succinic acid third, next to acetic acid and lactic acid. They made a correlation between the antibacterial activity of organic acids and the concentration of undissociated molecule. Ikawa (1985) mentioned the antibacterial activity of 0.1% concentration of fumaric acid and succinic acid against 19 strains of bacteria. Recently, Matsuda et al. (1994) reported on the minimum inhibitory concentration of malic acid against bacteria, molds and yeasts.

Mechanism of antimicrobial activity of organic acids may be explained from two aspects: attack of undissociated molecule, and suppressing of pH. In the first aspect, *Z. rouxii* V-19 is said to withstand the attack of organic acid molecules since it is producing these compounds. In the second aspect, it still grows in the medium with pH 2.0 (Fig.4-7 in section 4 - 4). Accordingly, in the antibacterial assays, it grew well while killing the bacteria.

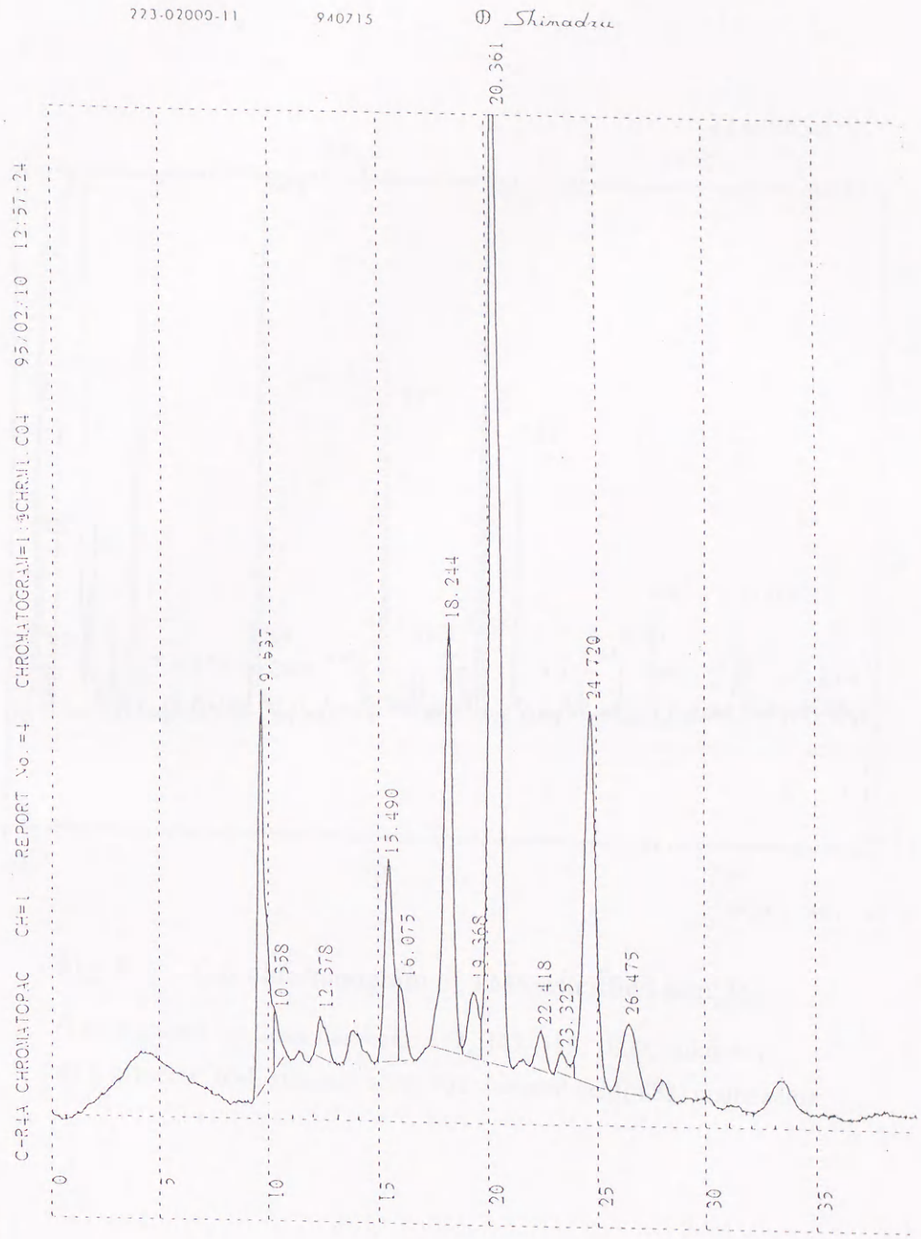


Fig. 8-1. HPLC chromatogram of partially purified acidic fraction
(The numbers are retention times in min: 15.490, L-malic acid; 18.244, succinic acid; 20.361, fumaric acid; 24.720, unknown)

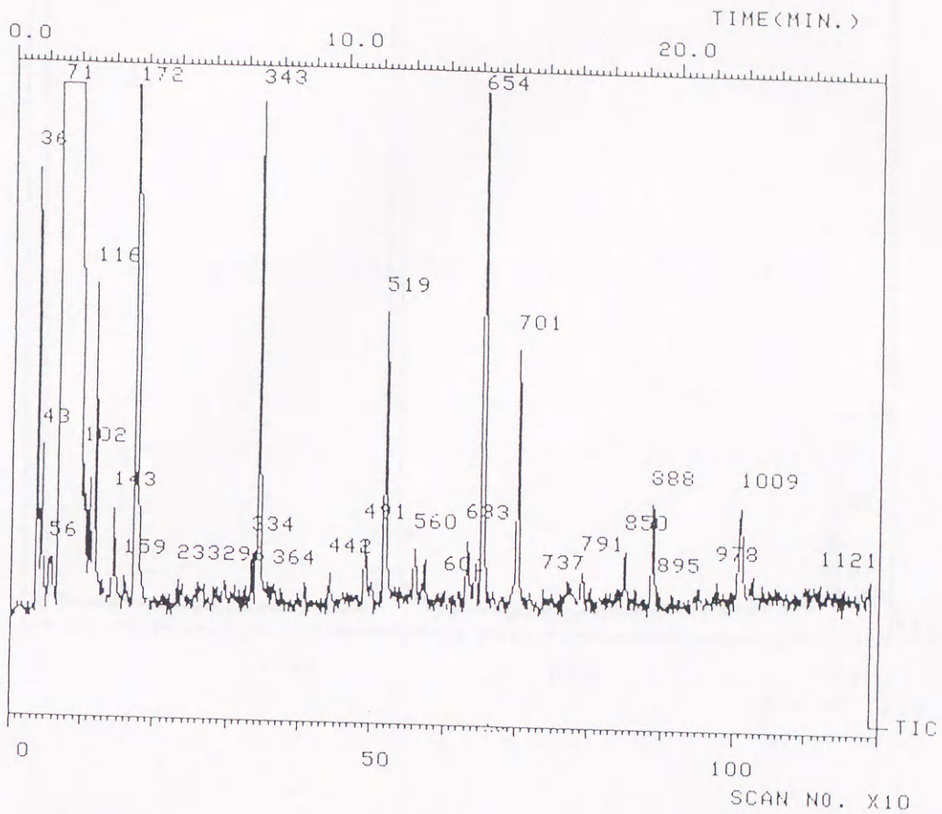


Fig. 8-2. Gas chromatogram of TMS esterified sample

(The numbers are scan numbers: 172, 343, 519, 1009, unknowns; 633, glycerol; 654, succinic acid; 701, fumaric acid; 888, malic acid)

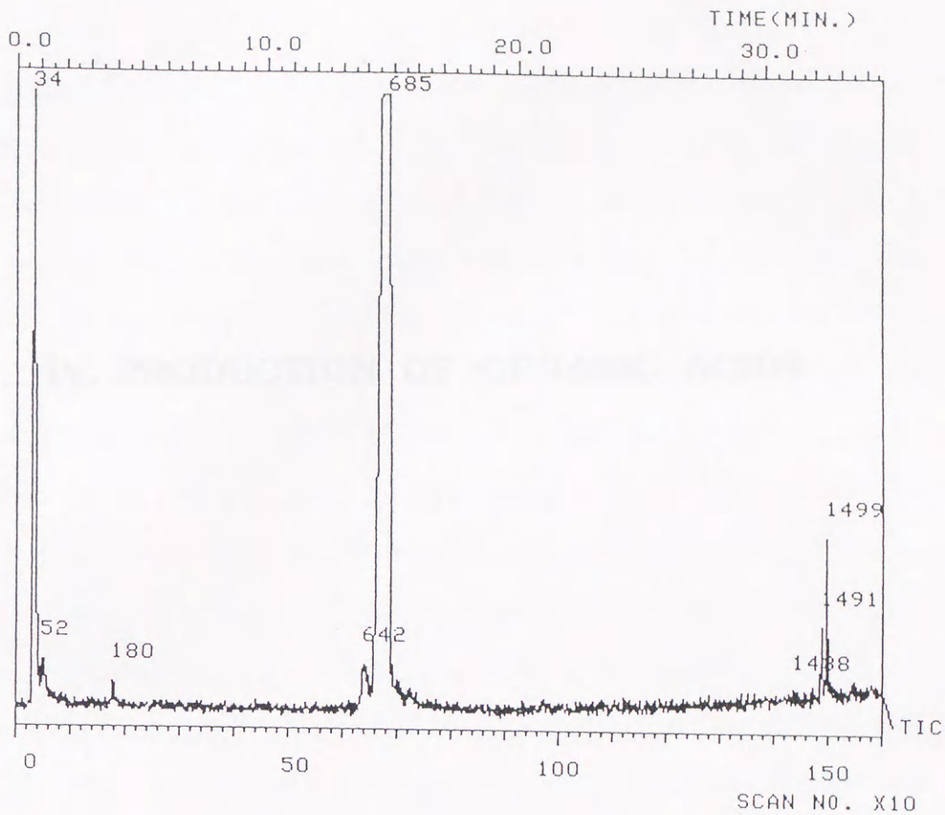


Fig. 8-3. Gas chromatogram of methylated sample

(The numbers are scan numbers: 642, fumaric acid;

685, succinic acid; 1499, unknown)

IV. PRODUCTION OF ORGANIC ACIDS

9. INTRODUCTION

Organic acids are used widely as an acidulant in soft drinks, and as chelating agents in a variety of other products. Acetic, malic, succinic, tartaric and citric acids contribute largely to flavour of yeast-fermented products such as wine, beverages and soy-sauce. Besides from the acids that are derived from the fruits employed as raw materials in wine making, other organic acids are produced during fermentation (Radler, 1993). There are reports on formation of citric acid by *Candida guillermondii* and *C. lipolytica* (Atkinson and Mavituna, 1983), acetic acid by *Zygosaccharomyces rouxii* (Young et al., 1980), L-malic acid by *Saccharomyces cerevisiae* (Schwartz and Radler, 1988), fumaric acid by *C. hydrocarbofumarica* (Yamada et al., 1970), and succinic acid by *S. cerevisiae* (Thoukis, 1964; Herde and Radler, 1978). Formation and possible pathways of some dominant organic acids in yeasts have been investigated by many researchers. Burden and Evenleigh (1990) claimed that yeasts have potential application for the production of a wide range of organic acids. On the other hand, Radler (1993) concluded that quantities of organic acids, especially malic and succinic acids, were such a quite low that industrial application was not practical.

