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DIFFERENCES BETWEEN THE FLOCCULENT YEAST AND THE NON-FLOCCULENT YEAST

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ABSTRACT

Differences between the flocculent yeast and the non-flocculent yeast, grouped by Helm's method, were investigated as a central question: the ability of adsorbing tannin, the content of phosphate compounds of various forms, and the content, property, and form of the "Zymocasein" fraction (essential component is nucleoprotein or ribosome) in the cells were chiefly discussed. The results showed the ability of adsorbing tannin and the contents of nucleic acid-, lipid- and protein-phosphorus were much the same in both yeasts. Only the contents of acid-soluble phosphorus were different, especially the difference in cold acid-soluble phosphorus was notable. Flocculent yeast was tolerably higher than non-flocculent yeast in its content. The "Zymocasein" fractions of both yeasts were nucleoprotein, and 85% of their phosphorus were nucleic-acid phosphorus, and their molecular weight were much the same, too. Their behabiours to Ca^{++} were also the same, and they were precipitated at once by Ca^{++}. The contents of the "Zymocasein" fractions of both yeasts are considered to be almost the same since no difference in the contents of nucleic acid of both yeasts were observed. However, the experimental results which in order to know the form of the "Zymocasein" fraction in cell and its degree of exudation, both yeast cells were examined, demonstrated that the "Zymocasein" fraction of the flocculent yeast cells was difficult to exude and it firmly associated with the cytoplasmic membrane system. It was also found that nucleic acid was present in the cell wall fraction of flocculent yeast. The form of the "Zymocasein" fraction in the cell was found to be remarkably different in both yeasts. Hence, it can be interpreted that the flocculent yeast cell surface presents the property of the "Zymocasein" fraction, owing to the fact that the "Zymocasein" fraction combines firmly with the cell membrane system, and, as the result, flocculating ability was shown.

Introduction

When the flocculating activity of bottom fermentation yeast is measured by Helm's method[1], namely, by measuring the volume of yeast cells settled down when yeast cells were allowed to stand for 10 min. after they were suspended in the acetate buffer of pH 4.5 containing Ca^{++}, regardless of the age of cells, the yeasts are usually grouped either into the one that flocculates, or the one that does not flocculate throughout. The variety of non-flocculent yeast, on the occasion of wort fermentation, has a large amount of suspending cells till the primary fermentation terminates, and so consequently accelerates the wort fermentation remarkably. Of the flocculent yeast, the mechanism of the essential flocculation and the flocculation during wort fermentation succeeded in becoming clear as reported previously[2,3,4].

Nevertheless, the problem of which flocculent yeast cells behave as nucleoprotein is still unknown, and in order to resolve this problem the investigation into the differences between non-flocculent yeast and flocculent yeast was taken. In the present paper, the results will be written in order.

Experimental Procedure and Results

I *Phosphorus Content and the Ability of Adsorbing Tannin of Flocculent Yeast and Non-Flocculent Yeast*

As already reported[2,3,4], the formation of flocculence of flocculent yeast was successfully explained by taking the problem of the negative charge on the yeast cell into consideration. However, the suspensibility of non-flocculent yeast has no relation with charge density, and even when there is no charge on the cells, the yeast cell fails to flocculate. As has been recognized by Wiels[5] and Eddy et al.[6], this circumstance can not be explained through yeast charge problem. Concerning this, the author has previously found that the flocculating ability of flocculent yeast was attributable to the "Zymocasein" fraction present in the cell, its negative charge depends on the phosphate compounds, and the changes of charge density have parallel relation with the amount of acid-soluble phosphate compounds, and, in the occasion of wort fermentation, the tannin-protein complex in wort adsorbs on the yeast cells and accelerates the yeast flocculation. Therefore the author started investigations with the studies of these relations. Flocculent and non-flocculent yeasts were separately inoculated into synthetic media and incubated. Having been perfected in fermentation, the grown yeasts were estimated in their ability of adsorbing tannin, their flocculating activity, and amount of nitrogen and phosphorus, according to the previously reported method[2,3,4]. The non-flocculent yeast and the flocculent yeast are called yeast A and yeast B respectively.

Table 1. The Flocculating Activity, the Ability of Adsorbing Tannin, and the Contents of Phosphorus and Nitrogen of Non-Flocculent Yeast A and Flocculent Yeast B Grown in Synthetic Media

Test yeast	Yeast A	Yeast B
Solution suspended yeast	Volumes of settled down yeast (ml)	
Fermenting wort adjusted to pH 4.6	0.0	4.9
Acetate buffer of pH 4.6	0.0	3.3
The above solution+0.005% $CaCl_2$	0.0	5.2
Acetate buffer of pH 4.6+0.03% tannin	0.0	14.3
The above solution+0.1% $CaCl_2$	0.3	—
Quantity of tannic acid adsorbed (mg/g of dry yeast)	53.0	46.3
Phosphorus content (mg/g of dry yeast)	12.7	16.4
Nitrogen content (〃)	72.6	69.6

Yeast A and yeast B had been inoculated into Wickerham's medium[7] containing 10% of glucose, 1% of polypeptone and various vitamins, and were cultured at 8°C. When the degree of Balling of each medium fell to 1.7° and 0.9°, yeast A and yeast B were collected respectively and used for experiments.

