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THE MECHANISMS OF FLOCCULATION OF FLOCCULENT YEAST DURING THE WORT FERMENTATION

PART II

THE SUBSTANCES ENDOWING THE NEGATIVE CHARGE TO YEAST CELLS AND THE MECHANISM OF THE ESSENTIAL YEAST FLOCCULATION (SECOND "BRUCH")

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ABSTRACT

It was found that the negative charge density on the yeast cells closely related to the quantity of a series of the substances related with nucleic acid including phosphate as the essential component in the yeast cells. Among these substances, nucleic acid is, however, a substance participating in the flocculation of yeast cells as the element constituting "Zymocasein". Consequently, it is considered that yeast charges are endowed by those phosphate compounds locating near the surface of yeast cells except nucleic acid. Majority of those phosphorous components are related to the energy metabolism, and their amounts dynamically change in accordance with the age of the cells, the nutritional condition of the medium and other various factors which internally or externally give a change in the physiological action of the cells. Consequently, in the wort fermentation, when its pH becomes about 4.5, yeast cells can hold a suspending state depending on their physiological condition: during a condition in which the activity of energy metabolism is vigorous, the negative charge density of the yeast cells is high, and the yeast cells keep a free, suspending state resisting the flocculating action of the cells. And when the activity of the energy metabolism of cells reduced, their negative charge density fall and so the yeast cells flocculate.

Introduction

Colloid-chemical explanation of the formation of essential flocculence of yeast cells (either the yeast flocculation determined by Burns's method[1], or the formation of the second "Bruch" proposed by the author[2]), has been concluded to be impossible, since the yeast cells do not form flocculence between pH 2 and 3, which is recognized to be their isoelectric point. So this conclusion has been supported by many investigators. However, the author[3] considered from the electrophoresis figures investigated by Silbereisen[4] that this isoelectric point of the yeast cells, as believed by the investigators heretofore, was not the so-called isoelectric point

which was discussed in the studies of protein, but rather it was an isocharge point at which the magnitude of both the negative and the possitive charges of charged components in the cell became equal, and that the substances that endowed the negative charge to the yeast cells from the state which converted the charge of the yeast cells would be attributed to a series of the compounds related to nucleic acids including phosphate as the essential component. Under this consideration it becomes clear that yeast protein is retained in considerably lower pH than its isoelectric point, as mentioned in Part 1, and then changes into a hydrophility depending on an increase in the hydration grade. Consequently, the yeast cell is stabilized and can not form the flocculence even if it made itself electrically neutral. Contrarily, in the buffer solution of pH 4.6, the yeast cells naturally become able to flocculate for the possession of character as mentioned in Part 1 (it was an hypothesis at that time) when the contents of the substances related to nucleic acids decreased. As already had indicated[5], these considerations made the colloid-chemical explanation possible on the problem of yeast flocculation. The author also considered that the contents of substances related to nucleic acid including phosphate as the essential component dynamically change in accordance with the age of the cells, and with the internal or external factors giving the changes in the physiological action of the cell in fermentation process. Consequently, as already had reported[5] it became possible the explanation of the biological theories which were hitherto proposed for yeast flocculation (namely, the deficiency of vitamins and nutrients caused by the contamination of lactic acid bacteria[6], the deficiency of growth factors[7], the deficiency of fermentable sugars[8], a toxic substance such as furfural[9], generation of yeast[10], the deficiency of inositol[11], and the other problems such as the incubating temperature, the light, the culture condition, or aeration being observed in experiments). In the previous experiments on these problems, in order to investigate the relation between the velocity of wort fermentation and the contents of substances mentioned above (for the worts made a difference in the contents of formol-nitrogen), the phosphorus contents of the yeast cells at various stages in the fermentation process were determined by the intensity which had been stained with toluidine blue, and their charge densities were measured by the amounts of added wort required to flocculate the yeast cells suspended in the buffer solution of pH 4.6, respectively. And so, the explanation has (at first sight) been regarded as appropriate[12].

In the present experiment, phosphorus contents of the cells were directly determined by chemical method, its appropriateness was reconfirmed, and then the mechanism of the essential flocculation of yeast cells was established.