Most of the above-mentioned works concerned with the yeast *S. cerevisiae,* which is the main species employed in wine industry. Apart from it, there are some works on transport of malic, succinic and other dicarboxylic acids in the yeast *Hansenula anomala* (Corte-Real and Leao, 1990), in *Schizosaccharomyces pombe* (Osothsilp and Subden, 1986), and in *S. bailii* (Kuczynski and Radler, 1982). *Zygosacchsaromyces rouxii* is one of the osmotolerant yeasts associated with foods with low water-activity such as

soy-paste, soy-sauce and other high-sugar foods. Although there are many works on production of polyalcohols by *Z. rouxii* (Onishi, 1960; Groleau et al., 1995), little has been known about formation of organic acids by the yeast. Young et al. (1980) reported on the production of acetic acid during anaerobic fermentation from glucose by a soy yeast *Z. rouxii* , which was a salt-tolerant strain. Nowadays, it is generally accepted that salt-tolerance and sugar-tolerance are different aspects of halophillism (Onishi, 1957; Tokuoka et al., 1985; Taing Ok and Hashinaga, 1997). In the previous work on detection of antibacterial action of sugar-tolerant yeasts, described in the above section, it was found out that some strains of *Z. rouxii* produced organic acids in such a considerable amount that bacteria were killed during their co-incubation with these yeast strains (Taing Ok and Hashinaga, 1995). This result and lack of information so far prompted to investigate the formation of organic acids by sugar-tolerant strain of *Z. rouxii* V19. This section deals with study on effects of type of cultural media and cultural conditions on the production of acids were also studied. Effects of precursors were also investigated and their possible metabolic pathway was discussed. Percent yield based on sugar consumption was calculated.

10. MATERIALS AND METHODS

10- 1. Cultural media and incubation

In the first part of the study, YPG10 liquid medium (yeast extract, 0.5; polypeptone, 1.0, glucose 10; all in %w/v; pH adjusted to 6.0) was used as control. YM medium (yeast extract, 0.3; malt extract, 0.3; polypeptone, 0.5; glucose, 10; all in %w/v; pH, 6.0) and Potato medium (peeled potato dice were leached in boiling water for 15 min and filtered through gauze and to

this, 10% w/v of glucose was added; pH, 6.0) were also used for comparison. To determine the effect of added inorganic nitrogen compounds, 3% w/v each of NH_4Cl, NH_4NO_3, $(NH_4)_2HPO_4$ or $(NH_4)_2SO_4$ were added to YPG10 control medium. Glucose in YPG10 medium was replaced with sucrose, galactose, maltose or fructose to investigate the effect of type of sugars. YPG media with different glucose concentrations from 10 to 50% w/v were also employed. To study the effect of initial pH on the production of acids, initial pH of YPG10 medium was varied from 4.0 to 8.0, using 1N HCl or 1N NaOH. To find out the effect of added minerals, 0.1% each of KH_2PO_4, $CaCl_2.2H_2O$, or $MgSO_4.7H_2O$, or 10 ppm each of $ZnSO_4.7H_2O$, $MnSO_4.4H_2O$ or $FeSO_4.7H_2O$ were supplemented to YPG10 medium. Moreover, YPG medium (2% glucose) with added NaCl or KCl (0.5M each) were also employed. Five mL each of the medium was placed in 15-mm test tube fitted with silicon plug. Freshly grown inoculum (0.1 mL), containing approximately 4 x 10^6 cells/mL was inoculated to each of the medium. Incubation was carried out for 140 h at 25°C. To study the effect of incubation temperature, test tubes containing YPG10 medium were placed also at 20, 30, 35 or 37°C.

In the subsequent second part of the study, YPG10 medium that contained 10% w/v glucose, with initial pH adjusted to 5.0 was used as control medium. To study the effect of precursors and to investigate the possible metabolic pathways, media containing succinic, malic, fumaric, glutamic or aspartic acid (to control medium, 0.3% each of corresponding acid was added and pH of each medium was adjusted to 5.0) were employed. Each type of medium consisted of a number of test tubes of 15-mm dia., covered with silicon plug, each containing 5 mL of medium. Inoculation was carried out as described in the first part. Incubation was carried out at 25°C until 30 days during which period the test tubes were taken out occasionally for analysis.

In the third part of the study that followed, the effect of concentration of selected precursors on the formation of malic and succinic acids was studied. To YPG30 medium that contained 30% w/v glucose, 0.3 to 0.7% each of glutamic, malic and succinic acids were added respectively and the initial pH was adjusted to 5.0. Inoculation was carried out as described in the first part. Incubation was carried out for 15 days at 25°C.

10 - 2. Analytical methods

Growth of the yeast was determined by measuring absorbence at 660 nm in the first and the second parts of the work. In the third part, wet weight of cell was measured. Initial and final pH of cultural broths were measured by Horiba pH meter. Initial and final values of total titratable acids were determined by titrating cultural broths before and after incubation respectively with 0.1N NaOH until the pH reached 8.1. Remaining glucose in fermenting broth was determined by AOAC method (1980).

10 - 3. Quantitative determination of organic acids

After incubation, cultural broth was centrifuged (10 min, 7000 x *g*) at 4°C. Part of the supernatant was used for determination its total acidity and the rest was filtered through membrane filter of 0.5 μm pore size followed by freezing at -18°C until use. One mL of this sample was ion-exchanged with amberlite IR-120 [H^+] and amberlite IR-45 [OH^-] resins in two connected columns successively. The latter column was eluted with 2N NH_3 solution followed by washing with water and the combined eluate was concentrated under vacuum at 40°C. The precipitate was dissolved in 10 mL of distilled water and the solution was injected to HPLC for quantitative determination of organic acids. The column and the conditons of HPLC analysis were the same as those described in section 7 - 2.

11. RESULTS AND DISCUSSION

11 - 1. Production of acids in different cultural conditions

Formation of malic and succinic acids by *Z. rouxii* V19 in various media is described in Table 11-1. Growth was the best in potato medium. On the other hand, amount of malic and succinic acids formed in potato medium was the least. Added inorganic nitrogen sources had little effect on the growth. In YPG control medium, concentrations of malic and succinic acids were 2.81 and 1.52 g/L respectively. Schwartz and Radler (1988) reported that the quantity of malate produced by 51 strains of *Saccharomyces*, *Saccharomycodes*, *Zygosaccharomyces* and *Schizosaccharomyces* varied between 0.1 and 2.6 g/L. Again, Heerde and Radler (1978) have described that succinic acid was formed in amounts of 0.2-1.7 g/L by fermenting yeasts of the genus *Saccharomyces*. Amount of acids produced by *Z. rouxii* V19 lay in the upper ranges of these reported values. Addition of ammonium chloride and ammonium hydrogen phosphate slightly increased the acid formation while inclusion of ammonium nitrate and ammonium sulphate had an adverse effect. Final pH could not be used as an indicator for quantity of acid produced since the type of medium was different from each other. Molar ratio of malic acid to succinic acid in all media was between 1.6 and 1.7. Schwartz and Radler (1988) mentioned that the molar ratio of the two acids varied from 0.06 to 3.6 depending upon the species and the strain.

Table 11-1. Formation of malic and succinic acids in various media [a]

Medium[b]	Absorbance at 660 nm	Final pH	Acid conc., g/L		Molar ratio of malic to succinic
			Malic	Succinic	
YPG, Control	1.31	4.21	2.81	1.52	1.63
Potato	1.52	3.35	1.56	0.84	1.64
YM	1.35	3.27	1.65	0.89	1.63
NH_4 Cl	1.32	3.83	2.88	1.54	1.65
NH_4 NO_3	1.37	3.86	2.75	1.51	1.61
$(NH_4)_2HPO_4$	1.24	4.52	3.11	1.62	1.69
$(NH_4)_2SO_4$	1.29	3.89	2.65	1.45	1.61

[a] 140-h still culture at 25°C in 10% glucose medium with initial pH 6.0.

[b] Concentration of supplement nitrogen compounds, 3% w/v.

Table 11-2. Formation of malic and succinic acids in media with different sugars [a]

	Absorbance at 660 nm	Final pH	Acid conc., g/L		Mole ratio of malic to succinic
			Malic	Succinic	
Glucose, contr	1.32	4.21	2.83	1.51	1.63
Sucrose	0.42	5.29	0.41	0.13	2.78
Maltose	1.15	4.25	2.53	1.41	1.58
Galactose	0.63	4.63	1.22	0.65	1.65
Fructose	1.21	4.19	2.41	1.23	1.73

[a] 140-h still culture at 25°C with initial pH 6.0.

[b] Concentration of sugars, 10% w/v.

Formations of malic and succinic acids in media with different type of sugars are described in Table 11-2. Glucose was the best for growth and acid production, followed by maltose or fructose. Sucrose and galactose had adverse effects on growth. The two sugars obviously suppressed the formation of malic and succinic acids; more severe with the latter. This explained the apparent increase in molar ratio of malic to succinic acid. In Table 11-3, effect of supplemented minerals on production of organic acids is shown. Among the added minerals, potassium hydrogen phosphate enhanced growth as well as acid production. Growth of yeast V19 was markedly enhanced by addition of KCl in the medium; however the acid production was hindered.