As shown in Table 1, yeast B had the typical property of flocculent yeast, and was aggregated by tannic acid and Ca^{++}. Contrarily, yeast A was not aggregated by tannic acid, not to mention Ca^{++}. Nevertheless, it is found that both yeasts had no difference in their ability to adsorb tannic acid. Accordingly the fact, that non-flocculent yeast failed to flocculate during the wort fermentation, was understood to be attributable to the fact that yeast A cell itself essentially had not the ability of flocculation, and not that the ability of non-flocculent yeast to adsorb tannic acid was lower than that of flocculent yeast. Under the circumstances, however, it is known that both yeasts have a difference in their phosphorus contents. And moreover, as has already been pointed out by Schoen[8] the phosphorus contents of flocculent yeasts are higher than that of non-flocculent yeast. Concerning the matters related to phosphate compounds, Masschelein et al.[9] have found that flocculent yeast had a sensitively adsorbing ability for Ca^{++} and was able to taken up Ca^{++} from even a solution of low concentration of Ca^{++}. And further, Kijima[10] has revealed that in the glucose fermentation, the flocculent yeast was shown to be fairly higher in the ratio of the moiety inhibited by UO_2^{++} to total fermentation velocity than that of non-flocculent yeast.

II *Changes in the Contents of Phosphorus of Various Forms of the Non-Flocculent Yeast and the Flocculent Yeast During Wort Fermentation*

The phosphorus amount of yeast cells changes as their physiological condition differed, therefore, only from the results mentioned in Table 1, it is unsuitable to conclude that the phosphorus contents of yeast A were lower than that of yeast B. Previously the flocculating ability of flocculent yeast was found to be attributable to the "Zymocasein" fraction, and yet, there existed a close relationship between the phosphorus amount of the cells and the formation of flocculence. So, for both yeasts, the contents of various forms of phosphorus in the cells at various

Table 2. Changes in Contents of Phosphorus Taking Various Forms of Yeast A During Wort Fermentation

Fermentation period (days)	2	4	6		9
Degree of Balling of fermenting wort	8.45	5.45	3.72		2.70
pH of fermenting wort	4.55	4.32	4.25		4.23
State of yeast cell	Suspending	Suspending	Suspending	Settled down	Settled down
Cold acid-soluble P(mg/g of dry yeast)	11.2	7.8	7.6	7.8	7.6
Lipid-P (〃)	1.2	0.8	0.9	0.9	0.9
Nucleic acid-P (〃)	7.8	5.7	5.2	5.3	5.3
Hot acid-soluble P (〃)	0.7	1.2	1.5	1.3	1.1
Protein-P (〃)	0.5	0.4	0.3	0.3	0.3
Total	21.4	15.9	15.5	15.6	15.2
Yeast yield (g/700 m*l*)			4.21	4.60	8.08 (0.82)

The wort of Balling 10.6° inoculated with 4 g of pressed yeast per 1 *l* of it, was poured into the glass tubes of about 2.5 cm in diameter and 50 cm in height, and was fermented at 8°C. The figure mentioned in parenthesis expresses the amount of suspending yeast. The analyses were carried out combining four glass tubes in which worts were being fermented.

Table 3. Changes in Contents of Phosphorus Taking Various Forms of Yeast B During Wort Fermentation

Fermentation period (days)	2		4		6
Degree of Balling of fermenting wort	8.34		4.63		3.65
pH of fermenting wort	4.80		4.47		4.38
State of yeast cell	Suspending	Settled down	Suspending	Settled down	Settled down
Cold acid-soluble P (mg/g of dry yeast)	15.0		11.7	10.9	10.7
Lipid-P (〃)	1.4		1.1	1.1	1.1
Nucleic acid-P (〃)	7.7		5.5	5.5	5.3
Hot acid-soluble P (〃)	1.5		2.0	2.3	2.7
Protein-P (〃)	0.6		0.5	0.5	0.5
Total	26.2	21.8	20.8	20.3	20.3
Yeast yield (g/525 ml)	3.70	0.88	3.09	3.30	5.71 (0.78)

Cultivating conditions are the same as in Table 2, but the degree of Balling of the original wort was 10.3°. The figure mentioned in parenthesis expresses the amount of suspending yeast. The analyses were carried out combining three glass tubes in which worts were being fermented.

stages during wort fermentation were compared and estimated in proportion to the method[3] described previously. Results of this for yeast A, in Table 2, and for that of yeast B, in Table 3 are shown respectively.

