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THE MECHANISMS OF FLOCCULATION OF FLOCCULENT YEAST DURING THE WORT FERMENTATION

PART III

THE ACTION OF TANNIN-PROTEIN COMPLEX IN WORT TO ACCELERATE THE YEAST FLOCCULATION, AND THE MECHANISM OF YEAST FLOCCULATION DURING THE WORT FERMENTATION

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ABSTRACT

It was found that wort proteins adsorbed on the surface of yeast cells as a form of tannin-protein complex, and accelerated yeast flocculation. This was not an effect due to an electric neutralization reaction, but was understood to be attributable to the tannin residue of tannin-protein complex (which reacted on the protein radical of the yeast cells), adsorbed on yeast cells, and so strengthened the characteristic of tannin to precipitate protein, and restrained the motion of the yeast cells. Accordingly, the higher the molecular weight of protein constituting tannin-protein complex became, the stronger the activity to accelerate yeast flocculation became. In the case of practical wort fermentation, though, it seems unnecessary to adhere too closely to this problem. Therefore the mechanism of yeast flocculation during the wort fermentation is essentially identical to that of the essential yeast flocculation. However, since the tannin-protein complex in wort acts on the yeast cells as an accelerator to flocculate, the yeast cells begin the flocculation before the appearance of the essential yeast flocculation in spite of a high negative charge density. Accordingly, the sugar concentration of fermenting wort at the beginning of the yeast flocculation is high, and further, its state of appearance becomes remarkably clear. Therefore, various factors which accelerate or depress the yeast flocculation in wort fermentation are, utterly equal to those of the essential yeast flocculation.

Introduction

In the experiment of wort fermentation, it was always observed that yeast begins to flocculate at higher sugar concentration than that of its essential flocculation described in Part II[1]. The author hence distinguished this flocculation during the wort fermentation from the essential yeast flocculation, and called it the first "Bruch", and searched for the substances causing this. In the results, she concluded[2] that the substances necessary to accelerate yeast flocculation are protein and the higher derivatives decomposed from protein. This observation was due to

the fact that the substances were well adsorbed by active charcoal and Japanese acid clay, and the wort treated with them decreased in nitrogen content. As for the mechanism of acceleration, she had admitted Johnston's result[3] which indicated that the isoelectric points of wort proteins were over pH 6.0 and subsequently modified[5,6] his yeast flocculation theory[4] as follows: When pH of wort falls with the progress of fermentation, wort protein adsorbs on the surface of the yeast cells owing to an increase in the density of the positive charge of wort protein, consequently the density of the negative charge on the yeast cells decreases and the yeast cells flocculate before they begin the essential flocculation in spite of a high level in the negative charge density of the yeast cells themselves. However, even under this modified theory[6], the phenomenon of yeast flocculation was difficult to explain perfectly. Namely, after the middle period of fermenttation, pH of wort falls already to about 4.5. The higher negative charge density the yeast cell has, the larger amounts of wort protein naturally adsorbs on the yeast cell. It follows that the more vigorous metabolism the yeast cell has, the larger amounts of wort protein the yeast cell adsorbs, and such yeast cells must begin to flocculate more rapidly than others. This is a hypothesis which contradicts the fact. To solve this inconsistency, the wort protein neutralizing the yeast charge must be assumed to have a considerably large volume[6]. Furthermore, Silbereisen[7] has found that the yeast cell, well flocculated in beer, kept moving to the anode as well as the yeast cell suspended in buffer solution. This contradicting phenomenon can be seemingly explained, if an adsorption between the wort protein and the yeast cell were remarkably weak. However, even with this explanation, some doubts still remain. In the present work, the mechanism which accelerated yeast flocculation by wort protein was re-investigated.