Effect of glucose concentration in the medium is shown in Fig. 11-1. Optimum growth was observed at 20% glucose but maximum acid production was achieved at a glucose concentration of nearly 30%. However, Schwartz and Radler (1988) stated that malic acid formation was maximum at 20% glucose. With all glucose concentrations, more malic acid was formed than succinic acid; molar ratio of the former to the latter remained almost constant at 1.61-1.63. Effect of initial pH is shown in Fig. 11-2. Initial pH 4 was the best for growth but pH 5 was the most suitable for acid formation. This result agreed with previous work on *S. cerevisiae* (Schwartz and Radler, 1988). Here again, more malic acid was produced than succinic acid and the molar ratio was almost constant at about 1.63. Effect of incubation temperature is shown in Fig.11-3. Growth increased with increase in temperature up to 35^{o}C; however maximum quantities of both acids were achieved at 25^{o}C.

Table 11-3. — Effect of added minerals on acid formation[a]

Supplement mineral		Absorbance	Final	Acid conc., g/L		Mole ratio of
Compound	Conc.	at 660 nm	pH	Malic	Succinic	malic to succinic
Control		1.31	4.22	2.81	1.52	1.63
KH_2PO_4	0.1%	1.18	4.25	3.21	1.72	1.64
$CaCl_2.2H_2O$	0.1%	1.21	4.08	2.86	1.52	1.66
$MgSO_4.7H_2O$	0.1%	1.19	4.15	2.53	1.39	1.61
$ZnSO_4.7H_2O$	10 ppm	1.28	4.13	2.67	1.45	1.62
$MnSO_4.4H_2O$	10 ppm	1.42	4.11	2.94	1.45	1.79
$FeSO_4.7H_2O$	10 ppm	1.43	4.11	2.83	1.48	1.68
NaCl	0.5 M	0.47	4.32	0.15	0.02	6.61
KCl	0.5 M	1.48	4.76	0.52	0.18	2.54

[a] 140-h still culture at 25°C in 10% glucose medium with initial pH 6.0.

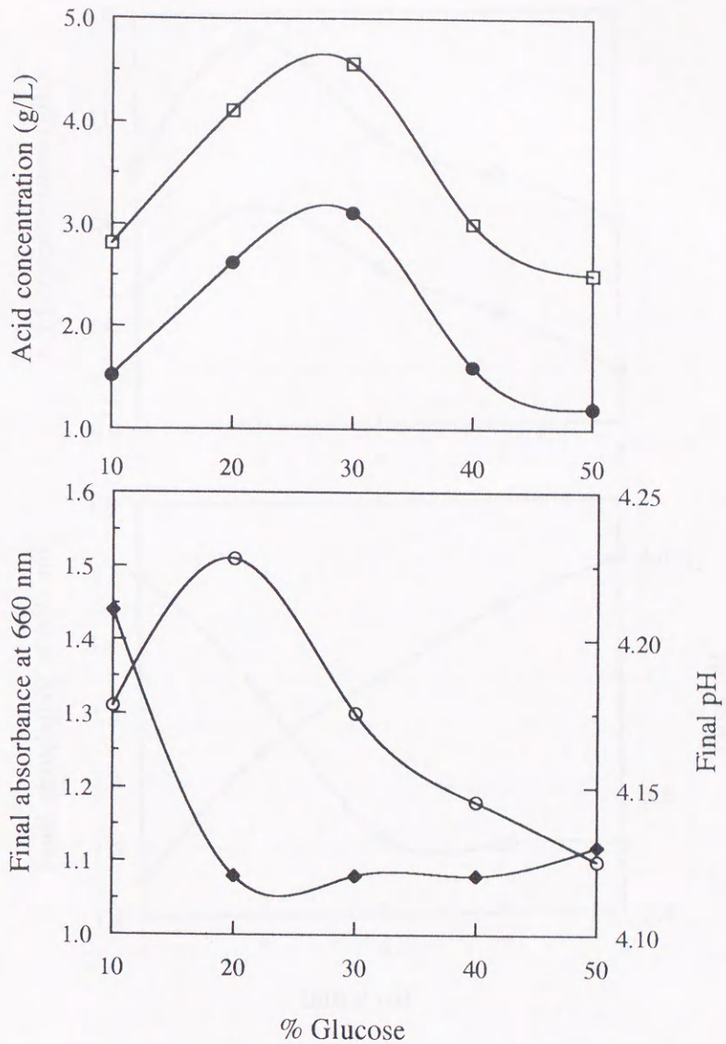


Fig. 11-1. Effect of concentration of glucose

□ Malic acid ● Succinic acid

○ Final OD at 660 nm ◆ Final pH

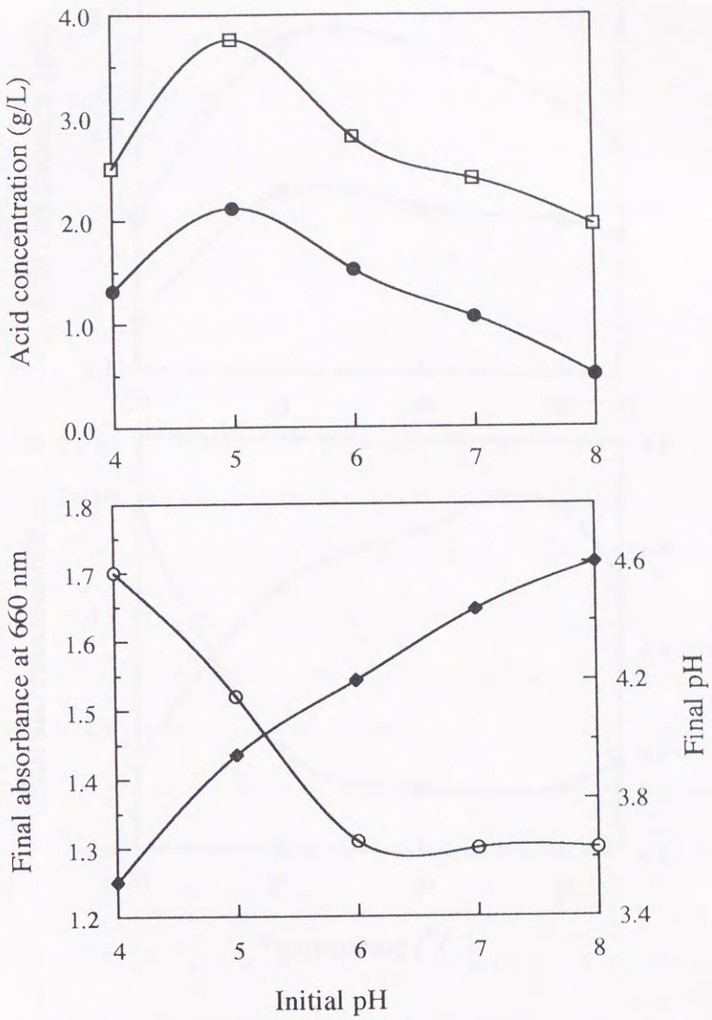


Fig. 11-2. Effect of initial pH

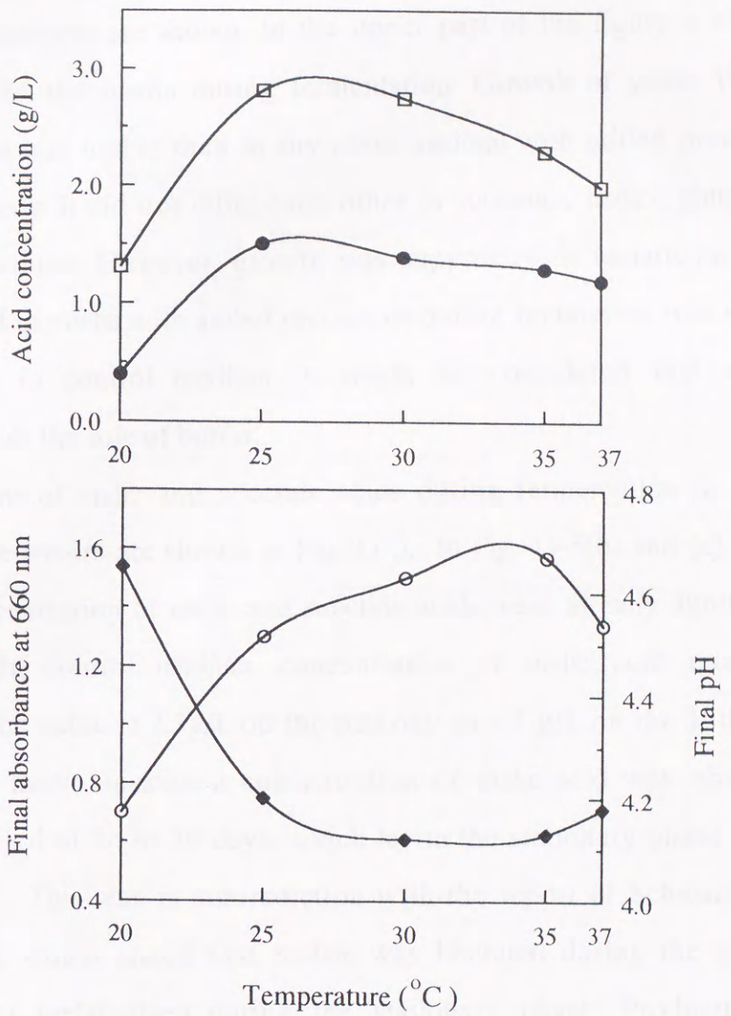


Fig. 11-3. Effect of incubation temperature

□ Malic acid ● Succinic aid

○ Final OD at 660 nm ◆ Final pH

11 - 2. Effect of added precursors

In Fig.11-4, growth curves of strain V19 in control medium and in media with added precursors are shown. In the upper part of the figure is shown change of pH in the media during fermentation. Growth of yeast V19 in control medium was higher than in any other medium with added precursor compounds. Growth did not differ each other in succinic-, malic-, glutamic-, and aspartic-medium. However, growth was suppressed in fumaric-medium. Decrease of pH in media with added precursors during incubation was not as sharp as that in control medium. It might be considered that added compounds took the role of buffer.

Formations of malic and succinic acids during fermentation in media with added precursors are shown in Fig. 11-5. In Fig. 11-5(b) and (c), initial values of concentration of malic and succinic acids were already subtracted respectively. In control medium, concentration of malic acid increased steadily from the value of 2.5g/L on the first day to 4.5 g/L on the 13th day. Also in other media, maximum concentration of malic acid was observed during the period of 16 to 20 days, which lay in the stationary phase of the growth curve. This was in contradiction with the report of Schwartz and Radler (1988), which stated that malate was liberated during the growth phase and not metabolized during the stationary phase. Production of succinic acid followed a similar pattern as that of malic acid; maximum concentration was obtained just beyond the exponential growth phase. This also did not agree with the results of Heerde and Radler (1978), which described that succinic acid was formed by yeasts of the genus *Saccharomyces* during the exponential growth phase. With succinic-medium, delayed or acquired formations of both malic acid and succinic acid itself were observed; maximum concentrations were obtained only after 20 days of incubation.