As seen in Table 2 and Table 3, the total phosphorus contents of yeast A were surely lower than that of yeast B through all stages during the wort fermentation. These lower contents were nevertheless understood to result from being in small quantity in acid-soluble phosphorus contents, since the phosphorus contents of the nucleic-acid form and of other forms were not difference in both yeasts. Particularly, both yeasts during the later period of fermentation were noticed to be much the same in their content of phosphorus of nucleic acid.

III *Properties of the "Zymocasein" Fraction of Non-Flocculent Yeast and Flocculent Yeast*

1) *Phosphorus Composition of the "Zymocasein" Fraction of Yeast A and Yeast B*

As already reported[2], the author discussed that the flocculating ability of flocculent yeast might be attributed to the "Zymocasein" fraction being present in the cell. She thought that there were probably some differences in properties between the "Zymocasein" fractions of both yeasts and she isolated respectively the "Zymocasein" fractions from both yeasts at the later period of wort fermentation and investigated their phosphorus composition by means of the method[3] already reported. The "Zymocasein" fraction was prepared from the cleaned yeast cells mixed with 3 volumes of water as mentioned previously[2]. The cell walls were broken down by repeating the following treatment four times: agitating the mixture for 30 min. under a reflux of iced water in a sonic disintegrator with 20 KC of frequency and 200 W in output. Through this procedure the intracellular components were educed, and subsequently, subjected to centrifugation. The cen-

trifugal residues were further washed with water through centrifuging. The supernatants and the washings of the residues were adjusted to pH 4.5 with acetic acid separately. The components precipitated from both were gathered together including all the granular components and the others which were present in cell. Accordingly, although this fraction is not always the same substance as the old "Zymocasein" of yeast protein, the fundamental constituent components of the former will be presumably the same as that of the latter, considering the fractionation method. The "Zymocasein" fraction became a pellet when it was frozen in acetate buffer solution of pH 4.6 and it denatured into completely insoluble form in the same buffer. So in the present work, the "Zymocasein" fraction isolated through the procedure of being frozen in acetate buffer solution of pH 4.6 and washed four times with the same buffer solution were used for the experiment. The results are shown in Table 4.

Table 4. Phosphorus Composition of the "Zymocasein" Fraction Obtained from Yeast A and Yeast B, and the Ratios of Nitrogen to Phosphorus

Forms of phosphorus \ Yeast strains	Yeast A	Yeast B
Cold acid-soluble P	3.4 %	2.6 %
Lipid–P	7.6	5.6
Nucleic acid–P	84.6	85.5
Hot aicd-soluble P	1.8	4.0
Protein–P	2.6	2.3
Total	100.0 %	100.0 %
Nitrogen/Phosphorus	5.55*	5.08

* Of the "Zymocasein" fraction of yeast A purified through a procedure which having been disolved in a alkaline solution, "Zymocasein" fraction is re-precipitated with acetic acid, the ratio of nitrogen to phosphorus was 5.41.

In Table 4, it was shown that the "Zymocasein" fractions of both yeasts at the termination of wort fermentation were the same in phosphorus composition and that about 85% of them were nucleic-acid phosphorus. From the results mentioned above, the "Zymocasein" fraction might be considered to be nucleoprotein. It was also shown that the "Zymocasein" fractions present in the cells had no difference in regard to their phosphorus compositions, regardless of the flocculating ability or the non-flocculating ability of the parent yeasts.

2) *Distribution of the Molecular Weight of the "Zymocasein" Fraction of Yeast A and Yeast B*

Measuring Procedure: For the original centrifugal supernatant of cytoplasmic educt obtained from the yeast cells by the sonic disintegrating treatment, and for the supernatant after precipitating the "Zymocasein" fraction from the original supernatant, the distribution of their molecular weight were respectively examined by means of Gel-filtration method through Sephadex. After that, the distribution