Experiments and Discussion

I *Isoelectric Point of Wort Protein and Ability of Wort Protein to Flocculate Yeast*

1) *Isolation of Wort Protein*

An unhopped wort of Balling 11.7° had been boiled for 1.5 hours; it was subsequently allowed to stand overnight at about 1°C, and after that the coagulated protein was excluded from the wort; a precipitate brought by an addition of ammonium sulfate in proportion of 3 g per 10 m*l* was separated. The precipitate was dialyzed against distilled water, and then was separated into two components, depending on their solubility in water. A water-soluble compound was called wort albumin. A water-insoluble compound was further dialyzed with 1 % NaCl and from this, 1 % NaCl-soluble component was separated. The separated component was called wort globulin. The wort removed the precipitate was added to ammonium sulfate to make a solution of 6 g/10 m*l*. The precipitate was separated, dialyzed against distilled water, and called a wort proteose. Since the wort proteose had a high viscosity, it was judged to contain a large amount of dextrine.

2) *Isoelectric Point of Wort Protein*

(a) *In the case of the electrophoretic method*: For wort albumin, electrophoretic diagrams in the range of buffer solution of pH 4.0 to 7.5 of 0.2 ionic strength were taken through a Tiselius electrophoresis apparatus, and from the diagrams the isoelectric point was judged. In the results the isoelectric point of albumin of

boiled wort was mainly at about pH 4.5, and a protein which had isoelectric point over pH 5.0 was hardly detected, but the protein which had an isoelectric point about pH 4.0 was contained in small quantity. In the case of the wort globulin and proteose the same results as above were obtained.

(b) *In the case of the turbidity method*: An aliquot part of wort albumin solution was added to a range of buffer solutions of pH 3.6 to 7.5 and allowed to stand at 8°C, for 3 hours. After that a turbidity was estimated by a photometer, and from the turbidity an isoelectric point was judged. In the result the turbidity at pH 4.5 was the strongest, therefore the isoelectric point of wort albumin could be surmised to be about pH 4.5.

From both of the results mentioned above, it was found that the isoelectric points of proteins of boiled wort were about pH 4.5. Although the results were different from Johnston's results[8], they were acceptable considering that Johnston had found that the wort protein readily denaturated to "protein 0" of which isoelectric point was at pH 3.9 during the boiling. Those proteins were understood to be the intermadiate products in the process of denaturation to "protein 0". However, in any case, the isoelectric points of proteins of practically fermented wort will be about pH 4.5, and so it might be impossible that the protein of the fermenting wort adsorbed on the surface of yeast cell by an electric neutralization reaction.

3) *Ability of Wort Protein to Accelerate Yeast Flocculation*

The method of determination of the ability of yeast flocculation: The centrifugally separated yeast was suspended in 20-fold volumes of the test medium. After agitating for one min., 25 m*l* of yeast suspension was poured into graduated glass tubes about 1 cm in diameter and allowed to stand for 10 min. Then number of m*l* of the settled down yeast was measured. The magnitude of the yeast-flocculating activity was investigated by the quantity of the setted down yeast (all the operation were performed at below 10°C). Albumin, the largest in quantity of all, represented the wort proteins. The ability of albumin to flocculate yeast was measured with the yeast grown in the various media. Table 1 expressed the results.

As shown in Table 1, concerning the yeast suspension in which yeast cells fermenting wort were suspended in the acetate buffer solution of pH 4.6, the ability of wort albumin to flocculate yeast was found to be weak. Contrarily, that of fermenting wort diluted to ten times the volume (in order to avoid the effect of sugars, the wort was diluted to ten times) was tolerably strong. On the other hand, tannic acid showed a prodigious precipitating action to the flocculent yeast as well as to the general protein. So it could be surmised that some relationships were present among those three. Then it was shown that the ability of tannic acid to flocculate yeast, when tannic acid was added to the fermenting synthetic medium with sugar of high concentration, notably weakened. From these circumstances it is understood that in fermenting wort, the ability of the original wort to flocculate yeast was weaker than that of the wort diluted to ten times, was attributed to the existance of sugar that prevented flocculation. This inhibiting action of sugars on the yeast flocculation has been found by Eddy[9], Gilliland[10] and Lindquist[11]. From this fact explanation becomes possible on the phenomenon that the flocculating ability was lost when the well flocculated yeast cell was pitched into an original wort.