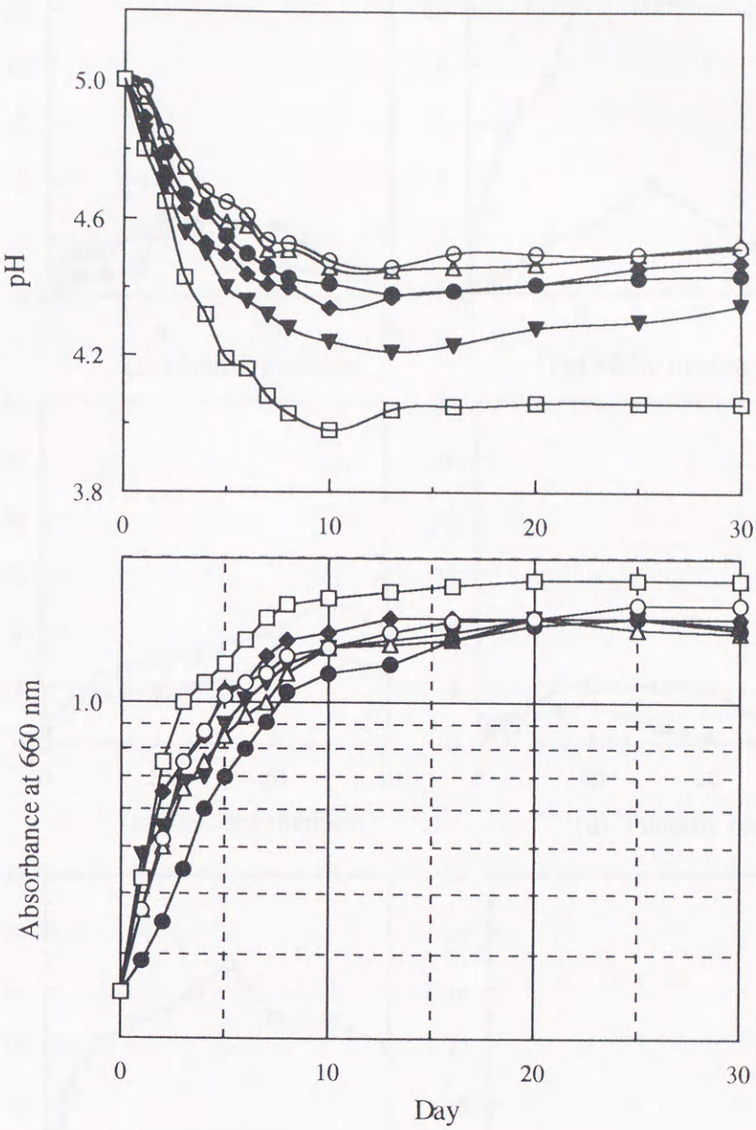


Fig. 11-4. Growth and change of pH in semi-aerobic culture of strain V19 in media with added precursors

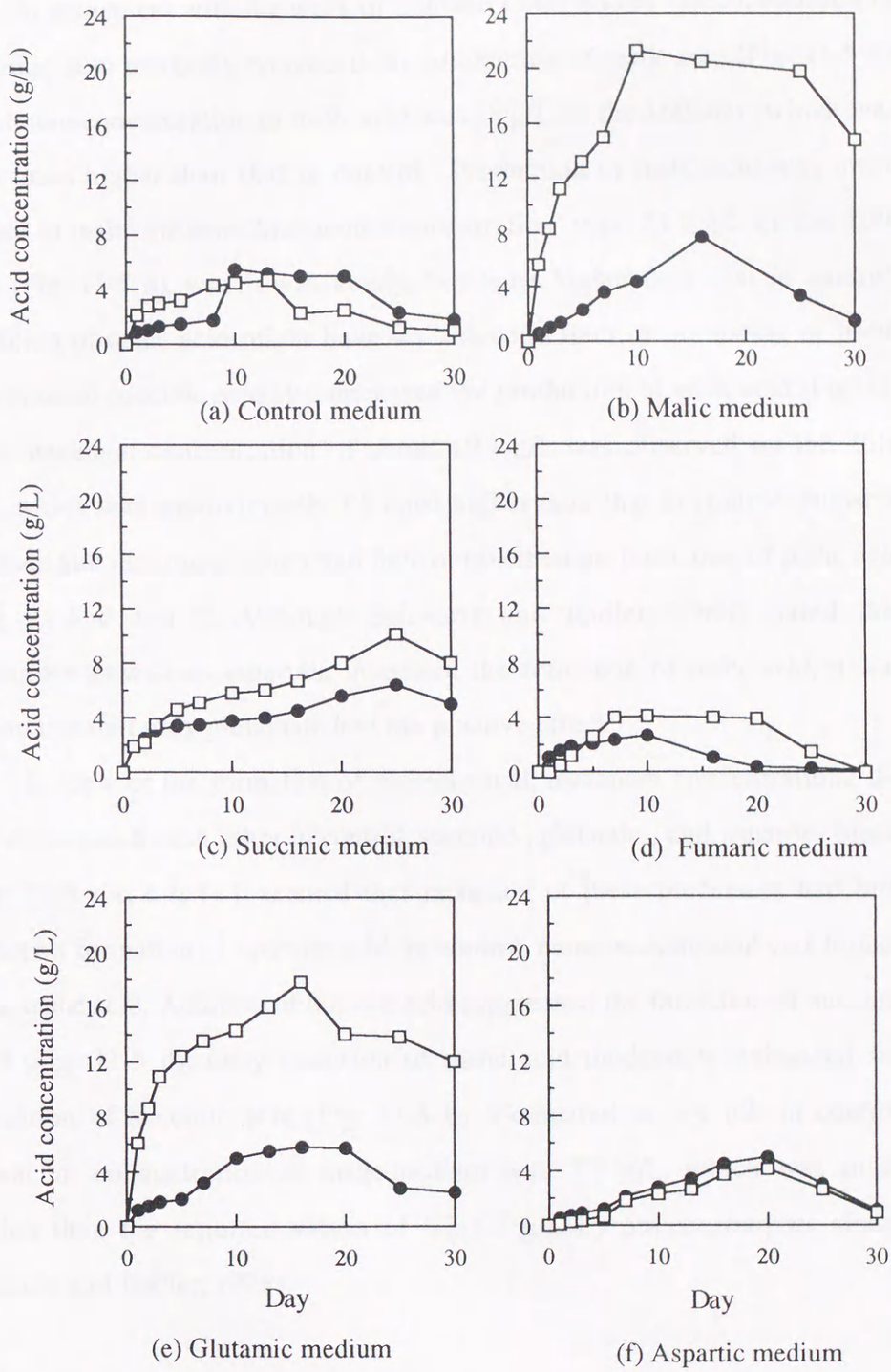


Fig. 11-5. Production of malic and succinic acids in media with added precursors

In agreement with the work of Schwartz and Radler (1988), addition of glutamic acid markedly enhanced the production of malic acid (Fig. 11-5 e). Maximum concentration of malic acid was 18 g/L on the 16th day, which was four times higher than that in control. Production of malic acid was much higher in malic-medium. Maximum concentration was 21.2 g/L on the 10th day (Fig. 11-5 b), which was nearly five times higher than that in control. Addition of malic acid might have an induced effect on formation of itself. Inclusion of succinic acid also increased the production of malic acid (Fig. 11-5 c); maximum concentration of about 10.1 g/L was observed on the 25th day, which was approximately 2.5 times higher than that in control. Fumaric-medium and aspartic-medium had little or no effect on formation of malic acid (Fig. 11-5 d and f). Although Schwartz and Radler (1988) stated that glutamate as well as aspartate increased the formation of malic acid, it was found out that only glutamate had the positive effect.

In view of the formation of succinic acid, maximum concentrations did not differ much each other in control, succinic-, glutamic-, and aspartic- media (Fig. 11-5 a, c, e & f). It seemed that inclusion of these precursors had little effect on formation of succinic acid. In control, more succinic acid was formed than malic acid. Addition of fumaric acid suppressed the formation of succinic acid (Fig. 11-5 d). Only inclusion of malic acid moderately enhanced the liberation of succinic acid (Fig. 11-5 b). Compared to 5.4 g/L in control, maximum concentration in malic-medium was 7.7 g/L, which was much higher than the reported values of 0.2-1.7 g/L by *Saccharomyces* strains (Heerde and Radler, 1978).

11 - 3. Effect of concentration of precursors

Effect of glutamic acid concentration in the medium on the production of malic and succinic acids is shown in Fig. 11-6. Growth (wet weight of cells)

increased with increase in glutamic concentration up to 0.5%, beyond which it was almost unchanged. Final pH slightly increased up to glutamic acid concentration of 0.4%, after which it was almost constant. Production of malic acid was directly proportional to glutamic concentration up to 0.5%; it increased nearly 2.5 fold from about 30.1 to 74.9 g/L. This value was more than 16 times higher than that in control medium (Fig. 11-5 a). Use of optimum glucose concentration (30%) and optimum initial pH (5.0) were considered to be the factors that increased the acid formation but the effect of glutamic acid was the most dominant. On the other hand, production of succinic acid was not enhanced by increase in glutamic acid concentration; indeed, the concentration was slightly reduced. As formation of malic acid was increased while that of succinic acid was decreased or remained unchanged, the molar ratio of the former to the latter increased from about 4 to nearly 12.

In Fig. 11-7, effect of initial concentration of malic acid is shown. In the figure, initial values of malic acid concentration were already subtracted. Similar results were observed as with the case of glutamic acid. Amount of malic acid increased almost directly with increase in initial malic acid concentration up to 0.5%. The increment was not as sharp as in glutamic medium and the final maximum concentration was only about half of that in glutamic medium. Concentration of succinic acid in malic-medium was, as in the second part of our work, higher than that in glutamic-medium. Maximum production of succinic acid was achieved (6.8 g/L) in the medium with 0.5% malic acid. Addition of more malic acid in the medium had little effect on formation of succinic acid. Molar ratio of malic acid to succinic acid remained almost unchanged.

