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Extension of Chronological Life in *Saccharomyces cerevisiae* under Ethanol Stress by Thermally Processed Rice *Koji* Extracts

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Abstract

The effect of thermally processed rice *koji* extracts on survival of yeast *Saccharomyces cerevisiae* was examined in comparison with non-heated *koji* extract. In chronological life span (CLS) tests on high-sugar fermentation, the survivals of the yeast cells grown with heated *koji* extracts were higher than non-heated *koji* extract. Heat treatment of the extracts by autoclaving led to a loss in most of amino acids due to the Maillard reaction, although histidine contents slightly increased. In glucose-arginine mixtures, arginine was partly converted to histidine by autoclaving and the addition of histidine prolonged the CLS of yeast cells. The yeast cells grown with the non-heated extracts were more resistant to oxidative stress whereas the antioxidant activities were lower than those of the heated extracts. The yeast cells grown with the heated extracts were more tolerant to ethanol and had a higher reduction capacity in the late stationary phase when the cells were incubated in the presence of ethanol. Maillard reaction products elevated the levels of reactive oxygen species to yeast cells grown under ethanol stress in the late stationary phase. These results suggest that thermally processed *koji* extracts can act as a protectant against ethanol stress during the late stationary phase of yeast growth and extend the CLS due to the increase of histidine contents by autoclaving.

Keywords

***Saccharomyces cerevisiae*, *Koji* Extracts, Life Span, Maillard Reaction, Histidine**

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1. Introduction

Rice *koji* has a long history of use in the manufacturing processes of fermented foods. In sake making, rice *koji* which is exclusively cultivated on rice by yellow mold, *Aspergillus oryzae*, contributes to the saccharification of starch materials and alcohol fermentation simultaneously by yeast in sake mash. Rice *koji* has various enzymes such as *α*-amylase, glucoamylase, and carboxypeptidase. Following this characteristic, the materials cultivated with the addition of *koji* mold can easily be digested by these enzymes and the resultant enzymatic products thus become important nutrients containing sugars, amino acids, and minerals for yeasts. In addition to being a good supply of nutrients to yeast, much attention has recently been paid to its antioxidant activities [1] [2]. Particularly, heat-processed *koji* enhances its antioxidant activity in accordance with the Maillard reaction [3].

The Maillard reaction, a well-known non-enzymatic browning reaction involving reducing sugars and amino acids, plays a vital role in the formation of flavor compounds and color to food, microbiological media, and various human diseases [4]-[6]. In *Saccharomyces cerevisiae*, the Maillard reaction products (MRPs) inhibit growth and alcohol production [7] [8], while the inhibitory effect is not detectable at low pH [9]. However, the positive effects of MRPs on yeast physiology are much less studied. Hereby, special attention in our work was given to the effects of thermally processed *koji* extracts including the MRPs on the survival of yeast cells. In the midst of high-sugar fermentation, yeast cell growth and viability are affected by ethanol reaching lethal concentrations [10] [11]. Thus, ethanol stress-related aging is progressive and cumulates to yeast cell death.

According to a recent report by Yamaoka *et al.* [12], mixed cultures with *Kluyveromyces lactis* and *S. cerevisiae* had an extended CLS of *S. cerevisiae* on high-sugar fermentation and found that amino acids metabolism was quite relevant to the CLS. In this work, the potential of an alternative way for the extension of *S. cerevisiae's* life span was examined by the use of rice*koji* extracts including various amino acids without the aid of non-*Saccharomyces* yeasts. In the course of the present study, changes of amino acids composition and the generated MRPs in *koji* extracts caused by thermal processing were of much note. In this work, we have revealed a uniquely positive effect of thermally processed *koji* extracts on yeast cells grown in the late stationary phase in the presence of ethanol while making a comparison between heated and non-heated *koji* extracts.

2. Materials and Methods

2.1. Yeast Strain and Culture Medium

A widely available sake yeast strain, *Saccharomyces cerevisiae* Kyokai No. 701 (K-701), was distributed by the Brewing Society of Japan. The yeast strain was grown on either YPD medium (1% yeast extract, 2% polypeptone, 2% glucose) or YNB medium (0.67% yeast nitrogen base [Difco], 2% glucose). Solid media contained 2% agar. All components and chemicals were from Wako Pure Chemical Industries, Ltd., Osaka, Japan, unless described particularly.

2.2. Preparation of Water Extracts from Rice *Koji* and Their Heat-Treatment

Dry *koji* made with 60% polished rice was used to prepare *koji* extracts from Tokushima Seikiku Co. in Anami, Japan. Distilled water was added at 3 times volume to the weight of rice *koji*. The suspension was kept at 60˚C for 6 h and then centrifuged at 4400 × g for 15 min at room temperature. The supernatant was used as a*koji* extract (KE). The *koji* extracts were autoclaved at 121˚C for 15 or 30 minutes. Non-heated *koji* extract (KE0) was filter-sterilized with 0.2 μm cellulose acetate membrane and used for the comparison of thermally processed *koji* extracts. Samples of the *koji* extracts were stored at −20˚C until used in all the tests. The pH of KE0 was 4.5 ± 0.1.