Table 1. The Abilities of Wort Albumin and of Tannic Acid to Flocculate Flocculent Yeast

Culture media	Wort*		Asparagine-medium**		Ammo. sulfate-medium***	
Solutions suspending yeast / Tested substances and amounts added	Fermenting wort adjusted to pH 4.6	Acetate buffer of pH 4.6	Fermenting asparagine-medium adjusted to pH 4.6	Acetate buffer of pH 4.6	Fermenting ammo. sulfate-medium adjusted to pH 4.6	Acetate buffer of pH 4.6
O (control)	1.1 ml	0.01 ml	0.01 ml	1.2 ml	0.2 ml	0.05 ml
Wort albumin, 9 mg%		0.05 ml	0.01 ml	2.5 ml	2.4 ml	0.05 ml
〃 18 mg%		0.05 ml	0.02 ml	3.0 ml	3.2 ml	0.05 ml
〃 27 mg%		0.05 ml		3.0 ml	3.2 ml	0.05 ml
〃 36 mg%		0.05 ml		3.0 ml		0.05 ml
Fermenting wort, 10%		2.8 ml		5.0 ml		
Tannic acid, 7 mg %		12.3 ml****	2.5 ml	9.2 ml****		12.5 ml****

Notes: *) A wort of Balling 11.7° was fermented and when degree of Balling of it went down to 6.6° the fermenting wort and the grown yeast were used for the experiments.

**) Wickerham's medium[8] of 10% glucose, containing asparagine as source of nitrogen was used having been supplied with vitamins, and when degree of Balling of it went down to 6.2°, the fermenting medium and the grown yeast were used for the experiments.

***) Ammonium sulfate had been used in place of asparagine of the asparagine-medium, and when its sugar content fell to 0.7% the fermenting medium and the grown yeast were used for the experiments.

****) Yeast cells settled down completely and superior liquid was clear.

II *Ability of Hops Tannin and Malt Husk Tannin to Flocculate Yeast and the Increase in Ability of Malt Husk Tannin to Flocculate Yeast Depending on Their Combination with Protein*

From the preceding experiment, it is found that isoelectric points of wort proteins are about pH 4.5 and also that their ability to flocculate yeast is weak. The action of wort protein on yeast flocculation, however, has been observed by Lange[12] in 1907. Since then this question has been discussed in various ways and so, it is impossible to be ignored. On the other hand, it has been found[13] that in wort, a large amount of tannin is present. Accordingly, it is naturally considered that protein is present in wort as tannin-complex. In the preceding section, it was found that tannic acid has a powerful ability to flocculate yeast. This is also recognized by Chevallier et al.[14] and there is no room for doubt. Since it was considered that some relationship is present between wort protein, tannin, and the ability of wort to accelerate yeast flocculation, this point was investigated.

1) *Isolation of Hops Tannin*: Fifty g of hops was added to 1 *l* of boiling water. Hops tannin was extracted by boiling for 1.5 hours, and then the spent hops was separated through filter paper. The filtrate was concentrated under diminished pressure. The resins were removed by treatment with ether. From the tannin extract obtained, tannin was precipitated by the addition of lead acetate solution as lead salt. The lead salt was washed carefully with distilled water, and then suspended in distilled water, the lead removed with hydrogen sulfide. After

that a hops tannin solution was obtained by means of concentration under diminished pressure.

2) *Isolation of Malt Tannin Fractions*: Malt tannin was extracted from malt husk through treatment with 50 % ethanol. Then ethanol was removed from the extract by means of concentration under diminished pressure and the resins were removed by treatment with ether. The extract treated with ether had been concentrated under diminished pressure, thereby removing the water, and was treated with 70 % ethanol to be separated into two parts; 70 % ethanol-soluble part and insoluble part; ethanol was removed from each part. Then tannin fractions were isolated through the procedure of lead salt as described above concerning the case of hops. They were named malt tannin fraction 1 and malt tannin fraction 2 (Isolation of tannin fraction from malt husk were carried out in according to Harris's method[15]). The ability of hops tannin and malt tannin fractions to flocculate yeast were measured in the previously mentioned method. Table 2 showed the results of the experiment.