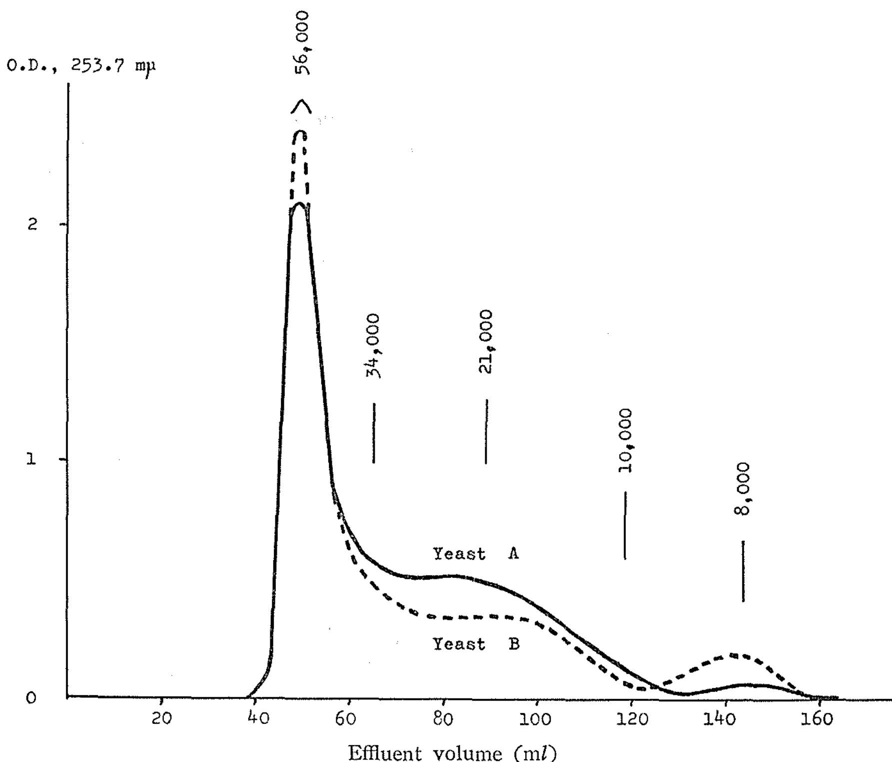

Fig. 1. Distribution of Molecular Weight of the "Zymocasein" Fraction of Yeast A and Yeast B
Sephadex G–75.
Column: $\phi 30 \times 165$ mm.
Eluting sol.: 0.1 M Na–Acetate, 0.4 M NaCl. (pH 6.0, $\mu=0.494$)
Effluent velocity: 1.4 ml/min.
Original supernatant: 1 ml.

of the molecular weight of the "Zymocasein" fraction was calculated as the difference between the two. Figure 1 was the summation of the results for yeast B and yeast A.

In Figure 1 it was shown that the distribution of the molecular weights of the "Zymocasein" fraction of both yeasts were more or less different in each section, and yet, a constitution seen from the division of the molecular weights was thought to be almost identical. Furthermore for a division of over 56,000 in the molecular weight excluded with Sephadex G–75, fractionation were attempted using Sephadex G–200. Similar continual patterns, however, were obtained even between the division of high molecular weight, and were not observed to be different.

3) *Behaviours of the "Zymocasein" Fraction of Yeast A and Yeast B to* Ca^{++}

From the experiments completed up to the preceding section, it was known that the "Zymocasein" fraction of yeast cell, regardless of the flocculating ability or the non-flocculating ability of the parent yeasts, the essential component was

nucleoprotein and, size of the constituent molecule were much the same. However, the facts that the non-flocculent yeast is insensitive to Ca^{++} and can not be flocculated with Ca^{++} are well known. In a related problem it was found that the natural nucleoprotein[11] extracted from *Streptococci* did not flocculate with Ca^{++}, and that the sol-phase nucleoprotein and microsomal nucleoprotein were different[12] in their sensitivities to Ca^{++}. Hence the "Zymocasein" fraction of both yeasts were examined to determine their ability to be aggregated with Ca^{++}. Namely, the "Zymocasein" fractions immediately after being fractioned through the preceding experiments were suspended in the acetate buffer solution of pH 4.5, at the rate of 5%. Subsequently $CaCl_2$ was added to these solution in the proportion mentioned in Table 5. After mixing well 20 ml of them were poured into graduated glass tubes 1 cm in diameter and were allowed to stand at 10°C. Then the volume of the upper clear liquid was measured at regular intervals. Table 5 indicated the results.

Table 5. Ability of the "Zymocasein" Fraction Obtained from Yeast A and Yeast B to Precipitate by Ca^{++}

Yeast strains	Yeast A		Yeast B	
Standing times / $CaCl_2 \cdot 2H_2O$ added	10 min.	30 min.	10 min.	30 min.
0 (control)	0.0 ml	0.0 ml	0.0 ml	0.0 ml
0.2 %	0.8	4.5	0.5	4.0
0.3	1.0	13.0	0.9	12.5

Quantities mentioned in Table express the volumes of clear supernatants.

In Table 5, contrary to expectation, it was shown that the "Zymocasein" fraction of the non-flocculent yeast, as well as that of the flocculent yeast, was well flocculated with Ca^{++}.

IV *Difference Between the Form of the "Zymocasein" Fraction of the Non-Flocculent Yeast and the Flocculent Yeast in the Cells*

From the results mentioned above, it was thought that both the "Zymocasein" fractions obtained from yeast A and yeast B were nucleoprotein and were different neither in quantity nor in quality. However, each of the yeasts showed a different behaviour: yeast A could not be flocculated by Ca^{++} contained in the external solution, but yeast B flocculated. In the ability of yeast to flocculate, Masschelein et al.[9] have thought the effect to be attributable to a granular basic structure of cell wall which was composed of glucan and chitin (recently the content of chitin was found[13] to be very small). Concerning this problem the author[2] has, however, found its nature to be originated in the intracellular "Zymocasein" fraction, as already reported. Considering this, the modes of the presence of the intracellular "Zymocasein" fraction of both yeasts, namely, the forms in the cells were considered as the problem and this point was therefore investigated.