Experimental Procedure and Discussion

I *Changes of Phosphorus Contents in Yeast Cells During the Wort Fermentation and the Effect of the Contents of Formol-Nitrogen in Wort on the Cells*

As the contents of formol-nitrogen in wort increased, the charge density of the grown yeast cell increased in proportion to the increased quantity, which resulted in the depression of the yeast flocculation, and consequently accelerated the wort fermentation. Though this fact have already been reported, the phosphorus contents of the grown yeast cell were chemically measured, compared and investigated

this time by using two sorts of malts with a different quantity of fomol-nitrogen.

(1) *Experimental Procedure*: From both malts with a different quantity of formol-nitrogen, two kinds of wort were prepared. Into both worts the purely cultivated flocculent yeasts were inoculated as follows; 4 g of the centrifugally separated yeast to 1 *l* of worts. Following the conventional method, the worts were poured into glass tubes 2.5 cm in diameter and 50 cm in height and they were fermented at 8°C. Then the contents of phosphorus of various forms were determined for the yeast of the 2nd day, of the 4th day, of 6th day and of the 10th day in fermentation. At the same time, the yeast yield, the degree of Balling of the fermenting wort, and the amount of sediment yeast were determined. After this, the grown yeast cells were washed three times with two volumes of a physiological solution of sodium chloride at 0°C used for the experiments. The determination of phosphorus depended on the modified Allen's method. The various forms of phosphorus were determined as follows; for the cold acid-soluble phosphorus, 1 g of the cleaned centrifugally separated yeast cells were extracted three times with 5 m*l* of 10 % trichloroacetic acid iced, at intervals of 5 min.; for the lipid-forming phosphorus, the residues were extracted two times with 5 m*l* of 70 % ethanol, at intervals of 2 hours at room temperature, and then they were extracted three times with 5 m*l* of the mixtures of ethanol and ether (3 : 1) at 40°C; after that, the two extracts were combined, and the extracted residues were extracted three times with 5 m*l* of 1 N perchloric acid, at intervals of 20 min. at 70°C as the hot acid-soluble fraction, in this way, were fractionated respectively. In the hot acid-soluble fraction, the content of phosphorus forming nucleic acids was calculated from the degree of light absorption at 260 mμ in wave length, and then the amount of hot acid-soluble phosphorus was made of the difference between the total phosphorus amount of hot acid-soluble fraction and the amount of phosphorus forming nucleic acids. The phosphorus contents in the final residues were determined as protein-forming phosphorus. In another experiment, the changes of the phosphorus contents in the cells were investigated by the M−T staining method[13], and the results were compared with the results obtained by the chemical analyses. The results were respectively summarized in Table 1 and Table 2.

Moreover, as was shown in both Tables, the total phosphorus contents of the yeast cells were in complete accord with the view of them considered from the tests by the M−T staining method.

As shown in Table 1, in the case of wort containing a large quantity of formol-nitrogen, the phosphorus contents of fermenting yeast cells hold a higher level to the termination of fermentation, and the amounts of suspending yeast cell were high, too. In the case of the wort shown in Table 2, containing a small quantity of formol-nitrogen, though the phosphorus contents of fermenting yeast at the early stage in fermentation were rather more than that shown in Table 1 (it probably took part in an effect in which the incubated yeast cell was high in the phosphorus content), with progress in the fermentation, it is recognized that the phosphorus contents of the yeast cells decreased early. In parallel with that, the yeast cell formed the flocculence early. Accordingly, it might very likely be that the phosphorus contents of the grown cell were influenced by the dependence on the contents of amino acids or composition of amino acids in wort, and at the same time, the yeast cells might change their flocculation time.