2.3. Preparation of Model MRP Systems

Glucose (18%, w/v) as approximated from *koji* extracts and amino acids (100 μmol) were dissolved in 100 mL of a 50 mM K-PO4 (pH 4.5). Four amino acids; arginine, histidine, glutamic acid, and lysine, were chosen from amino acid analysis of KE0 and these amino acids either distinctly increased or decreased after autoclaving. The amino acid-glucose mixture was autoclaved for 30 min at 121˚C. Samples of the mixture were stored at −20˚C until used in all the tests. For a control, 18% glucose solution (w/v) filter-sterilized was used to compare with the amino acid-glucose mixture.

2.4. Measurement of Browning

The browning intensity of *koji* extracts was measured at 420 nm using a UV-1800 spectrophotometer (Shimazu, Kyoto, Japan). When necessary, appropriate dilutions were made to have an optical density of less than 1.0.

2.5. Determination of Antioxidant Activity

The effect of the *koji* extracts on the DPPH radical was determined according to the modified method of Lijun *et al.* [13]. Each extract (0.3 ml) was mixed with 0.2M MES buffer (0.3 ml, pH 6.0) and ethanol (0.6 ml) to which 1.2 ml of 0.2mM DPPH (2,2-diphenyl-1-pycrylhydrazyl) solution in 0.1M MES buffer (pH 6.0) containing 50% ethanol was added. The mixture was kept at the room temperature for 30 min, and then the absorbance at 520 nm was measured. Trolox, a water soluble analog of Vitamin E, was used as a standard and the ability of scavenging the DPPH radical was expressed as the equivalent concentration of Trolox (nmol-Trolox/ml).

2.6. Chronological Life Span Measurements

Five days prior to the start of the chronological life span (CLS) experiments, *S. cerevisiae* K-701 was grown in YPD medium at 30˚C for 2 days under static conditions. Then the static culture was inoculated into YNB medium at 0.01 volumes and grown at 30˚C for 3days under static conditions. The yeast cultures were diluted 1/100 into the high-sugar medium (YNB20, 0.67% yeast nitrogen base, 20% glucose) containing 10% (v/v) *koji* extracts. The CLS experiment was then started at 30˚C for 2 weeks under static conditions. To determine the age-dependent mortality, cell viability at different days of growth was examined by spreading serial dilutions (1:10) of the cultures on solid YPD plates followed by incubation at 30˚C for 2 days.

2.7. Oxidative Stress Sensitivity

Yeast cells were grown at 30˚C for 2 days or 5 days in YNB medium with or without *koji* extracts (10%, v/v) under static conditions. For each cell, a series of 10-fold dilutions was prepared in sterile water over a range of concentrations from 10^{-1} to 10^{-5}, relative to the initial culture. 5 µl of each dilution were spotted sequentially onto the YPD media containing 2.5 mM H_2O_2 or 2.5 mM paraquat. The cells were then grown at 30˚C for 2 days before being photographed.

2.8. Ethanol Tolerance Test

Yeast cells were grown at 30˚C for 2 days or 5 days in YNB medium with or without *koji* extracts (10%, v/v) under static conditions in the presence or absence of ethanol (6%, v/v). The cells were collected and washed with sterile water and suspended with 20% ethanol (v/v) at an initial optical density of 600 nm of 2 (approximately 2×10^7 cells/ml). The suspensions were incubated at 30ºC and the number of surviving cells was determined by counting colony-forming units on YPD plates at regular intervals during incubation.

2.9. Cellular Reductive Capacity with WST-8

The cellular reductive capacity was measured with a Cell counting kit-8 (Dojindo, Laboratories, Kumamoto, Japan) by the use of a reductive reaction from a WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt] [14] [15]. The yeast cells were harvested by centrifugation, washed with sterile distilled water once, and then resuspended in distilled water to obtain the initial OD 600 nm = 0.2. 100 µl of the cell suspension were seeded into 96-well plates, added 10 µl of a WST-8 reagent solution and incubated at 30˚C for 1 hour. The absorbance at 450 nm was recorded by a microplate reader (Thermo Scientific MultiskanFC, Vantaa, Finland). The number of CFU/ml in the used cell suspensions was determined on YPD media after 2 days of incubation at 30˚C. The reductive capacity was expressed as ΔOD450/6 log CFU/ml.

2.10. Measurement of Intracellular ROS Levels

For intracellular ROS detection, the yeast cells were harvested by centrifugation at 3000 rpm for 5 min, and washed with sterile distilled water twice, and then resuspended in sterile distilled water at an initial optical density

of 600 nm of 0.2 (approximately 2×10^6 cells/ml). A dye, 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA, Molecular Probes) was added at the final concentrations of 10 µg/ml from a stock solution of 2.5 mg/ml in ethanol [16]. After incubation of 1 hour at room temperature, samples were analyzed using flow cytometry on a BD FACS Calibur HG™ (Becton-Dickinson, Japan), equipped with a 15 mW argon laser (488 nm). Ten thousand cells per sample were analyzed. The green fluorescence (FL1, 514 - 564 nm) was gated in a scatter-gram of log SS × log FS in fluorescence measurements.

2.11. Analysis

Glucose concentrations were determined using a HPLC (Class-LC10, Shimazu, Japan) with a NH2P-50 4E column (4.6 × 250 mm, Showa Denko K. K., Japan) connected to a RI detector. The column was eluted with a degassed mobile phase, acetonitrile: H_2O (75:25), at 40˚C and at a flow rate of 1.0 ml/min. Furfuraldehydes were modified by the procedure of Tu *et al.* [17]. Aliquots of 1 ml of the *koji* extracts were placed in test tube and 1.7 ml of concentrated hydrochloric acid and 1.4 ml of 30 mM TBA (2-thiobarbituric acid) solution were added. The mixtures were heated to 30˚C for 30 min. The content of furfuraldehydes was measured spectophotometrically at 414 nm with furfural as a standard. Amino acids were measured by the post-column fluorimetric detection of their o-phthalaldehyde derivatives by the method of Joseph and Davies [18] using a HPLC (LC-VP, Prominence, Shimazu, Japan) with a Shim-pack Amino-Na column.