1) *Experimental procedure*: A majority of the nucleic acid present in the cells of microorganisms has been known to be linked to protein as nucleoprotein. By applying this information, the investigation was performed. The ratio of nucleic-acid phosphorus to the total phosphorus in the cells of cleaned test yeast

was first determined. It was taken to represent the containing ratio of nucleic-acid phosphorus of all the "Zymocasein" fraction which was held in the cells. Then the cell wall of the same test yeast was broken down through the sonic treatment described previously. After that, the centrifugal supernatant and the centrifugal supernatant of washings with water were obtained. For both the supernatants, amounts of the total phosphorus and the phosphorus of the "Zymocasein" fraction were determined respectively. From these values the containing ratio of the nucleic-acid phosphorus of the "Zymocasein" fraction was calculated. (Under this sonic treatment, the yeast cells were broken or fissured semicircle in their cell walls and had reached the degree of being educed as cytoplasmic tissue into the external liquid. Under this condition it was possible to completely fractionate into the cell wall fraction and cytoplasm by centrifugation without breaking down the cell wall structure. On one side, the cells with damaged cell walls were between about 70% and 80% of the whole cells, but to know the exact ratio of the damaged cells was impossible. The containing ratio of nucleic-acid phosphorus of the "Zymocasein" fraction, for discussing the degree of liberation of the "Zymocasein" fraction, therefore, was based on the total phosphorus content in the educt from the cell in place of the total phosphorus content in cell. And yet, it seems that the value would serve as a standard in the investigation to find the degree of liberation of the "Zymocasein" fraction). The containing ratio of the nucleic-acid phosphorus for the yeast cells and the containing ratio of the nucleic-acid phosphorus of the "Zymocasein" fraction for the educts thus obtained have been compared and the present state of the "Zymocasein" fraction in the cells was discussed.

Yeast A, cultured in laboratory, and yeast B, immediately after it terminated its primary fermentation at brewery, were measured by means of the procedure mentioned above to determine the degree of liberation of the "Zymocasein" fraction in the cells. It was found that the degree of liberation of the "Zymocasein" fraction from yeast B was about 50% of that from yeast A. Yeast A and yeast B were further examined to determine the condition which liberated the "Zymocasein" fraction from the yeast cells in the progress of wort fermentation. The

Table 6. Liberating Degree of the "Zymocasein" Fraction from Yeast A in Progress of Wort Fermentation

Fermentation period (days)	3	6	9
Degree of Balling of fermenting wort	8.82	4.35	2.30
pH of fermenting wort	4.45	4.14	4.14
$\frac{\text{Nucleic acid–P}}{\text{Total P in cell}} \times 100$ (A)	32.8	32.0	31.4
$\frac{\text{"Zymocasein"–P}}{\text{Total P in educt}} \times 100$ (B)	34.9	35.2	37.0
(B)×0.846 (C)	29.7	29.8	31.3
$\frac{(C)}{(A)} \times 100$	90.6	93.1	99.7

Wort was fermented in KI tubes[14] at 8°C. The treated yeast (10 g) was washed four times with two volumes of water.

Table 7. Liberating Degree of the "Zymocasein" Fraction from Yeast B in Progress of Wort Fermentation

Fermentation period (days)		3	7	10
Degree of Balling of fermenting wort		9.8	5.5	4.5
pH of fermenting wort		4.60	4.30	4.27
$\frac{\text{Nucleic acid-P}}{\text{Total P in cell}} \times 100$ (A)		29.1	28.8	28.5
$\frac{\text{"Zymocasein"-P}}{\text{Total P in educt}} \times 100$ (B)	Original	28.3	15.1	14.5
	Washings	2.4 (3)	12.4 (10)	8.0 (10)
(B) × 0.855 (C)		26.1	23.4	19.2
$\frac{(C)}{(A)} \times 100$		89.8	81.3	67.4

Wort was fermented in KI tubes at 8°C. Figures in parentheses express number of times of washings with water for the treated yeasts.

results were summarized in Table 6 and Table 7 respectively. From the preliminary experiment it was expected that in yeast B it would be difficult to educe the "Zymocasein" fraction from the cells. In the experiment mentioned in Table 7, the yeasts treated through the sonic treatment were repeatedly washed with water untill there was no detection of the "Zymocasein" fraction in the centrifugal supernatant of washings. It is considered, however, that to wash the treated yeast cells too much may result in extracting the low molecular phosphate compounds from the yeast cells which did not have broken down cell walls thereby mixed with the whole cell. Consequently, the containing ratio of the "Zymocasein" fraction might go down. This question was investigated to estimate the containing ratio of phosphorus of the "Zymocasein" fraction in each of the original yeast liquids by performing sonic treatments and washings on them. This question was found to be considered unnecessary.