Table 2. Abilities of Hop Tannin Fraction and of Malt Tannin Fractions to Flocculate Flocculent Yeast, and Contents of Tannin and of Protein in Them

	Yeast-flocculating activity		
Samples / Amounts added	Hop tannin fraction	Malt tannin fraction 1	Malt tannin fraction 2
0 (control)	0.0 m*l*	0.0 m*l*	0.0 m*l*
1 m*l*/100 m*l*	0.3 m*l*	0.05 m*l*	2.0—3.0 m*l*
2 m*l*/100 m*l*	2.9 m*l*	0.1 m*l*	3.5—4.0 m*l*
3 m*l*/100 m*l*		1.0 m*l*	
4 m*l*/100 m*l*		1.6 m*l*	

Test yeast: A flocculent yeast was collected from a fermenting wort of Balling 1.35°, and washed twice with acetate buffer solution of pH 4.6, after that it was tested. (Flocculating activity of this yeast against the fermenting wort was 5.5 m*l*)

	Contents of Tannin and of Protein		
Tannin	44.2 mg %	28.9 mg %	8.7 mg %
Protein-forming N		2.9 mg %	10.6 mg %
Peptide-forming N		2.5 mg %	6.4 mg %
Total N	8.7 mg %	11.6 mg %	28.2 mg %
Raw protein (N×6.25)	54.4 mg %	72.5 mg %	176.3 mg %
Tannin : raw protein	1 : 1.2	1 : 2.5	1 : 20.3

Contents of tannin, by Stone and Gray's method[16], and contents of protein-forming- and peptide-forming nitrogen, by Lundin's method[17] were respectively determined.

Table 2 shows that each fraction of these tannins has a powerful ability to flocculate yeast. However, it is noted that in the malt tannin fractions, the tannin content of fraction 2 was one third of that of fraction 1, and inversly the yeast-flocculating ability of fraction 2 showed a four-fold strength of that of fraction 1. Reason of this stronger yeast-flocculating ability of malt tannin fraction 2 seems to be in its combination with proteins, as was indicated by a comparison between the constituents of both fractions.

III *Action of Wort Proteins to Accelerate Yeast Flocculation Caused by the Formation of Tannin-Protein Complex*

The action of tannin-protein complex to accelerate yeast flocculation as seen in the preceding section was investigated to determine whether the action was present during the practical wort fermentation.

1) *Adsorption of Tannin-Protein Complex in Wort by Resting Yeast Cell*

A hopped wort was allowed to stand overnight at 0°C. All the coagulated substances in wort were completely removed by centrifugation. Five hundred m*l* of this wort was pitched with 5 g of yeast (A flocculent yeast, in a brewery washed four times with 0.1 % of NaOH, at 6°C, cleaned with water, and pressed), stirred sufficiently, and left standing at 2°C. After standing for four hours, the yeast was separated from the wort by centrifugation. About the supernatant wort, the contents of nitrogen in various forms were determined, and at the same time the content of tannin was determined by Harris's method[18] of nylon. On the other hand, the centrifuged yeast, after being washed once with water, was treated three times with 0.1 % NaOH, and about the alkaline washings, the amounts of nitrogen and tannin were determined. Table 3 summarizes the results. In the control group, for the wort adjusted to pH 4.5, the experiments were performed, too.