3. Results

3.1. Effect of Heated *Koji* Extracts on Yeast Cell Survival

High-sugar fermentation which would be expected of outstanding yeast cell death was carried out in the presence of *koji* extracts (10%, v/v) with or without heat treatment. As shown in **Figure 1**, the significant difference between heated and non-heated *koji* extracts was observed in the growth curves after 10 days of fermentation. For instance, *S. cerevisiae* grown in media containing non-heated *koji* extract (KE0) was completely dead at 12 days, while the survivals of the yeast cells grown in media with KE15 and KE30 were 3.18log CFU/ml and 3.21log CFU/ml, respectively. Thus, the longer the heating time of *koji* extract was, the higher the survival of *S. cerevisiae* cell was. These results show that thermal processing by autoclaving of *koji* extracts was acknowledged to be beneficial in keeping the survival of yeast cells at the late stationary phase with high ethanol concentrations. Incidentally, the ethanol concentrations produced in all the used media were almost the same at approximately 10% (v/v).

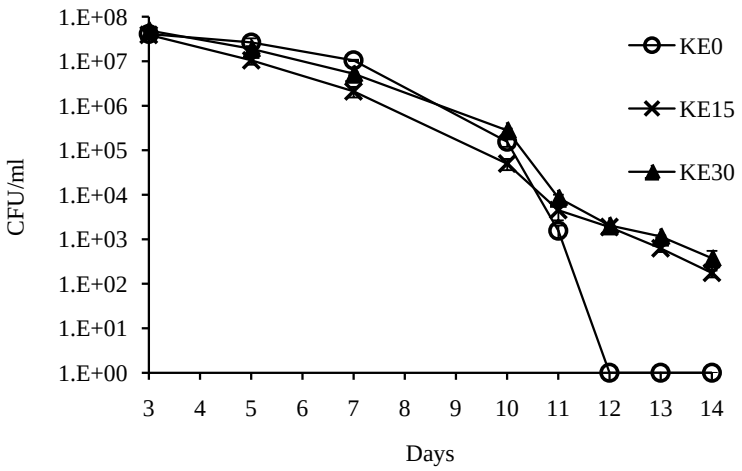

Figure 1. Transition of yeast populations (*S. cerevisiae* K-701) during fermentation in YNB20 medium with or without *koji* extracts (KE). Different heat-processed (0, 15, and 30 min at 121˚C) *koji* extracts were added at a final concentration of 10% (V/V). Fermentation was performed with the inoculation of precultures grown in YNB medium at 0.01 volumes to each medium at 30˚C. Each plot shows the average and standard deviation of duplicate experiments.

3.2. Effect of Thermally Processing on Rice *Koji* Extracts

During the autoclaving of *koji* extracts, the glucose concentrations decreased and furfuraldehydes containing furfural, one of the Maillard Reaction products (MRPs), increased (**Table 1**). The contents of furfuraldehydes were relatively low (94 µg/ml) even after autoclaving for 30 min and this value did not exceed 1 mg/ml which is sufficient to inhibit *S. cerevisiae* growth [7]. The browning reaction at 420 nm progressed and the antioxidant activity increased with increasing the heating time. The results were well line with the known fact that the intensity of the Maillard reaction increases with increasing temperature and time.

3.3. Changes in Free Amino Acids Content of *Koji* Extracts by Thermally Processing

From the results of **Table 1**, amino acid loss was expected by heat treatment due to the occurrence of the browning reaction between reducing sugars and amino acids. Most of the amino acids in non-heated *koji* extract except for histidine and lysine decreased during autoclaving (**Table 2**). In order to identify the cause of increased histidine and lysine, a mixture of glucose and histidine or lysine was heated for examining the amino

Table 1. Main chemical compounds, browning, and antioxidant activity of heat-processed and non-heated *koji* extracts.

Heat processing	Glucose (g/l)	Furfuraldehydes (µg/ml)	Browning (A_{420})	DPPH radical scavenging activity (nmol/ml)
None (KE0)	182.6 ± 15.9	13.99 ± 0.35	0.314 ± 0.016	0.464 ± 0.067
121˚C, 15 min (KE15)	156.3 ± 2.7	52.89 ± 0.56	0.890 ± 0.047	0.940 ± 0.180
121˚C, 30 min (KE30)	150.3 ± 3.5	94.24 ± 2.10	1.491 ± 0.005	1.148 ± 0.140

DPPH radical scavenging activity was represented as nmol Trolox equivalents per 1 ml of *koji* extracts. Data are the average ± SD of at least two independent experiments.

Table 2. Amino acid concentrations of heat-processed and non-heated *koji* extracts. The concentrations are given in µmol/ml of *koji* extracts.