Discussing the results upon non-flocculent yeast A, which was shown in Table 6, its "Zymocasein" fraction was understood to be almost all liberated from the yeast cells through all the stages in the wort fermentation. Contrary to these, in the case of yeast B, as shown in Table 7, it was found that its "Zymocasein" fraction was tolerably well liberated from the yeast cells at an early stage of wort fermentation, but as the fermentation progressed the liberating degree of the "Zymocasein" fraction was found to go down. It is recognized that the form of the "Zymocasein" fraction in the cells, namely, the structure of the membrane system or the constituent composition of it, was clearly different in both yeasts.

Concerning the problem of there being a difference in the cell wall structure of both yeasts, Eddy et al.[15] have found that the cell walls, in the preparation of yeast cells possessing abilities to agglutinate, possessed the ability to agglutinate quite similar to those shown by the parent yeasts. And the ability of the cell walls to agglutinate was progressively lost by treatment with papain, since in this case the release of "material A" composed of a carbohydrate (as mannose) of 85%, of nitrogen of 1.2%, and of phosphorus of 0.37%, followed a roughly parallel course. Furthermore, from the results investigated of the properties of the cell

walls of 13 strains of yeasts including top and bottom, they[16] found that the cell walls prepared from mature cells reproduced the same flocculating behaviours as their parent yeast cells, although that of the young cells did not. The results of Eddy et al. agreed with a phenomenon in which the degree of the liberated "Zymocasein" fraction from the cells went down as the fermentation age progressed on yeast B. Accordingly, the results known for yeast B indicated this to be common to all of the flocculent yeasts. Another question related with these matters concerned ribosome in *Bacillus subtilis* which was present in a free state in an actively growing cell, and was present in a state associated with the membrane structure in a period immediately before turning into a resting state[17].

V *Presence of Nucleic Acid in the Cell Wall Fraction of Flocculent Yeast*

Masschelein et al.[9] had reported that the cell walls of the flocculent yeast cells did not contain nucleic acid. The cell wall fractions used for the experiment by them were, however, substances gathered only partly, since they were not stained with methylene blue nor toluidine blue nor with Gram-staining, from the finely broken down cell walls. Though the activity of staining with basic dye such as toluidine blue and methylene blue were matters of course as well as Gram-staining activity, the staining activity has been found[18,19] to be in a close relationship with ribonucleic acid. Therefore, if nucleic acid was not detected in this unstained part, there was no substantial evidence that nucleic acid was not present in the cell wall system itself. On the one hand, a basic layer of cell wall, composed of glucan, was shown to be a fundamental seat for the appearance of flocculence[9]. This suggested that they were associated with ribosome, since they had an appearance of a granular structure. And moreover, this layer had been shown[20] to have significant amounts of phosphorus. Recently, Eddy et al.[21] have found that the cytoplasmic membrane is a major portion of the cell wall system and it contained ribonucleic acid and deoxyribonucleic acid. Hauge et al.[22] have also found it contain high amounts of ribosome in larger particles isolated from the French pressure cell homogenates of a yeast cell. On the other hand, as a problem related with this matter, the recent studies have demonstrated that ribosome or nucleoprotein were associated with various bacterial cell membranes[17,23~32]. As from the results obtained by the preceding experiment, the presence of nucleic acid in the cell membrane system of flocculent yeast was detected, and so the cell wall fraction was isolated from yeast B at a later stage in wort fermentation to determine the presence of nucleic acid in the cell wall fraction according to the previously mentioned method[3]. The results were given in Table 8 and Figure 2.

Isolation of the Cell Wall Fraction from Yeast B: Cleaned yeast cells mixed with three volumes of water were successively treated three times in a sonic disin-

Table 8. Contents of Phosphorus of Various Form in Cell Wall Fraction of Yeast B

Cold acid-soluble P	0.07 mg/g of dry cell wall fraction
Lipid–P	0.07 〃
Nucleic acid–P	1.96 〃
Hot acid-soluble P	0.28 〃
Protein–P	0.42 〃