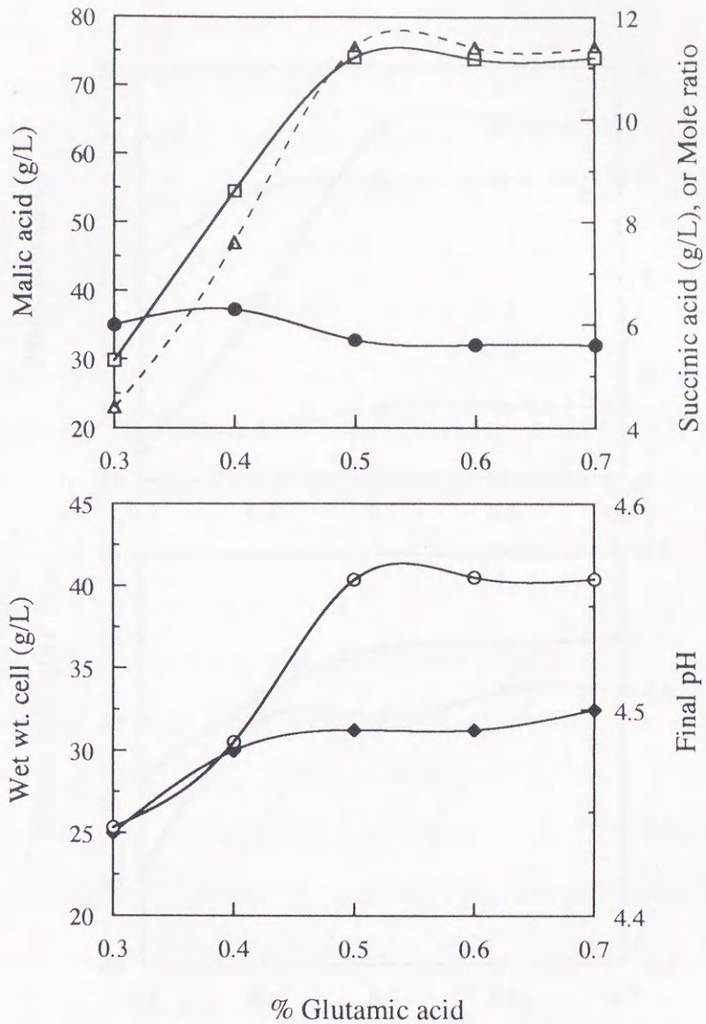


Fig. 11-6. Effect of initial concentration of glutamic acid

□ Malic acid ● Succinic acid △ Molar ratio of malic to succinic

○ Wet wt. cell ◆ Final pH

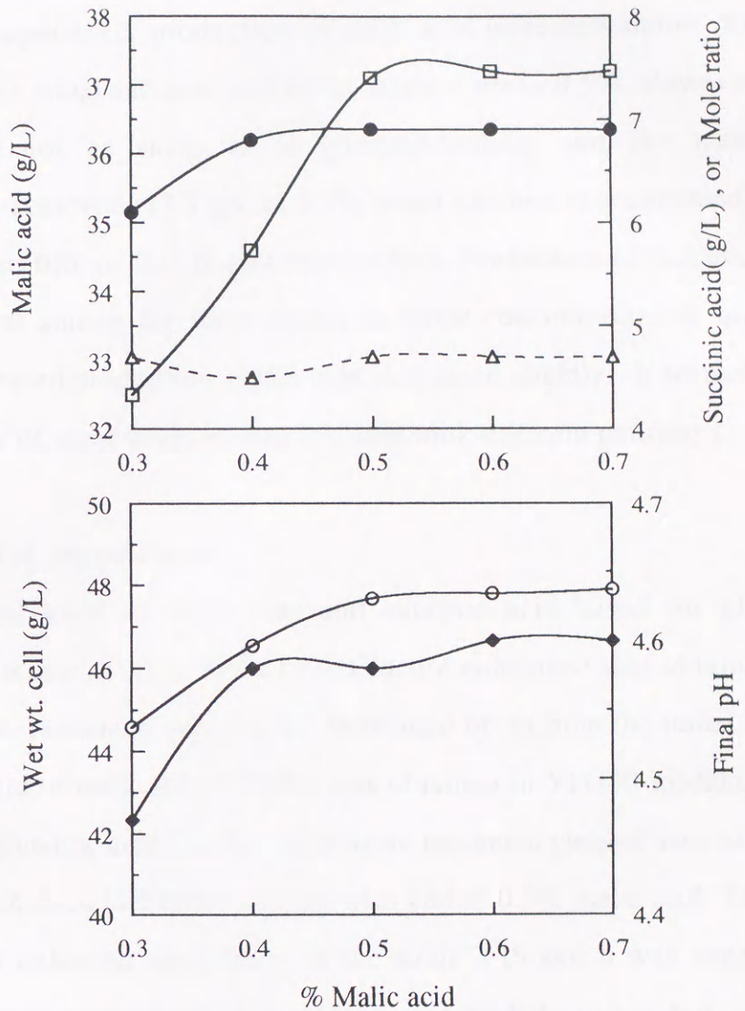


Fig. 11-7. Effect of initial concentration of malic acid

□ Malic acid ● Succinic acid △ Mole ratio malic to succinic

○ Wet wt. cell ◆ Final pH

Effect of initial concentration of succinic acid is shown in Fig. 11-8. Unlike the previous two media, increase in initial concentration of succinic acid in the medium had a detrimental effect on the growth. Even though the growth was suppressed, production of malic acid increased almost directly with increase in initial succinic acid concentration up to 0.5%. However, the increase was not as sharp as in glutamic-medium and the maximum concentration achieved (13.3 g/L at 0.5% initial succinic concentration) was only about one fifth of that in glutamic-medium. Production of succinic acid was the lowest among the three media; as initial concentration of succinic acid was increased production of this acid decreased slightly. It seemed that in *Z. rouxii* V19, malic acid and succinic acid took different pathways.

11 - 4. Yield of organic acids

Individual yield of malic acid and succinic acid based on glucose consumption is described in Table 11-4. Glucose consumed was obtained by subtracting the remaining sugar in the fermented broth from the initial value. Maximum yield of malic acid (32.8%) was obtained in YPG30 medium with added 0.5% glutamic acid. On the other hand, maximum yield of succinic acid (8.1%) was achieved in YPG10 medium with added 0.3% malic acid. There is a potential of industrial application of the strain V19 and it was suggested that further study on production of acids on mass scale be proceeded.

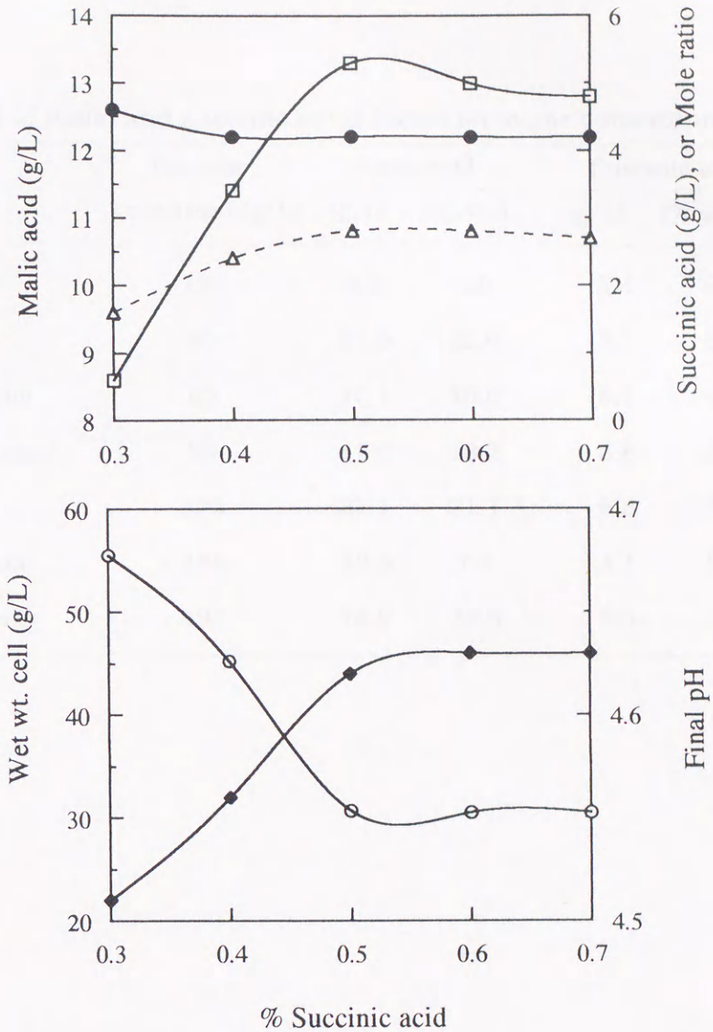


Fig. 11-8. Effect of initial concentration of succinic acid

□ Malic acid ● Succinic acid △ Molar ratio of malic to succinic

○ Wet wt. cell ◆ Final pH

Table 11-4. Yield of malic and succinic acids based on sugar consumption

Medium	Glucose consumed (g/L)	Malic acid		Succinic acid	
		(g/L)	(%yield)	(g/L)	(% yield)
YPG10, conrtol	100	4.5	4.5	5.4	5.4
YPG10, 0.3% malic	95	21.5	22.6	7.7	8.1
YPG10, 0.3% succinic	95	10.1	10.6	6.1	6.4
YPG10, 0.3% glutamic	98	17.8	18.2	5.8	5.9
YPG30, 0.5% malic	175	37.1	21.1	6.8	3.9
YPG30, 0.5% succinic	180	13.3	7.4	4.1	2.3
YPG30, 0.5% glutamic	193	74.9	32.8	5.5	2.8

V. COLLAGENOLYTIC ACTIVITY OF ISOLATED YEASTS

12. INTRODUCTION

Microbial collagenases are proteinases that are capable of hydrolyzing native collagen *in vitro* under physiological conditions (Mandl, 1961). Production of collagenase or collagenase-like proteases from various bacteria have been reported by many workers. Among the collagenase-producing bacteria, *Clostridium histolyticum* is the most studied microorganism and crude or purified collagenase from this bacteria are now available commercially. There were works on collagenase from the mold *Trichophyton shoenleinii* (Rippon, 1968) and *Aspergillus oryzae* (Nordwig and Jann, 1968). However, reports on the presence of collagenolytic activity in yeasts are very rare. In the book, "The Yeast: a taxonomic study", it is stated that with the exception of *Crypotococcus* species, few yeasts have the ability to liquefy gelatin which is a denatured form of collagen (Kreger-van Rij, 1984). Through the identification of sugar-tolerant yeasts isolated from high-sugar foods, a few strains of *Zygosaccharomyces rouxii* that possessed the activity of gelatin liquefaction were incidentally found out. Although the enzyme active against gelatin may not be active against collagen, as pointed out by Merkel et al. (1975) , the results were encouraging and it would be beneficial to investigate the presence of collagenolytic enzyme from a different source.