KE0	KE15	KE30
2.07 ± 0.49	2.01 ± 0.24 (97)	2.12 ± 0.30 (102)
1.94 ± 0.11	1.86 ± 0.01 (96)	1.87 ± 0.02 (96)
2.74 ± 0.03	2.37 ± 0.03 (87)	2.08 ± 0.16 (76)
1.82 ± 0.52	1.44 ± 0.04 (79)	1.44 ± 0.08 (79)
3.33 ± 0.18	3.02 ± 0.32 (91)	3.02 ± 0.28 (91)
2.34 ± 0.48	1.94 ± 0.02 (83)	1.92 ± 0.02 (82)
1.49 ± 0.88	1.06 ± 0.45 (71)	0.91 ± 0.40 (61)
1.52 ± 0.22	1.32 ± 0.00 (87)	1.30 ± 0.02 (86)
4.12 ± 0.15	3.60 ± 0.14 (87)	3.43 ± 0.02 (83)
1.88 ± 0.24	1.70 ± 0.49 (90)	1.69 ± 0.02 (90)
2.11 ± 0.19	1.95 ± 0.08 (92)	1.89 ± 0.10 (90)
2.12 ± 0.94	2.56 ± 0.85 (121)	2.57 ± 0.82 (121)
0.73 ± 0.02	0.79 ± 0.01 (108)	0.85 ± 0.03 (116)
3.77 ± 0.14	2.71 ± 0.04 (72)	2.21 ± 0.22 (59)
31.98 ± 0.29 (100)	28.31 ± 0.24 (90)	27.30 ± 0.22 (88)

The values of blankets were represented as the percentage of KE. Data are the average ± SD of at least two independent experiments.

acids loss. As the result, both of the amino acids decreased during autoclaving at similar pH 4.5 of *koji* extract (data not shown). On the other hand, the relatively high-arginine content in *koji* extract notably decreased by heat processing. The arginine loss was examined by autoclaving a mixture of arginine and glucose as well. Interestingly, the arginine concentrations decreased by autoclaving with increase in histidine contents depending on glucose concentrations (**Figure 2**). Thus, the increase in histidine contents of heated *koji* extracts seemed to be connected to the products from arginine by heating in the presence of glucose. For lysine, there was no explicit explanation about the increase.

Given that the amino acids concentrations such as arginine changed due to heat processing, it occurred that any amino acid would affect the improved survival of *S. cerevisiae* in media with KE30. Additionally, is there a positive effect of MRPs from thermally processing on the survival of yeast cells? We conducted the CLS experiments when four different amino acids (arginine, glutamic acid, histidine, and lysine) were added to YNB20 medium. As the result, histidine was the best amino acid being attributed to better survival in *S. cerevisiae* of all the four amino acids (**Figure 3**). The survival of the yeast cells grown with histidine was 4-fold higher compared to those grown with control media at 14 days incubation. Following this result, it was investigated whether heated arginine-glucose mixture could improve the survivals of yeast cells compared to the non-heated mixture due to the partial conversion of arginine to histidine, and also whether either heated or non-heated histidine-glucose mixture could render any contribution to the improved survivals. As shown in **Figure 4**, it was obvious that the heated arginine-glucose mixture showed a definite improvement on the CLS, whereas the thermally processed histidine-glucose mixtures showed no sign of positive effect. Besides, the contents of arginine and histidine decreased by 69% and 72%, respectively, in each amino acid-glucose mixtures through autoclaving. In the meantime, histidine was generated at the concentrations of 0.26 μmol/ml from autoclaving of arginine- glucose mixtures.

3.4. Response to Oxidative Stress

Due to the fact that *koji* extracts had antioxidant activities and the activities enhanced after autoclaving (**Table 1**), the response of yeast cells grown on the media with *koji* extracts to oxidative stress such as hydrogen peroxide and paraquat might be reinforced with their activities. It was obvious that the *S. cerevisiae* cells grown with media containing *koji* extracts had an increase in resistance to oxidative stress compared to yeast cells grown in YNB medium (**Figure 5**). Unexpectedly, heated *koji* extracts conferred to the sensitivity to reactive oxygen species as opposed to rather than increased resistance. As in common, when yeast cells entered the late stationary phase at 5 days, they became more tolerant to paraquat and less tolerant to hydrogen peroxide irrespective of growth media.

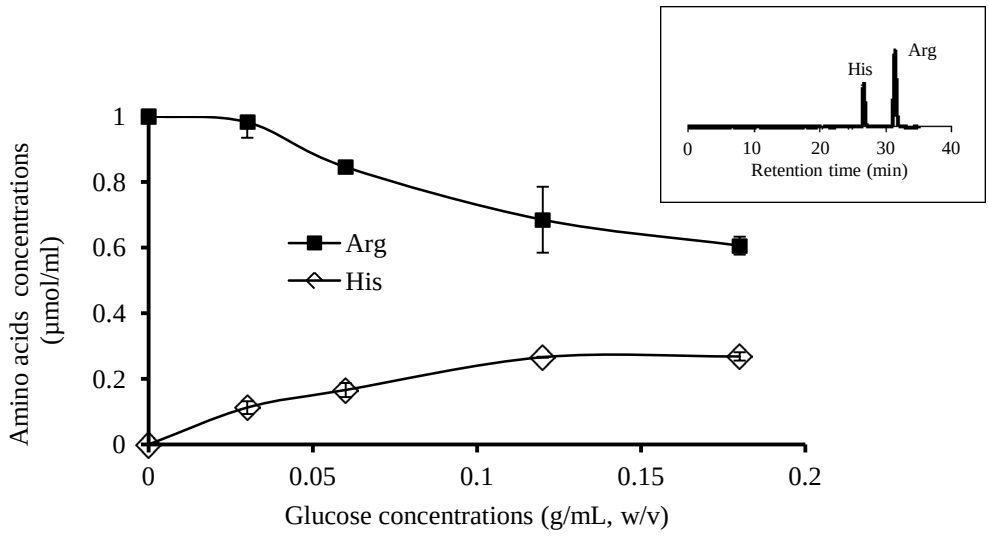

Figure 2. Effect of glucose on arginine loss and histidine gain in a glucose-arginine solution autoclaved for 30 min at 121˚C at pH 4.5. The inlet shows the elution profile of the solution of 1 mM arginine containing 18% glucose (w/v) after autoclaving. Each plot shows the average and standard deviation of duplicate experiments.