Table 1. The Phosphorus Contents of Yeast Cells Grown in the Wort Mashed with the Malt Containing Large Amounts of Amino Acids

Fermentation period (days)	0	2		4		6		10	
Test yeast	Inoculated yeast	Suspending yeast	Sediment yeast	Suspending yeast	Sediment yeast	Suspending yeast	Sediment yeast	Suspending yeast	Sediment yeast
Amount of yeast (Multiple to amount of inoculated yeast)	—	1.83	0.47	1.93	1.19	0.95	2.87	0.09	3.71
Degree of Balling	10.40	8.34		5.00		2.64		1.64	
pH	5.56	4.80		4.60		4.52		4.50	
Cold acid-soluble P	11.6	14.6		12.1		11.8	11.1		10.9
Lipid-P	1.2	1.5		1.4		1.4	1.3		1.2
Nucleic acid-P	4.8	7.9		6.1		5.7	5.6		5.4
Hot acid-soluble P	2.5	1.0		2.8		3.2	3.0		3.2
Protein-P	0.5	0.5		0.4		0.4	0.4		0.5
Total phosphorus	20.6	25.5	22.1	22.8	21.4	22.5	21.4		21.2

The content of formol-nitrogen; 25.30 mg %. Degree of Balling of the wort fermented to the termination by *Sacch. oviformis*; 2.05°. Degree of Balling of the wort fermented completely by bottom fermentation yeast; 0.90°.

Phosphorus contents express mg per 1 g of dry yeast. The analyses were carried out combining three glass tubes in which worts were being fermented.

Table 2. The Phosphorus Contents of Yeast Cells Grown in the Wort Mashed with the Malt Containing Small Amounts of Amino Acids

Fermentation period (days)	0	2		4		6	
Test yeast	Inoculated yeast	Suspending yeast	Sediment yeast	Suspending yeast	Sediment yeast	Suspending yeast	Sediment yeast
Amount of yeast (Multiple to amount of inoculated yeast)	—	1.76	0.42	1.47	1.57	0.37	2.72
Degree of Balling	10.33	8.34		4.63		3.65	
pH	5.50	4.80		4.47		4.38	
Cold acid-soluble P	13.2	15.0		11.7	10.9		10.7
Lipid-P	1.1	1.4		1.1	1.1		1.1
Nucleic acid-P	4.5	7.7		5.5	5.5		5.3
Hot acid-soluble P	3.6	1.5		2.0	2.3		2.7
Protein-P	0.6	0.6		0.5	0.5		0.5
Total phosphorus	23.0	26.2	21.8	20.8	20.3	20.5	20.3

The content of formol-nitrogen; 15.88 mg %. Degree of Balling of the wort fermented to the termination by *Sacch. oviformis*; 2.30°. Degree of Balling of the wort fermented completely by bottom fermentation yeast; 1.39°.

The phosphorus contents express mg per 1 g of dry yeast. The analyses were carried out combining three glass tubes in which worts were being fermented.

II *The Substances Endowing the Negative Charge to Yeast Cells*

As seen in Table 1 and Table 2, the changes in the total phosphorus contents in the yeast cells at various stages which took place in the progress of fermentation completely agree with the phenomena that in the wort fermentation, the charge densities of yeast cells changed with progress of fermentation, and the sediment yeast cells and the suspending yeast cells were different in the charge densities, which were determined by means of the electrophoresis method by Jansen et al.[14]. The results were also accord with the phenomena that an inverse relationship was present between the electrophoretic mobility and the flocculating activity of yeast cells, since the electrophoretic mobility of yeast cells reduced correspondingly with the increase in the fermentation period, which were found by Eddy et al.[15]. Fukui et al.[16] had recently found that having been cultivated in a medium containing lactic acid, sake yeast readily formed the flocculence and showed a marked decrease in the content of NAD, CoA (they are nucleotide coenzymes) and cytochrome c as compared with the control yeast[17]. A phenomenon in which the yeasts cultivated in an inositol-deficient medium increased their flocculating activity was a fact known since long before, and in this case, it has been found[18] that the same phenomenon as mentioned above occurred in yeast cells. Recently, Challinor et al.[19] have found that although both the inositol-deficient yeast and the normal yeast had the same ribonucleic acid contents, inositol-deficient yeast cell walls were lower in phosphorus content than normal yeast cell walls. The author[3] has already considered that the negative charge on yeast cells might be attributed to a series of the compounds related to nucleic acid including phosphate as its essential component and from the pH value converting the yeast charge. Rosenburg[20] has already inferred that the presence of phosphorus in yeast cell walls, from the phenomenon which the fermentation velocity of yeast was inhibited by UO_2^{++}. Recently, Eddy et al.[21] had determined the phosphorus content in cell walls, and showed that the negative charge of yeast cells was atrributed to phosphorus. On the other hand, Lindquist[22] has surmised nucleic acid to be present on the yeast cell surface. Van Steveninck[23] has found that an inhibition of the uptake of glucose by yeast, which necessitated treatment with UO_2^{++}, was due to the binding of UO_2^{++} to polyphosphate groups at the cell surface. Besides, it has been made an established theory that the energy source for the active transport of biological membrane to cation is ATP. Also the presence of ATPase in a cell membrane of bacteria has been recognized[24]. Consequently, the possibility of the presence of compounds related to ATP and ATPase were discussed. Few et al.[25] discussed, in studing the cytoplasmic membrane of *Micrococcus lysodeikticus*, that its electrophoretic mobility decreased by treating it with UO_2^{++}, and that the compounds binding with UO_2^{++} were phosphatidic acids. Macfarlane[26] has detected cardiolipid, phosphatidyl-inositol, phosphatidyl-glycerol and some phosphatidic acid from phosphatidic acids present in the cell membrane of the same bacteria. Moreover, teichoic acids, which were closely related to nucleotides, were recently found in the cell wall of bacteria[27,28,29,30,31,32].