This section concerned with the screening of yeasts for gelatin-liquefaction, isolation of crude enzyme and determination of its activity against various substrates including insoluble collagen. Cultural conditions that affected the production of the enzyme were also described. Comparison was made between the yeast enzyme and *Cl. histolyticum* collagenase.

13. MATERIALS AND METHODS

13 - 1. Yeast strains and materials used

Ninteen strains of *Z. rouxii* and one strain of *Torulaspora delbrueckii* were used in screening tests. Gelatin used in the cultural medium and as substrate for enzyme assay was type B from bovine skin, of Sigma product. Insoluble collagen (type 1) from bovine Achilles tendon, acid soluble collagen (type 1) from kangaroo tail, N-carbobenzoxy-GLY-PRO-LEU-GLY-PRO (abbreviated hereafter Cbz-GPLGP), and N-(3-{2-Furyl}Acryloyl)LEU-GLY-PRO-ALA (abbreviated hereafter FALGPA) were all purchased from Sigma. *Cl. histolyticum* collagenase (type 1A) [EC 3.4.24.3], also a Sigma product, was a crude enzyme equivalent to first 40% ammonium sulfate fraction and was said to contain neutral protease activity. Casein (Hammarsten) and trypsin (2000 unit/g) [EC 3.4.21.4] were products of Merck and Wako, Japan, respectively. All other chemicals were special grades of Wako products. Unless otherwise stated, 0.05 M Tris-HCl buffer containing 0.1 M calcium acetate, pH 7.5 was used in dialysis and in determination of collagenase activity throughout this work.

13 - 2. Screening tests for activity of gelatin liquefaction and caseinase activity

Yeasts were screened out based on their activity of gelatin liquefaction. Five mL of Wickerham synthetic medium containing 0.5% glucose, 0.5% ammonium sulphate, approximately 10% sterilized gelatin in addition to trace elements and vitamins was used as the medium as described by Kreger-van Rij (1984). Actively grown yeast suspension (0.1 mL) was inoculated onto the surface of solidified medium in the same way as the procedure for test of

assimilation of carbon compounds. Inoculated media were incubated at 25°C and liquefaction of gelatin was observed after one, two and three weeks.

Caseinase activity was detected by the method described by Taing Ok (1982). Yeasts were directly inoculated to YM agar plates (yeast extract, 0.3; malt extract 0.3; polypeptone, 0.5; glucose, 2; all in % w/v) containing of 1% Difco skim milk. After incubation at 25°C for two weeks, transparent zone around the colony was checked.

13 - 3. Culture media and cultivation methods

Following media were used for cultivation: (a) YPG broth medium (yeast extract, 0.5%; polypeptone, 1.0%; glucose, 10%; all w/v); (b) YPGG broth medium (YPG medium + 0.5%, 1%, or 5% w/v gelatin). Unless otherwise stated, initial pH of the medium before autoclaving was adjusted to 7.0.

Selected strain V19 was cultured in 50 mL of medium in 250-mL Erlenmayer flasks, each inoculated with 1 mL of yeast inoculum freshly grown in YPG broth medium. With still cultures, the flasks were incubated at 25°C for 30 days. With shake cultures, the flasks were put on a reciprocal shaker and incubated at 25°C for 70 hours. Sample flasks were taken out occasionally and the enzyme activity was determined.

13 - 4. Isolation of crude enzyme

Unless otherwise stated, all procedures were carried out at 4°C. After incubation, the cultural broth was centrifuged under 7000 x *g* for 10 min. With YPGG medium, since undissociated portion of gelatin in the supernatant was gelled out during centrifuging, centrifuge tubes were allowed to stand for a while at room temperature so as to liquefy the gel again. Ammonium sulfate fractionation (70% saturated) was carried out for 24 h, after which the

suspension was centrifuged under 10000 x *g* for 10 min. With YPGG medium, dissociated portion of gelatin in the cultural supernatant precipitated out together with enzyme. The precipitate was dissolved in 5 mL of the buffer and centrifuged again under 3000 x *g* for 5 min. Precipitate, considered as undissociated gelatin, was discarded. The dissolved portion, which contained crude enzyme as well as dissociated portion of gelatin, was dialyzed against 400 fold of buffer for 24 h, with two changes of buffer. With YPG medium, it was no need to recentrifuge and the dissolved solution was dialyzed in the same way as with YPGG medium.

13 - 5. Determination of gelatin-liquefaction activity

Strain V19 was still-cultured in YPG medium for 3 weeks and the crude enzyme (70% ammonium sulfate feractionate) was collected as stated above. Three mL of 10% gelatin in hot distilled water (pH unadjusted) in 15-mm test tube, was sterilized at 15 psi for 15 min, and was made solidified at 25°C. To top of the solidified layer, 0.5 mL of crude enzyme was added aseptically and incubated at 25°C for 3 days after which the liquefied portion was poured down and measured by a measuring cylinder. Buffer solution (0.5 mL) was employed as control.

13- 6. Activity against insoluble collagen

Ten mg of insoluble collagen was suspended in 2 mL of Tris-HCl buffer, pH 7.5. It was incubated with 0.2 mL of enzyme at 37°C. Boiled enzyme solution was used as blank. After 18 h of incubation the reaction mass was filtered through Toyo No.2 filter. Color development of 0.5 mL filtrate was carried out with ninhydrin reagent according to the method described by Rosen (1957). One unit of enzyme was defined as that liberated peptides

from the substrate equivalent in ninhydrin color to 1.0 μ mole of L-leucine per h at 37°C at pH 7.5 in the presence of 0.1 M calcium ions.

13 - 7. Activity against soluble collagen

The activity was determined as the same as with insoluble collagen except that 2 mg of substrate was incubated with 0.2 mL of enzyme for 5 h. One unit of enzyme was defined as the same as that with insoluble collagen.

13 - 8. Activity against Cbz-GPLGP

The activity was determined according to the procedure described by Appel (1974). One unit of enzyme was defined as that liberated peptides from the substrate equivalent in ninhydrin color to 1.0 μ mole of leucine per min at 37°C at pH 7.5 in the presence of 0.1 M calcium acetate.

13- 9. Activity against FALGPA

The activity was determined by the method based on the procedure described by Van Wart and Steinbrink (1981). Three mL of 50 mM of FALGPA in Tris-HCl buffer (pH 7.5) was put into 1-cm cuvette and the cuvette was placed at 25°C for 2 min. At zero time, 2 μΛ of enzyme was added and OD at 345 nm was subsequently measured with UV spectrophotometer (UV-160A, Shimadzu). After incubating at 25°C for 3 min, OD was measured again. One unit of activity was defined as change in OD per min at 25°C at pH 7.5 in the presence of 0.1 M calcium ions.

13 - 10. Protease activity against casein

Caseinase activity was determined according to the procedure described by Hashinaga et al. (1986). One unit of protease was defined as one μmole of tyrosine liberated per h at 37°C at pH 6.5.

13- 11. Protein determination

Protein content of crude enzyme solutions were determined by biuret method (Layne, 1957) using bovine serum albumin as standard.

13 - 12. Effect of pH of culture medium on production of enzyme

Shake cultures were carried out in YPG media with initial pH of 6.0, 7.0 and 8.0. Cultural broths were harvested on 30, 50 and 70 h, respectively and the activity of crude enzyme was monitored using Cbz-GPLGP as substrate.

13- 13. Effect of reaction pH on enzyme activity

Crude enzyme obtained from 50-h incubation in YPG medium was used for this experiment. Substrate Cbz-GPLGP was dissolved in Tris-HCl buffer with pH ranging from 6.0 to 8.5. Activity was determined as described above with the substrate.

13 - 14. Effect of calcium ion on activity and stability of enzyme

Crude enzyme obtained from 50-h incubation in YPG medium was used for this experiment. One mL of crude enzyme in Tris-HCl buffer (pH, 7.5) containing 0.1 M calcium acetate was stored at 4^{o}C for one day. Another 1 mL of crude enzyme was dialyzed at 4^{o}C for one day against 400 fold of Tris-HCl buffer (pH, 7.5) containing no calcium ion. Buffer was changed twice during dialysis. One day-old dialyzed and undialyzed enzymes were tested for their activity against Cbz-GPLGP at 37^{o}C and pH 7.5, in the presence and absence of calcium ion.

14. RESULTS AND DISCUSSION

14 - 1. Gelatin-liquefaction activity of strain V19

In Table 14-1, results of screening tests are shown. None of the yeast strain showed caseinase activity. On the other hand, strain V9, V10, V19 and V20 showed the activity of gelatin liquefaction. Gelatin was partially liquefied in two weeks and totally liquefied in three weeks. From screening tests, strain V19, which showed the strongest activity was selected for further study. The activity was also observed when gelatin was incubated with crude enzyme from strain V19. After 3 days incubation at 25^{o}C, volume of gelatin liquefied was 3 mL/ mL of crude enzyme (0.32 mg protein/mL).

14 - 2. Activity against various substrates

Activities of crude enzyme from V19 against various substrates are shown in Table 14-2. Crude enzyme used was obtained from still culture in YPGG medium containing 5% gelatin incubated for 25 days at 25^{o}C (0.28 mg protein/mL). For comparison, the corresponding activities of commercial crude *Cl. histolyticum* collagenase (0.4 mg/mL) and trypsin (1 mg/mL) were also measured. Results revealed that yeast enzyme had the activity against soluble and insoluble collagen as well as against synthetic peptides, Cbz-GPLGP and FALGPA. The former peptide was described to be one of the most suitable substrates for collagenase (Nagai et al., 1960). The latter peptide was claimed as a specific substrate attacked only by collagenases (Van Wart and Steinbrink, 1981). Compared to *Cl. histolyticum* collagenase, the activity of *Z. rouxii* enzyme was low; the activities against insoluble collagen was about one-third and the activity against FALGPA was about two-fifths of the corresponding values of commercial enzyme.