Table 3. Adsorption of Tannin-Protein Complex in Wort by Resting Yeast Cell

Samples	Total N	Protein-N	Peptide-N	Tannin	Adsorbed tannin
Original wort (Balling 12.0°)	59.8 mg%	12.5 mg%	10.0 mg%	4.7 mg%	
Wort treated with yeast	55.0 mg%	7.7 mg%	10.2 mg%	3.9 mg%	4.0 mg/5g of yeast
Dilute alkaline washings of yeast treated with wort	5.4 mg	1.8 mg* + 1.9 mg	0.6 mg	1.8 mg	
Wort of pH 4.5	58.8 mg%	12.1 mg%	9.4 mg%	4.5 mg%	
Wort of pH 4.5 treated with yeast	55.0 mg%	8.6 mg%	9.3 mg%	3.5 mg%	5.0 mg/5g of yeast
Dilute alkaline washings of yeast treated with wort of pH 4.5	5.5 mg	1.3 mg* + 2.4 mg	0.5 mg	2.1 mg	

* It precipitated when the alkaline washings had been neutralized.

Table 3 shows that the worts were adsorbed both tannin and protein at the same time by the treatment with yeast. The worts treated with yeast became perfectly clear regardless of the pH value and remarkably changed its appearance.

2) *Adsorption of Tannin-Protein Complex by Yeast Cell and the Ability of Yeast to Flocculate During Wort Fermentation*: A hopped wort (made clear by filtration and centrifugation) was inoculated with yeast (cleaned by washing four times with 0.1 % Na_2CO_3) at the rate of 5 g to 1 *l*. It was fermented at 12°—11°C. About the fermenting worts and the washings of propagated yeast (after being washed once with water, was washed two times with 0.1 % Na_2CO_3), changes in the amounts of various forms of nitrogen and tannin taking place during the progress of fermentation were determined at regular intervals. A relationship between those and ability to flocculate yeast was also examined. The results are summarized in Table 4.

Table 4. The Relationship between the Adsorption of Tannin-Protein Complex by Yeast Cell and the Flocculating Activity of Yeast During Wort Fermentation

Items / Samples	Degree of Balling	Protein-nitrogen	Tannin	Yeast-flocculating activity in fermented wort	*Yeast-flocculating activity in acetate buffer of pH 4.5
Original wort	12.03	12.5 mg%	4.7 mg%		
Wort of 4th day in fermentation	6.97	11.1 mg%	4.2 mg%	0.1 ml	0.01 ml
Wort of 6th day in fermentation	3.67	9.3 mg%	4.1 mg%	2.2 ml	0.05 ml
Wort finished in fermentation	1.52	7.9 mg%	4.0 mg%	4.9 ml	0.7 ml
Wort finished in fermentation centrifuged after measuring the yeast-flocculating activity	1.52	10.1 mg%	4.5 mg%		

* Having had its flocculating activity measured, the yeast was again centrifuged, after that it was tested.

The Amounts of Protein-Forming Nitrogen, of Peptide-Forming Nitrogen and of Tannin in the Yeast Washings

Items	Tannin			Protein-nitrogen			Peptide-nitrogen		
Fermentation period(days) / Sorts of washings	4	6	Primary fer. for 6, stored for 15, at 2°C	4	6	Primary fer. for 6, stored for 15, at 2°C	4	6	Primary fer. for 6, stored for 15, at 2°C
Buffer solution of pH 4.6	0.6 mg	0.7 mg	0.7 mg	0.9 mg	0.5 mg	0.8 mg	0.2 mg	0.8 mg	0.7 mg
0.1% Na_2CO_3	0.5 mg**	0.4 mg**	0.5 mg**	1.3 mg*** + 1.9 mg	1.2 mg*** + 1.5 mg	1.4 mg*** + 1.1 mg	0.3 mg	1.2 mg	0.2 mg

Quantities mentioned in Table are calculated per 5g of the centrifuged yeast.

** These are determined for the filtrates removed the precipitates which occurred when the washings had been neutralized, and the practical amounts are considerably larger than these values.