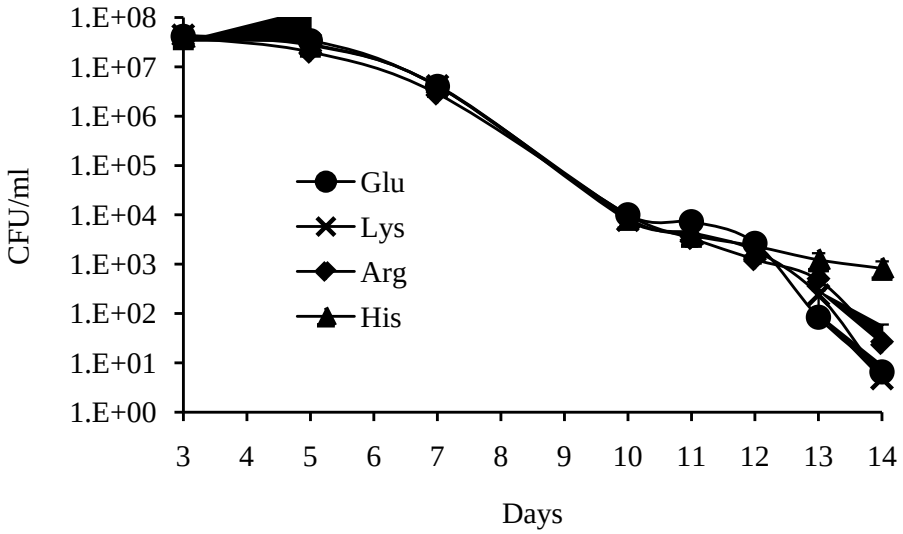

Figure 3. Transition of yeast populations (*S. cerevisiae* K-701) during fermentation in YNB20 medium with four different amino acids (arginine, glutamic acid, histidine, and lysine)—glucose mixtures without heat-treatment. The mixtures contained 1 mMamino acid and 18% glucose (W/V) at pH 4.5 and were added at final concentration of 10% (V/V) to YNB20 medium. Fermentation was performed with the inoculation of precultures grown in YNB medium at 0.01 volumes to each medium at 30˚C. Each plot shows the average and standard deviation of duplicate experiments.

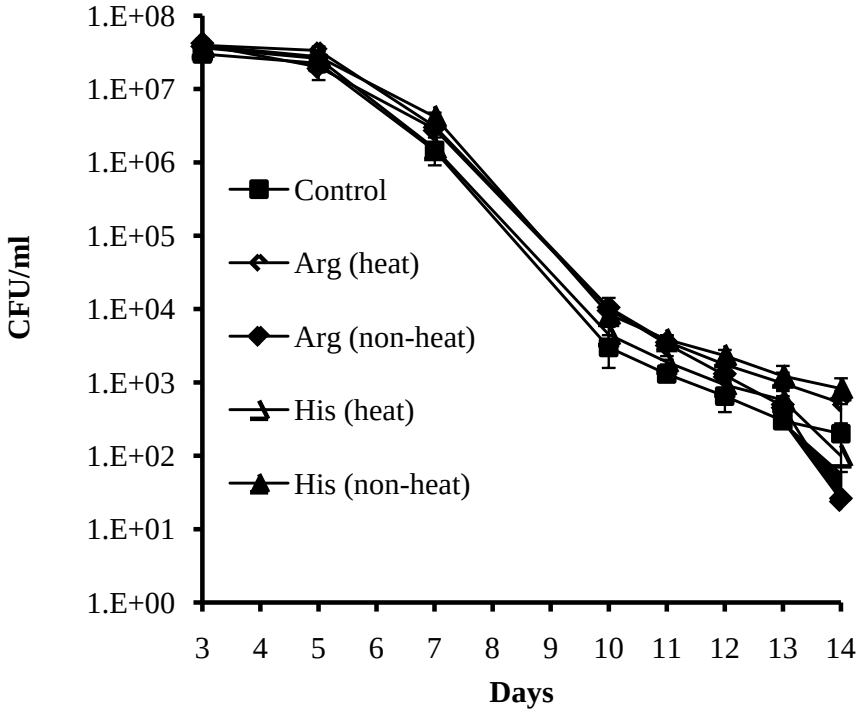

Figure 4. Transition of yeast populations (*S. cerevisiae* K-701) during fermentation in YNB20 medium with arginine-glucose mixtures or histidine-glucose mixtures with or without heat-treatment. Amino acid-glucose mixtures were autoclaved for 30 min at 121˚C) and added at a final concentration of 10% (V/V). Control was substituted for 18% glucose (W/V) instead. Fermentation was performed with the inoculation of precultures grown in YNB medium at 0.01 volumes to each medium at 30˚C. Each plot shows the average and standard deviation of duplicate experiments.