Table 14-1. Screening tests for caseinase activity and activity of gelatin liquefaction

Strain	Caseinase activity	Activity of gelatin liquefaction
Z. rouxii		
V1	–	–
V2	–	–
V3	–	–
V4	–	–
V5	–	–
V6	–	–
V7	–	–
V9	–	++
V10	–	++
V13	–	–
V14	–	–
V15	–	–
V17	–	–
V18	–	–
V19	–	+++
V20	–	+
V22	–	–
V24	–	–
V25	–	–
T. delbrueckii		
V16	–	–

Table 14-2. Activity of *Z. rouxii* enzyme and commercial proteolytic enzymes against various substrates.

Enzyme	Protein (mg/ml)	Activity, unit/mg protein				
		Collagen		Synthetic peptides		Casein [d]
		Insoluble [a]	Soluble [a]	Cbz-GPLGP [b]	FALGPA [c]	
Crude yeast enzyme	0.28	5.6	27.1	21.1	0.41	0.7
Crude *Clostridium* collagenase	0.40	18.3	70.4	25.0	1.03	65.7
Trypsin	1.00	1.3	6.4	0	0	84.9

[a] One unit liberates peptides from collagen equivalent in ninhydrin color to 1 mmole leucine per h at 37°C at pH 7.5 in the presence of 0.1 M calcium acetate.

[b] One unit liberates products equivalent in ninhydrin color to 1 mmole leucine per min at 37°C at pH 7.5 in the presence of 0.1 M calcium acetate.

[c] One unit is defined as change in OD at 345 nm per min at 25°C at pH 7.5 in the presence of 0.1 M calcium acetate.

[d] One unit hydrolyses casein to produce color equivalent to 1 mmole tyrosine per h at 37°C at pH 6.5.

Although it was regarded that trypsin had no activity against collagen (Hanada et al., 1971), it was found out that it had a slight activity against native collagen, which may be due to the presence of some soluble proteins in the collagen. Treatment of native collagen with trypsin before assay may be an alternative but as warned by Nordwig (1971), it might denature the collagen. On the other hand, it was found out that trypsin had no activity against Cbz-GPLGP and FALGPA.

In view of inclusion of nonspecific proteases, crude yeast enzyme showed only a slight activity against casein. The activity against casein was about 1/90 th and 1/120 th of the corresponding values of bacterial collagenase and trypsin respectively. This indicates that the yeast enzyme is relatively free of nonspecific proteases, though we have to admit that there needs to test with other nonspecific proteins.

According to Nordwig (1971), microbial collagenases can be categorized into three main types: (a) enzymes that are inactive against unspecific proteins such as casein but degrading specifically collagen, soluble or insoluble, and synthetic model peptides, (b) enzymes that attack native and denatured collagen such as gelatin or even unspecific proteins, but unable to cleave model peptides, and (c) enzymes that have a high affinity for synthetic peptides and capable of digesting denatured collagen, but not native collagen. Whereas the first two types were designated as true collagenases of type 1 and 2, respectively, the third one was regarded as pseudo-collagenase. According to this classification, the crude enzyme from *Z. rouxii* V19 would be designated as type 1.

14 - 3. Production of enzyme in different media by different cultural methods

In early days of microbial collagenase history, since the enzyme was usually associated with anaerobic bacteria, anaerobic cultures were carried out for the enzyme production. Later, there were reports on collagenases from aerobic microorganisms. In the present work, semi-aerobic still cultures were first accomplished. Strain V19 was incubated in YPGG medium containing 5% gelatin and the production of enzyme was monitored periodically up to 30 days employing insoluble collagen as substrate. Until 7th day, no activity was detected. Then the activity gradually increased and maximum activity of 1.6 unit/mL was measured on 25th day. On 30th day, there was no activity at all.

In Table 14-3, results of shake cultures of the yeast in both YPG (initial pH of 6, 7 and 8) and YPGG media (with 0.5% and 1% gelatin) are shown. Production of enzyme was checked periodically up to 70 h employing insoluble collagen and Cbz-GPLGP as substrates. Results of shake cultures confirmed that the enzyme could be produced aerobically and within a relatively shorter time. Maximum production was achieved on 50-h incubation. As in still culture, the enzyme was readily inactivated with prolonged incubation time.

Table 14-3. Production of collagenase in shake cultures

Medium	Incubation time (h)	Activity, unit/ml	
		Collagen [a]	Cbz-GPLGP [b]
YPG, pH 6	30	0	0
	50	0	0
	70	0	0
YPG, pH 7	30	0	0
	50	7.7	22.7
	70	1.1	1.9
YPG, pH 8	30	0	0
	50	5.1	10.9
	70	1.7	2.4
YPGG, pH 7 (0.5% gelatin)	30	0	0
	50	8.4	23.5
	70	1.5	1.7
YPGG, pH 7 (1% gelatin)	30	0	1.5
	50	7.1	19.8
	70	0	1.3

[a] One unit liberates peptides from insoluble collagen equivalent in ninhydrin color to 1.0 mmole of leucine per h at pH 7.5 at 37°C.

[b] One unit liberates products from Cbz-GPLGP equivalent in ninhydrin color to 1.0 mmole of leucine per min at pH 7.5 at 37°C.

Some workers claimed that production of bacterial collagenases was only achieved or enhanced greatly when the cultivation was induced by adding such substrates as casein or denatured collagen into the medium (Mandl et al., 1953; Merkel et al., 1975). In the present study with shake cultures, it was found out that there was no need to add gelatin into the medium. Activity of crude enzyme from YPG medium was quite comparable to those from YPGG media containing 0.5% or 1% gelatin. In fact, inclusion of gelatin in the medium made the isolation complicated; undissociated gelatin in the medium always gelled out during centrifuging at low temperature and dissociated gelatin also precipitated out together with enzyme after addition of ammonium sulfate. There might be a partial loss of enzyme when the precipitated was discarded. The enzyme obtained from YPGG medium was obviously pigmented even after dialysis. All these facts suggested that gelatin-containing medium was not preferable to YPG medium.

As can be seen from Table 14-3, production of enzyme was not favourable at initial medium pH less than 7.0. It seemed that rapid decrease of pH during incubation made the enzyme inactive. On the other hand, with initial pH greater than 7.0, the growth of yeast was surpressed and the crude enzyme was pigmented. Therefore pH 7.0 was considered to be the optimum pH for enzyme production.

14 - 4. Effect of pH on the activity

In Fig.14-1, the relative activities against Cbz-GPLGP at various pH are shown. The activity at pH 7.5 was taken as reference. The enzyme used was obtained from shake culture of YPG medium (pH 7) harvested after 50-h incubation. Although the enzyme used was not yet purified, the apparent optimum pH of the crude enzyme was about 8.2.

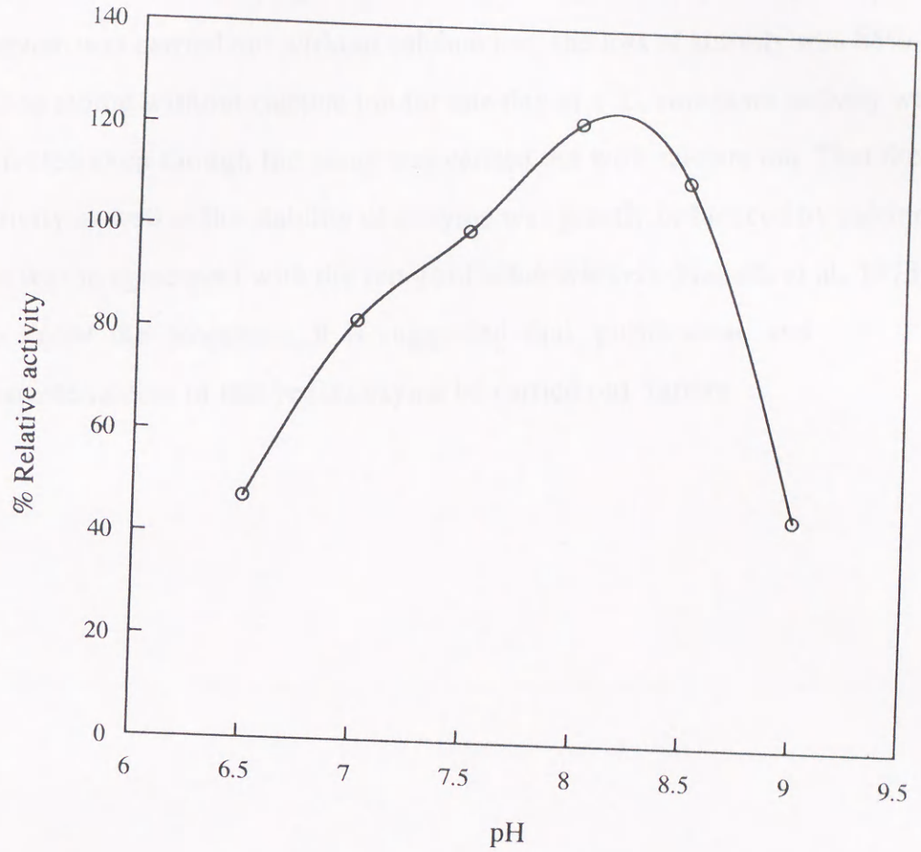


Fig. 14-1. Effect of pH on yeast collagenase

14- 5. Effect of calcium ion

After one day of storage at 4°C and pH 7.5 in the presence of calcium ion, 38% of the activity against Cbz-GPLGP was lost. When the assay of that enzyme was carried out without calcium ion, the loss of activity was 88%. When stored without calcium ion for one day at 4°C, almost no activity was detected even though the assay was carried out with calcium ion. That the activity as well as the stability of enzyme was greatly influenced by calcium ion was in agreement with the report of other workers (Hanada et al., 1973). To know the properties, it is suggested that purification and characterization of this yeast enzyme be carried out further.

VI. SUMMARY

High-sugar fermented vegetable extracts are novel health food products in Japan which employ sugar-tolerant yeasts during processing. In oder to understand their distribution in these foods and their role in functionality of such foods, sugar-tolerant yeasts were isolated from nine samples of products, together with one sample of fermented ume extract (Japanese apricot), and one sample of honey. Twenty three strains were identified as *Zygosaccharomyces rouxii* ; one strain as *Z. bailii* ; one strain as *Torulaspora delbrueckii* ; one strain as *Candida bombicola*. Nearly 90% of identified strains belonged to *Z. rouxii* with variations in fermentation and assimilation properties. All strains grew well on 50% w/w glucose medium and all but two strains grew on 60% w/w glucose medium. Sixteen strains belonged to strong sugar-tolerance type (poor or no growth at 1% and maximum growth at 30 or 40% w/w glucose); four strains to moderate type (grew well at 1% and maximum growth at 10 or 20% w/w glucose); seven strains to weak type (maximum growth only at 1% w/w glucose). One strain of *Z. rouxii* , strainV19, grew up to 80% (w/w) glucose in liquid medium. This strain grew well at pH 2.5. In view of salt-tolerance, only three strains belonged to moderate type (maximum growth at 0.5 or 1 M NaCl); the remaining all to weak type (maximum growth only at 0 M NaCl). It is suggested that sugar-tolerance and salt-tolerance of yeasts are in different aspects.