*** These precipitated when the washings were neutralized.

Table 4 showed that yeast adsorbed tannin-protein complex during wort fermentation. Accordingly, it is recognized that wort protein adsorbs on the yeast cells in a complex form combined together with tannin. And more, it is admitted that the tannin-protein complex acted as a factor to accelerate yeast flocculation. This was determined from the results; after the yeast at its sixth day in fermentation and the yeast which finished its fermentation had been measured in the flocculating activity, their flocculating activities decreased when they were suspended in the acetate buffer solution, and from the results summarized in Table 2.

IV *The Mechanism of Tannin-Protein Complex in Wort to Accelerate Yeast Flocculation*

The phenomenon of adsorption between tannin-protein complex and the yeast cell is a combination based on a mutual reaction between the tannin residue of the tannin-protein complex and the protein reaction radical on the yeast cell. There is

no effect on the yeast charge. An acceleration in the precipitating action of the yeast cell and a binding of its motion brought about by adsorption of tannin-protein complex on the yeast cell are the causes to accelerate yeast flocculation. This point is different from the previous report[6], yet, there is no change of view on the problem of acceleration of yeast flocculation by wort protein. As discussed previously[2], the activity of the tannin-protein complex to accelerate yeast flocculation is easily surmised to be due to a difference of size of the molecule of the protein constituting the tannin-protein complex. The greater the increase in the size of the protein molecule, the larger is its effect of binding on adsorption. The effect to accelerate flocculation will develop more notably in proportion to the increase in the viscosity of protein. Through the present work, phenomenon of the increase of the flocculating ability of the yeast cell through the process of wort fermentation, and the problem of the well-flocculated yeast in beer that move to anode, came to be explained reasonably.

Conclusion

The flocculent yeast cell behaves[19] like a particle of protein with the same property as "Zymocasein" (nucleoprotein and ribosomes) which is a main component in the cell. Consequently, the flocculent yeast cell becomes an unstable state in fermenting wort (pH 4.8—4.3). On the other hand, wort protein adsorbs on the yeast cell in a complex form combined together with tannin, and then the suspending state of yeast cell further moves to instability and is more ready to flocculate. However, at the early stage of fermentation, the yeast cell holds its charge density at high level. Owing to a preventive action of sugars toward yeast flocculation, the yeast cell is held in a suspending state. At the middle stage of fermentation when there is a decrease in the concentration of sugars, the charge density on the yeast cell decreases according to the reason described in Part II[1], and if the suspending activity attributable to the negative charge falls below a level of ability to accelerate yeast flocculation (depending on tannin-protein complex), the yeast cell begins to flocculate before the essential flocculation occurs, and settle down in spite of a high level of negative charge on itself. Accordingly, the yeast flocculation during wort fermentation is complex as in the case of the essential flocculation. Nevertheless, as far as the brewery fermentation concerns, the effect of high molecular protein on the fermentation velocity need not to be considered as recognized previously[20,21]. It seems that earliness or lateness of the occurrence of yeast flocculation during wort fermentation progresses in parallel with the activity of essential yeast flocculation. Accordingly if three factors described in Part II, namely, the constituents and the contents of wort amino acids, the degree of Balling of wort completely fermented by bottom brewery yeast and the condition of aeration for wort have been sufficiently studied, control of the fermentation velocity of wort will very likely be performed to some extent.

Summary

1) Isoelectric points of proteins of boiled wort were about pH 4.5 and their ability to flocculate yeast was weak.

2) Tannin fractions isolated from hops and malt husk revealed considerably powerful abilities to flocculate yeast, especially the malt tannin fractions whose

ability to flocculate yeast increases in parallel with the increase of the amount of protein combined.

3) The yeast cell cleaned with dilute alkaline solution adsorbed tannin-protein complex in wort, regardless of a resting or a fermenting state.

4) Proteins in wort adsorb on the protein reaction radical of yeast cell in a form of tannin-complex by the simple adsorption reaction which does not influence the charges on yeast cell, accelerate yeast flocculation.

5) The mechanism of yeast flocculation during wort fermentation was explained.

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