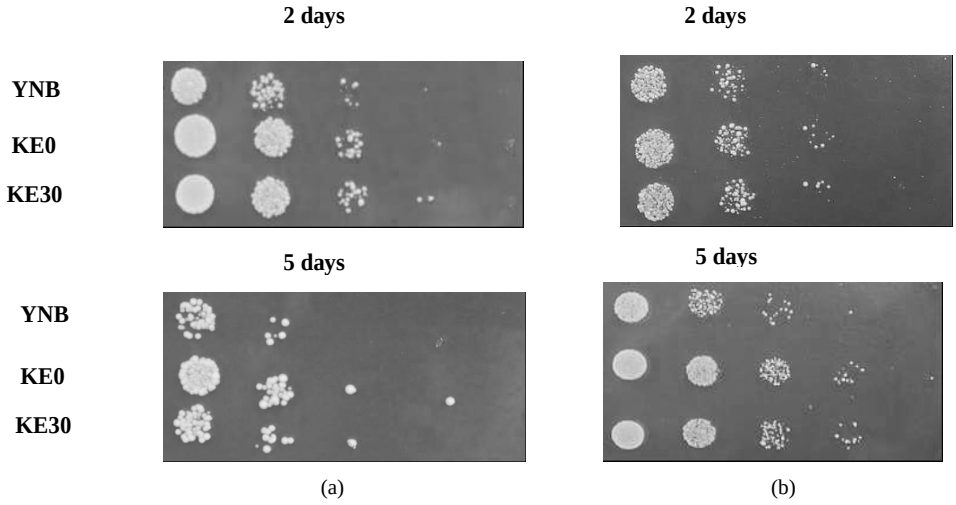

Figure 5. Oxidative stress tolerance of *S. cerevisiae* K-701 grown on different media. Cells were precultured at 30˚C for 2 or 5 days in YNB medium with or without KE. For each strain on different media, a series of 10-fold dilutions from 10-1 to 10-5 were prepared and spotted onto the YPD medium containing 2.5 mM H_2O_2 or 2.5 mM paraquat. (a) Hydrogen peroxide; (b) Paraquat.

3.5. Ethanol Tolerance

The heated *koji* extracts improved the survival of *S. cerevisiae* cells on high-sugar fermentation so it is natural to assume that the yeast cells grown on media containing heated *koji* extracts would have more tolerance to ethanol. To confirm this premise, we examined the ethanol tolerance of *S. cerevisiae* cells grown with or without *koji* extracts in the presence and absence of ethanol 6% (v/v). Preliminary experiments identified this ethanol concentration being inhibitory, but not lethal, for *S. cerevisiae* K-701 grown on all the used media.

In the absence of ethanol stress, the yeast cell grown on media containing KE0 after 2 days of incubation had the highest tolerance to ethanol of all the measured media (**Figure 6(a)**). There was no dominance of KE30 over ethanol tolerance. Surprisingly, the *koji* extracts were observed to not have a distinguishable difference in ethanol tolerance of the YNB-grown cells after 5 days of incubation (**Figure 6(b)**). In the presence of 6% (v/v) ethanol, the yeast cells grown with *koji* extracts were more tolerant to ethanol stress than those grown on YNB medium after 2 days of incubation (**Figure 6(c)**), while the viable cell population of yeast cells grown on YNB medium began to increase after 5 days of incubation (**Figure 6(d)**). This result was exactly opposite of the growth seen in the absence of ethanol. When KE0 and KE30 are compared, the yeast cells grown with KE30 were less sensitive to high ethanol concentrations. It appeared to indicate the survival behavior of *S. cerevisiae* cells on high-sugar fermentation (**Figure 1**). In addition, this positive effect of heated *koji* extracts was seen in the yeast cells grown in the late stationary phase in the presence of ethanol.

3.6. Cellular Reduction of Tetrazolium Salts (WST-8)

To investigate if the increase in ethanol tolerance of yeast cells grown on KE30 in the presence of ethanol is involved in reduction capacity, the activity of cellular reduction was analyzed by the use of WST-8, which is shown to be reduced extracellularly to its soluble formazan, via electron transport across the plasma membrane of living cells [14]. The degree of WST-8 reduction (ΔOD450) was the highest in KE0 after 2 days incubation, while the reduction capacity was significantly enhanced as yeast cells were grown on KE30 after 5 days incubation (**Figure 7**). The reduction capacities of yeast cells grown on YNB, KE0, and KE30 in the presence of ethanol 6% (v/v) increased 162%, 130%, and 183%, respectively, from 2 days to 5 days. During the transition from the exponential phase to the late stationary phase, yeast cells became more respiratory as well as more tolerant to higher ethanol concentrations on the various growth media.