On testing of antibacterial activity, strain *Z. rouxi* V19 showed the strongest. The active fraction of its metabolite was found to be organic acids; Malic, succinic and fumaric acid were identified out from cultural broth by HPLC as well as GC-MS analyses.

Glucose concentration of 30%, initial pH 5.0, and 25^{O}C were the best for maximum production of both malic and succinic acids by *Z.rouxii* in

semiaerobic culture in YPG medium. Sugars other than glucose, supplementary inorganic nitrogen sources and minerals had no remarkable effect. Addition of NaCl (0.5 M) had detrimental effect both to growth and production of acids. Addition of KCl (0.5 M) enhanced the growth but not acid formation. Acids were produced much at stationery phase. On the 13 th day of incubation in YPG (10% glucose), concentration of malic and succinic acids in the medium were 4.9 and 5.5 g/L, respectively. Inclusion of glutamic, malic and succinic acid in the medium increased the production of malic acid. However, only malic acid enhanced the formation of succinic acid. Inclusion of fumaric and aspartic acids in the medium had adverse effect both to growth and production of acids. Maximum amount of malic acid produced was 74.9 g/L (32.8% yield) in the medium with 0.5% glutamic acid and that of succinic acid was 7.7 g/L (8.1% yield) in the medium with 0.3% malic acid.

Through the screening tests for proteolytic activity, it was found out that strain *Z. rouxii* V19 possesed the activity of gelatin liquefaction. The activity of crude enzyme from this strain was determined against insoluble and soluble collagens, synthetic peptides and casein. Whereas the activities against insoluble collagen and synthetic peptide FALGPA were about one-third and two-fifths of the corresponding values of commercial *Clostridium histolyticum* collagenase [EC 3.4.24.3], the activity against casein was only about one-ninetieth. Production of enzyme was carried out in still and shake cultures at 25^{o}C, with and without gelatin in the medium. Maximum production of enzyme was achieved in 25-d still culture and in 50-h shake culture. Inclusion of gelatin in the medium did not enhance enzyme production. The optimum pH of medium for enzyme production was 7.0. Optimum pH for the enzyme activity against Cbz-GPLGP was about 8.2 at 37^{o}C. Calcium ion was essential for the activity and stability of crude yeast collagenase.

要旨

園芸生産物の発酵食品は耐糖性酵母を利用しており，これらの酵母の分布や役割が明らかになっていない．本研究では 9つの市販品および自家製の梅エキスと蜂蜜を原料として耐糖性酵母を分離し，さらに同定した．その結果，23 株の *Zygosaccharomyces rouxii*，1 株の *Z. bailii*，1株の *Torulaspora delbrueckii*，1 株の *Candida bombicola* であることが明らかになった．*Z. rouxii* は全株の約 90% に当った．すべたの株は 50%w/wグルコース濃度，2 株を除いた菌株は 60%w/w 糖濃度で生育した．16 株は高度耐糖性（1% では生育しないか弱く，30 から 40% が最適）を持ち，4 株は中度性耐糖性（1% でも生育するが，最適な濃度は 10 から 20%）で，7 株は低度耐糖性（最適な濃度は1%）を持っていた．中では *Z. rouxii* V19 は 80% w/w グルコースの入った液体培地でも生育できることが分かった．耐塩性を調べた結果，3 株だけが中度性耐塩性（0.5 から 1.0 M NaCl が最適濃度）で，残りの株は低度耐塩性（0 M NaCl が最適な濃度）を持っていた．このことは耐糖性と耐塩性は違った特性であることを示していた．

スクリーニングテストの結果，V19 株は最も強い抗菌性を持っていることが分かり，その活性物質は有機酸であることが明らかになった．HPLC および GC-MS 分析によってリンゴ酸，コハク酸，フマル酸が同定された．

次にリンゴ酸とコハク酸の生成に及ぼす培地や培養条件を調べた結果，30% グルコース，pH 5.0 および 25℃ が最適であることが分かった．他の糖や、YPG培地に添加した無機窒素化合物や無機物は影響がなかった．NaCl (0.5M) は酵母の生育および有機酸の生成を抑制した．一方，KCl (0.5M) は生育を促進したが有機酸の生産はきわめて少なかった．また YPG 培地に30日間静置培養した結果，有機酸の生産は log phase を超えて stationery phase に

入るとき（13 から 20 日の間）がもっとも多いことが分かった．YPG (10% glucose) 培地ではリンゴ酸とコハク酸の生産は 13 日目でそれぞれ 4.9 と 5.5 g/L であった．リンゴ酸の生産にグルタミン酸，リンゴ酸およびコハク酸がいちじるしく影響を与えているのに対し，コハク酸の生産にはリンゴ酸だけが影響した．フマール酸とアスパラギン酸は酵母の生育と有機酸の生産を抑制した．YPG (30% glucose) 培地にグルタミン酸を 0.5% 添加したとき、リンゴ酸の生産量は 74.9 g/L に達し，酵母が利用した糖に対しての回収率は 32.8% であった．またYPG (10% glucose) 培地にリンゴ酸を 0.3% 添加したとき、コハク酸の生産量は 7.7 g/L であり，回収率は 8.1% になった．

スクリーニングテストを行った結果，V19株はゼラチンの液化性を持っていることが分かった．酵母から未精製の酵素を抽出し，水溶性と不溶性のコラーゲン，合成ペプチドおよびカゼイン基質に対する活性を測定した．酵母の酵素は市販の*Clostridium histolyticum* コラゲナーゼ [EC 3.4.24.3] と比べ，酸可溶性のコラーゲンと合成ペプチド FALGPA に対する活性はそれぞれ 1/3 と 2/5 程度であった．それに対し，カゼインに対する活性は 1/90 に止まった．25℃における酵素の生産量は静置培養では 25 日目，振とう培養では 50 時間後に最も高かった．ゼラチン添加による酵素生産量の促進効果はなかった．また培地のpH は 7.0 が最適であることが分かった．Cbz-GPLGP 基質に対しては37 ℃での最適反応 pH は 8.0 から 8.5の 間であり，酵素の活性と安定化には Ca イオンが不可欠であると分かった．

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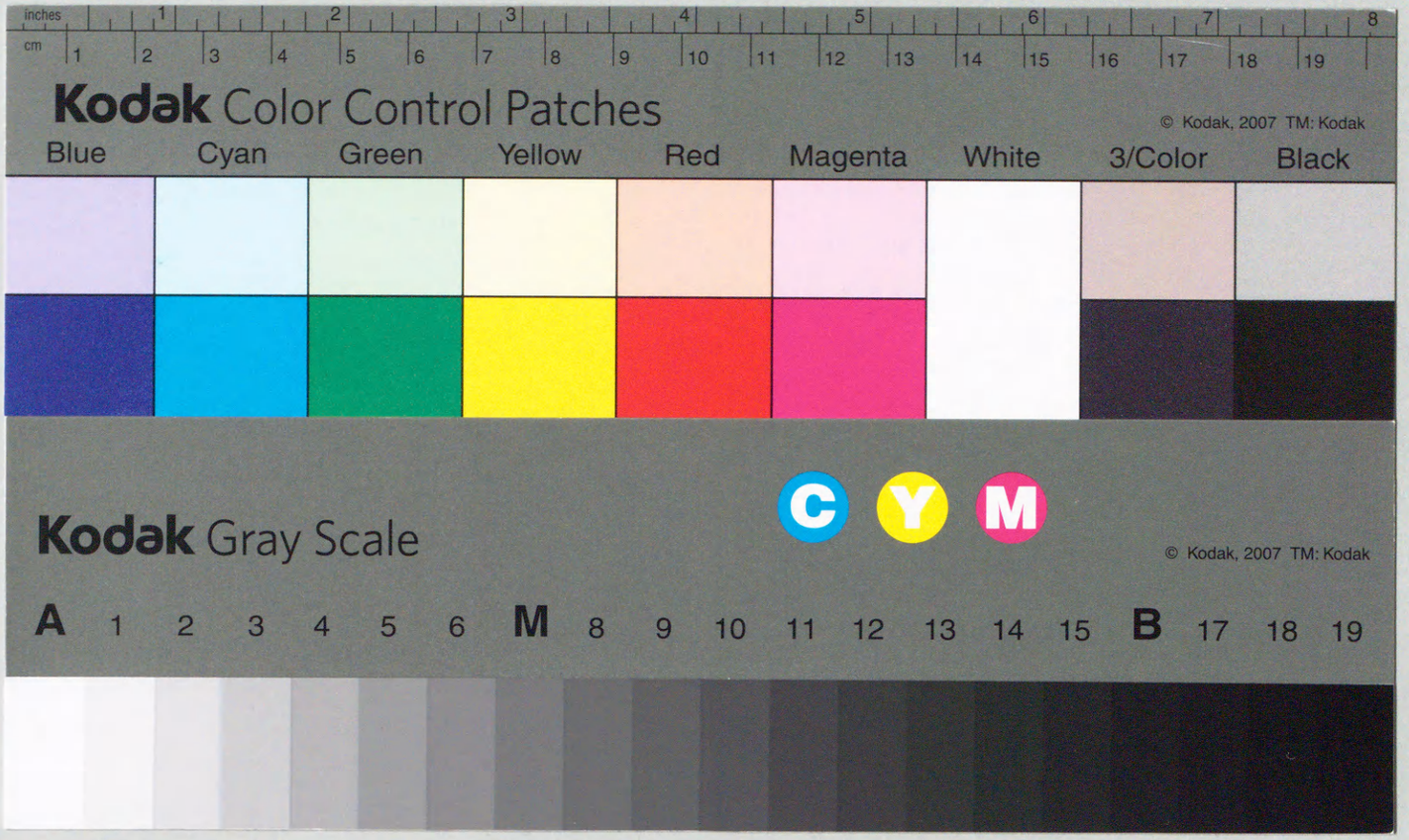
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