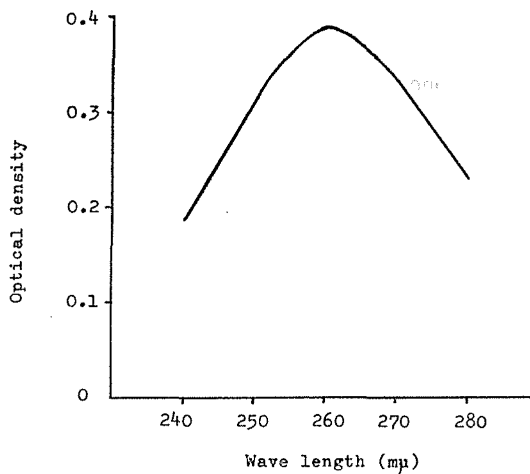

Fig. 2. Absorption Spectrum of the Extract from Cell Wall Fraction of Yeast B

tegrator at 1°C, for 30 min. The cytoplasms were removed by centrifugation. The sonic disintegration of the yeast cells and the removing treatment of cytoplasms were repeated untill the yeast cells were almost disintegrated. When the small fragments of the cell walls of yeast B were frozen, they showed the ability to flocculate which held even in a condition of very fine fragments, so that the cell wall fractions and cytoplasms could be well separated by centrifugation. The cell wall fraction isolated in this way was suspended in 10% sucrose, and was subsequently subjected to fractional centrifugation. The cell wall was completely freed from the cytoplasmic granule and was purified through washing with distilled water. Though the cell wall fraction did not contain cytoplasmic granule, they contained a trace of the whole cell. As for yeast A, the cell wall fraction acquired an ability to suspend as the cell walls disintegrated, and it was difficult to separate the cytoplasmic granule by means of the same procedure as isolating the cell wall fraction from yeast B, which made a notable difference between the two yeasts.

As shown in Table 8, the cell wall fraction isolated from yeast B contained about 0.2% of phosphorus of nucleic acid. This value corresponded to 37% of that (5.4 mg/g) of whole cell. In the cell wall fraction a trace of the whole cell was observed. Even if the cytoplasmic granules could not be detected in microscopy, the presence of an undetectable granule was doubtful. However, the containing ratio of nucleic acid in the cell wall fraction was such a high ratio that it was impossible to explain it through the contamination by them. Therefore it was concluded that the cell membrane system of yeast B contained nucleic acid; in other words, the cell wall system of yeast B was associated with nucleoprotein or ribosomes.

Discussion

From the results of the experiment achieved above, the difference between the flocculent yeast cell and the non-flocculent yeast cell is considered to be mainly a condition of the association between intracellular nucleoprotein and the cell wall

system when it contains cold-acid soluble and hot-acid soluble phosphate compounds. And thus, for the flocculent yeast cell, it may be interpreted that since the nucleoprotein is strongly associated with the cell wall system, or since the nucleic acid is associated with the protein[13] in the cell wall, the cell itself will behave as a nucleoprotein in an external solution. The so-called cell walls have been found to contain no nucleic acid, however, as mentioned above, the presence of nucleic acid or ribosome in cytoplasmic membrane has been found in various kinds of bacteria. The yeast cell wall, as well as that of general bacteria, is known[20,33] to have a pore structure and to possess the ability to permeate even considerably high molecular substances. A special permeability of nucleic acid to the cell wall has also been found because the transformation[34] of a yeast strain into a different yeast strain was possible through desoxyribonucleic acid, which was derived from a different yeast strain and added to the culture medium. Besides, nucleic acid-polysaccharides complex (the hydrolysate gave mannose) has been isolated[35] from a yeast. The evidences combined together with those facts and the results of the experiment described above will give the thorough appropriateness to render the conclusion mentioned above to yeast B. Concerning the relationship between yeast B and yeast A, in the reports related to the presence of nucleic acid or ribosome in the cell membrane, conflicting results are given even on the same strains of bacteria. As for *Micrococcus lysodeikticus*, Gilby et al.[36] could find no more than traces of nucleic acid in the protoplast membrane while Gel'man et al.[27] have recognized that it contained nucleic acid of 12.2% in the cytoplasmic membrane. The same conflict exists concerning *B. megaterium* strain M, as revealed in the results of Weibull et al.[37] and Wachsman et al.[30]. From those facts and the results of the experiments on yeast A, it is possible that in yeast A, the cell membrane system is stable to the external solution, and so the yeast cells are able to hold their suspending ability, because either the intracellular nucleoprotein is free from the cell membrane, or the power and amount of association is weak enough to allow them liberate state. This opinion of the author (the details will be shown later) is substantiated by Kijima[10] who has found that there is remarkable correlation between the ability to be inhibited on the fermentation rate of the bottom brewing yeast by UO_2^{++} and their flocculating character.

Masschelein et al.[38] have considered that the difference between the flocculent yeast and the non-flocculent yeast depended on a difference in the amount of mannan which is the main component of the yeast cell wall. The mannan content of the non-flocculent yeast was nearly constant through all the stages of the fermentation process. Contrary to the case of non-flocculent yeast, the mannan content of the flocculent yeast cell at the later half of the earlier period of fermentation process was twice as much as that of the non-flocculent yeast, and it decreased gradually accordingly as the fermentation progressed. At the later period when the yeast cell began to flocculate, the mannan content became half of that of the non-flocculent yeast. And after the mannan was removed by washing with alkaline solution, the non-flocculent yeast cell began to strengthen in its flocculating activity. And they have further thought that the ability of the yeast cell to flocculate was attributable to the glucan layer mentioned previously. Nevertheless, flocculent yeast can flocculate in the presence of Ca^{++} through all stages of the fermentation process, regardless of the amounts of mannan content in the yeast cell. This matter has been cleared by Helm et al.[1] and is also recognized through the present works by the author. It is known[39] that when a yeast cell which flocculated well was stored, its floccu-

lating activity gradually decreased. Those facts can not be explained by its lower content of mannan in its yeast cell than that of the non-flocculent yeast cell. The fact that a characterized optimal pH fixed for the flocculation of flocculent yeast is existent can not be explained if the fundamental substance of producing the flocculating ability is glucan.