Putting together the evidences mentioned above concerning the flocculent yeast cell, it is permissable to conclude that the substances endowing the negative charge to yeast cells, are a series of the substances related to nucleic acids with phosphate as the essential component, and the negative charge density on yeast cells decreases in parallel with the decrease of the substances related to nucleic acids. Further, in respect to this decrease, yeast cells form the flocculence. Among those substances,

nucleic acids are, however, as reported in Part I, recognized to participate in the formation of flocculences as the essential component of "Zymocasein" (Lindquist[22] has surmised that the negative charge of yeast cell is attributed to nucleic acid). In the wort fermentation, the phosphorus which give the negative charge to yeast cells will be mainly the acid-soluble phosphorus, and of this, only those which located near the cell surface will contribute. A special fine effect[33,34] of Ca^{++} on the acceleration of yeast flocculation will be a proof of the view.

The Mechanism of Essential Flocculation of Flocculent Yeast

Summing up the preceding section, the mechanism of the essential flocculation of flocculent yeast is able to be described as follows: the flocculent yeast cell is possible to behave itself as the yeast nucleoprotein through some unknown mechanism, and, due to the fact that pH 4.6 is the isoelectric point of nucleoprotein, the yeast cells became remarkably unstable and readily reached a precipitable state when they were suspended in the acetate buffer solution of pH 4.6. Nevertheless, in yeast cells, phosphate compounds, required for energy metabolism and for the life of the cells, are present. By those phosphate compounds yeast cells are endowed with negative charges; and depending on a quantitative relationship with those phosphate compounds, they are able to retain a suspending state or to flocculate. The yeast cells hold a suspending state during the condition in which the activity of the energy metabolism in the yeast cells is vigorous and contents of phosphate compounds in it are highest. When there is a fall in the fermenting activity, the charge density on the yeast cells decrease, and the yeast cells flocculate and settle down.

On the Various Phenomena of Yeast Flocculation Observed During the Wort Fermentation; and the Control of the Velocity of Wort Fermentation

The transformation of energy attending on the fermentation is performed through the phosphorus metabolism. Consequently, the phosphorus contents of yeast cells during the fermentation process change in accordance with aeration, process of the cultivation and other various factors that gives internal and external influences on the energy metabolism of yeast as well as the nutrients, growth factors, and mineral salts of the culture medium. However, in the case of wort fermentation, the cultivating condition is constant in principle, and so the fermentation velocity is mainly governed by the condition of the process of cultivation of yeast and the nutritional condition of wort. The condition of the cultivation process is a special matter and may be excluded; so that only the nutritional condition in the medium is concerned. As vitamins and mineral salts are richly contained in the wort, so it is thought that the energy metabolism of the yeast cells will progress without a hitch. The amounts of energy accumulated in the yeast cells are mainly controlled by the structure of the fermentable sugars. Nevertheless, as reported[12] previously, the amounts of energy accumulated in the yeast cells also change according to their dependence on the contents of amino acids in wort. In particular, in the case of wort fermentation, the latter was considered to be of importance. A wort was supplied with amino acids, and fermented. As the result, the wort added amino acids, in the fermentation the yeast began to flocculate more slowly, was more markedly accelerated in fermentation velocity