3.7. Intracellular ROS Levels

Confirming the idea that the difference of the cellular tolerance to ROS toxicity and ethanol stress in the yeast

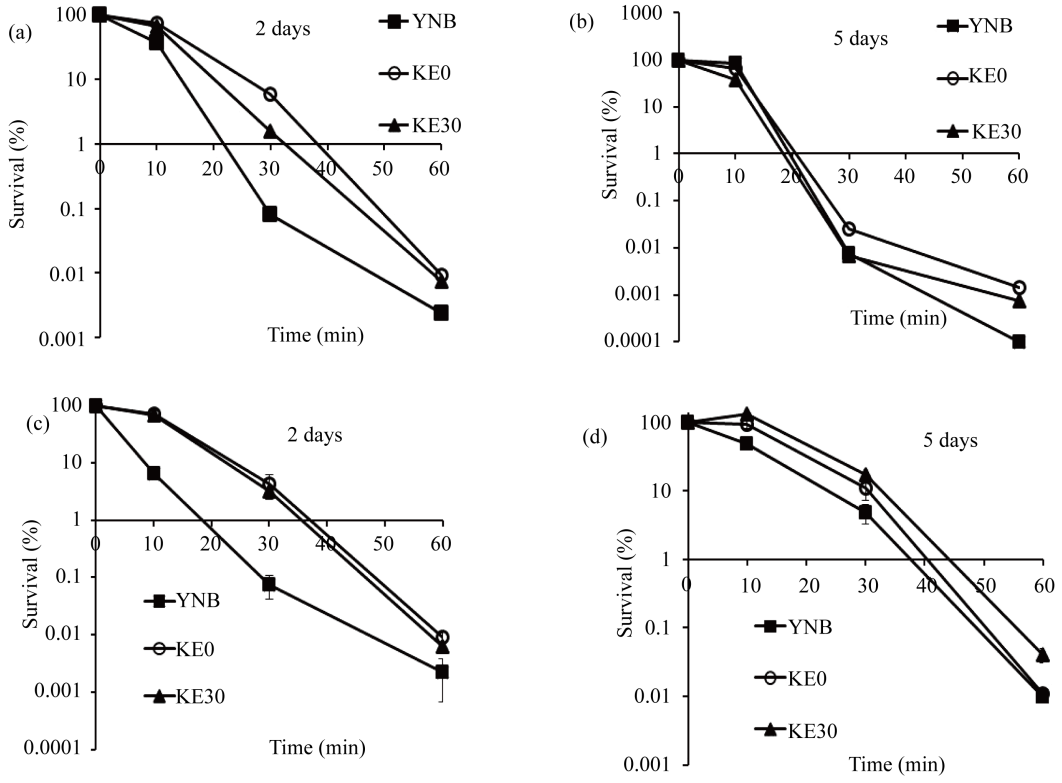

Figure 6. Ethanol tolerance of *S. cerevisiae* K-701 grown in different media in the presence or absence of 6% (v/v) ethanol under static conditions. Yeast cells were grown at 2 days (a) or 5 days (b) with or without *koji* extracts (10%, v/v) in the absence of ethanol, while they were grown at 2 days (c) or 5 days (d) in the presence of 6% (v/v) ethanol. Then yeast cells were collected at the days and were subjected to ethanol stress (20%, v/v) at 30˚C. Each plot shows the average and standard deviation of duplicate experiments.

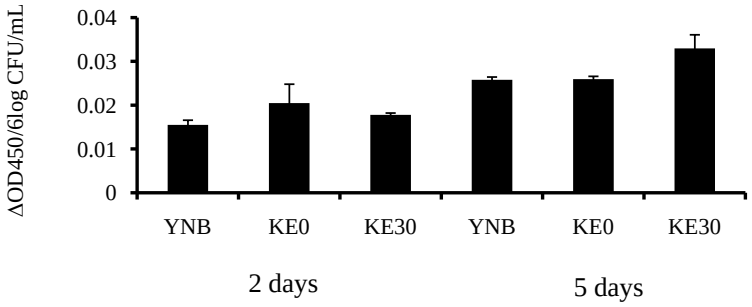

Figure 7. WST-8 reduction capacity of *S. cerevisiae* K-701 grown on different media. The cells were cultivated in the YNB or in the YNB with KE0 and KE30 (10%, v/v) in the presence of 6% ethanol (v/v) at 30˚C for 2 days or 5 days under static conditions. Each plot shows the average and standard deviation of duplicate experiments.

cells grown on media containing heated or non-heated *koji* extracts is involved in the intracellular ROS levels, the ROS levels were determined at various stages of growth in the presence or absence of ethanol. In the absence of ethanol, the order of ROS levels was KE0 (median, 164) > KE30 (median, 138) > YNB (median, 43) after 2 days incubation (**Figure 8(a)**). As well, that of ROS levels were KE0 (median, 74) > KE30 (median, 46) > YNB (median, 12) after 5 days of incubation. The longer incubation of yeast cells until the late stationary phase led to a decrease in the ROS levels in all the media. In respect of the oxidative response to paraquat, the decrease in the ROS levels seemed to increase in the tolerance to paraquat at 5 days (**Figure 5**). For H_2O_2, the decreased ROS levels between 2 and 5 days of incubation were likely to be detrimental to its response. It can