Kijima[10] investigated, for the brewery yeasts, the relationship between the extent of the inhibition of fermentation velocity by UO_2^{++} and the tendency to the flocculation. He has made it clear that there is an extremely remarkable correlation between the inhibition of fermentation by UO_2^{++} and the degree of flocculating ability. So, in proportion to the increase of the flocculating ability, the rate of an enzyme system sensitive to UO_2^{++} which was occupied in the total fermentation velocity in glucose fermentation, increases. For this correlation, Kijima, as well as Masschelein, has related it to the contents of polysaccharides in the cell wall of yeast. But if the cell wall has a pore structure and has the ability to permeate even tolerably high molecular substances, it can not be thought that the large obstruction against the permeation of UO_2^{++}, as seen in his experimental results, is brought about through a difference in the thickness of its exterior layer. And if, as in Kijima's opinion, this is dependent on the content of the polysaccharides in the cell surface in the case of flocculent yeast, a moiety of enzyme system sensitive to UO_2^{++} must be naturally changed according to the change of the mannan content which changes with the ripeness of the cell, as found by Masschelein et al. Kijima has, however, known that the correlation did not related to the degree of the ripeness of the cell. The author interpreted the problem as follows, namely, UO_2^{++} is known[33] for its specially strong affinity to polyphosphate. Van Steveninck[40] has recently discussed that an inhibition of the glucose uptake by the yeast cell, caused by UO_2^{++}, was attributed to an inhibition of phosphorylation which was due to the binding of UO_2^{++} to the polyphosphates present at the cell surface. Cirillo et al.[41] have reported that the yeast cell, washed with a solution of uranyl nitrate, prevented intracellular sugar loss (not consumption of sugar, but a reverse transport of sugar). If the action of UO_2^{++} belongs to either case, this effect of binding UO_2^{++} appears as the phenomenon of inhibiting fermentation. One of the substances binding UO_2^{++} is thought to be ATP which is a co-enzyme in glycolysis and also an energy source of active transport of the biological membrane to cation. On the other hand, ATP has been found to be an essential component of yeast ribosome by Ohtaka et al[42]. Being related to such matters, the correlation between the increase on flocculating ability of yeast and the increase in the moiety of enzyme system sensitive to UO_2^{++} represents a correlation between the increase in the flocculating activity of yeast and the increase in the content of ribosome associated with cell wall system.

Mill's theory[43] of yeast flocculation, in which the flocculent yeast cells are linked, joining two different cells by salt bridges with Ca^{++}, has recently been presented and Lycette et al'.[44] discussed that lipids or phospholipids were involved in the yeast flocculation: the former may connect with ribosomes present in the cell walls, and the latter may connect with the removal of ribosome from the cell membrane accompanying a destruction of the cell membrane structure which is attributed to the removal of lipid or phospholipid from the cell membrane. Further, Masschelein et al.[38] have observed that when yeast was grown in a medium containing NaF, the flocculating activity of the grown yeast increased. They consider this phenomenon to be a result of the inhibition of mannan synthesis by

NaF (but, it was written in the report that the inhibition of mannan synthesis was not certain). However, it is probably interpreted to be attributed that in this situation the inositol, present in the medium (the yeast extract is added to medium), antagonistically reacted to NaF[45], so the yeast cells naturally acquired properties of yeast grown in an inositol-deficient medium. The phenomenon activating the flocculation of the yeast cells grown in the inositol-deficient medium is known already[46,47] not only to be a simple decrease in the mannan-synthesizing amount, but also to be depended on its effect[47,48]. In addition, Masschelein et al.[38] have adduced that the flocculent yeast evolved carbon dioxide in larger amounts than the non-flocculent yeast evolved in the presence of 2, 4-dinitrophenol. But this phenomenon is able to be explained as follows. Flocculent yeast contains acid-soluble phosphorus compounds in larger amounts than does non-flocculent yeast.

Conclusion

The main differences between the flocculent yeast and non-flocculent yeast were found in the following two matters:

1) The content of acid-soluble phosphorus compounds of flocculent yeast is higher than that of non-flocculent yeast.

2) There is a difference in the associating condition of the intracellular "Zymocasein" fraction and the cell membrane system, namely, in flocculent yeast the nucleoprotein (mainly, ribosomes) is strongly associated with the base part of the cell membrane system, so the whole cell behaves like nucleoprotein.

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