than was the control wort, which has already been reported[12]. In the present paper, the influence of the difference of the contents of amino acids is recognized clearly. As shown in Table 1 and Table 2, both of the worts, during a period at which the fermentable sugars are present in the high amount at the early stage of the fermentation process, have the yeasts with the high phosphorus content and there are no differneces in phosphorus contents between the yeasts. And yet, as they progress to the middle period of fermentation, it is found that the yeast fermtened the wort containing small amounts of amino acids (as expressed in Table 2) and decreased rapidly in the phosphorus contents. It is thought that at this period in the wort, amino acids which are required for the turnover of enzymes, which are necessary for metabolism of the yeast cells, are exhausted[35]. Accordingly, the yeast must provide the required amino acids through synthesizing from the other amino acids or the decomposition of peptide ingredients. At this time, the condition supplying the energy which is necessary for this is markedly deteriorating owing to the decrease in the concentration of sugars, and at the same time, the energy source itself is markedly deteriorating, which is attributed to the increase in the containing ratio of sugar difficult in fermentation (such as maltotriose[36,37]). The yeast cell must therefore divert the energy accumulated in the interior of its own body for this purpose. Consequently, the negative charge density of the yeast cells rapidly decreases and the yeast cells flocculate, and settle down. As both the worts have little difference in the degree of Balling of the final wort fermented by *Sacch. oviformis*, it is thought that the contents of the higher molecular sugar of over maltotriose scarcely differ between the worts, and so the difference in the fermentation velocity between the worts is concluded very likely to be originally attributed to the difference in the contents of amino acids. On the effect of the quantity of amino acids of the medium on the fermentation velocity of this, long ago Malkow[6] found it to stimulated yeast flocculation in the case of a lack in nitrogen source at the fermentation of molasses, and Devreux[38] has likewise found this to occur in the use of the wort lacking in nitrogen source.

Above-mentioned is a discussion in which the control of the fermentation velocity of wort is considered from the view point of the composition of wort, and yet, the condition of aeration to wort is also a factor able to control in large part the fermentation velocity[39]. It is well known that aeration of wort affects greatly the phosphorus content of the yeast. Furthermore, it has been found that yeast cells grown under fully aerobic condition are larger in electrophoretic mobility[40] and their suspending activity becomes more powerful[41] than those obtained from microaerophilic cultures. The pathway acquiring the energy, taking into consideration the above circumstances, seems to be natural. Accordingly, it seems very likely that in the case of the wort with the same composition, the fermentation valocity depends on the aerating condition of the wort. Therefore, principally, the three factors described above have been discussed sufficiently, and measures to meet the situation have been taken, so the control of wort fermentation seems very likely to become possible to a certain extent.

Summary

1) The worts were prepared from the malts with a different quantity of amino acids. After that, for the flocculent yeast, the relationship between the ability of flocculating yeast and the changes in the contents of phosphorus of various

forms in grown yeast, noting the progress in fermentation and the contents of amino acids in wort, were investigated. The results showed the contents of amino acids in wort affected the phosphorus contents of yeast after the middle period of fermentation. In the case of the wort containing a small quantity of amino acids, the phosphorus content of yeast decreased rapidly and yeast formed the flocculence at an early stage. In the case of the wort with a large quantity of amino acids, the decrease in the phosphorus content of grown yeast, progressed slowly, so, the considerably large amounts of the yeast cells were held in the suspending state to the termination of the primary fermentation. It was thus recognized that the yeast cell changed its flocculation time, depending on the contents of the amino acids in wort.

2) It was known that the phosphorus content of the yeast cells and its flocculation activity were in a correlative relationship. Accordingly, it was considered that phosphorus content of yeast also had a correlative relationship with the negative charge density of the yeast cells.

3) It was discussed that the factor giving the effect on the charge density of the yeast cells was presumably the acid-soluble phosphorus compounds located near the cell surface among the substances related to nucleic acid.

4) Yeast flocculation which was brought about in the acetate buffer solution of pH 4.6, namely, the mechanism of the essential yeast flocculation, was discussed.

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