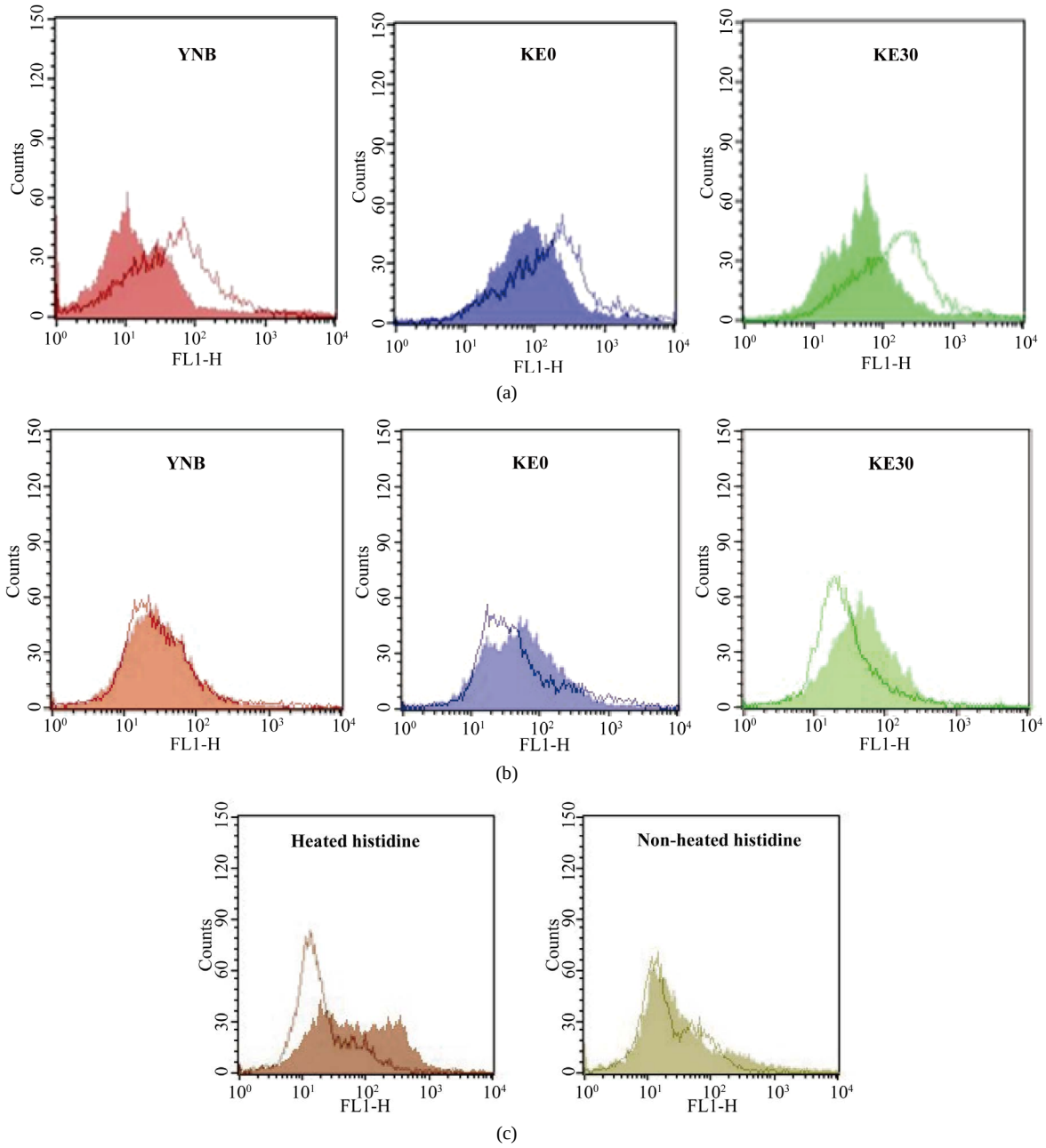

Figure 8. Assessment of intracellular ROS levels. The *S. cerevisiae* K-701 was grown in YNB medium with or without KE at 2 or 5 days at 30˚C under static conditions in the absence (a) or presence (b)of ethanol (6%, v/v). KE was substituted for a histidine-glucose mixtures (c). The ROS levels of the yeast were measured by FACS using the probe H2DCF-DA. The lines indicate yeast cells grown after 2 days of incubation and the filled histograms indicate yeast cells grown after 5 days of incubation. (a) In the absence of ethanoll; (b) In the presence of ethanol; (c) In the presence of ethanol (Histidine).

only be said that the ROS levels were higher in KE0 than in KE30 and that the higher tolerance to oxidative stress required the higher ROS levels in yeast cells regardless of the growth phase. In addition, it is worthwhile to note that decreased ROS levels in the late stationary phase accompanied a high sensitivity to ethanol stress.

In contrast to the absence of ethanol, when yeast cells were grown on *koji* extracts in the presence of ethanol (6%, v/v), increased ROS formation was observed between 2 and 5 days of incubation (**Figure 8(b)**). The increased rates of fluorescent intensity on the basis of median in the histograms were 106%, 143%, and 185% from 2 days to 5 days in YNB, KE0, and KE30, respectively. This is a distinguishable difference between heated

and non-heated *koji* extracts.

In order to resolve what caused heated histidine to show no effect in the CLS experiments, we analyzed the intracellular ROS levels of yeast cells grown on media containing histidine-glucose mixture with or without heat treatment in the presence of ethanol (**Figure 8(c)**). For the non-heated histidine, the changes in the ROS levels were negligible between 2 days and 5 days of incubation. On the contrary, the ROS levels of the heated histidine increased in the late stationary phase with 5 days of incubation.

4. Discussion

Koji extracts which were prepared in our study contained high glucose concentrations (18%, w/v) and approximately 30 mM free amino acids at pH 4.5. The hallmarks of the Maillard reaction such as browning, glucose loss, amino acid loss, and furfuraldehydes production were observed in *koji* extracts after thermally processing by the use of autoclaving (**Table 1** and **Table 2**). In the Maillard reaction, Amadori rearrangement products (1-amino-1-deoxy-2-ketoses) can easily undergo deamination and dehydration at low pH [4] [5]. To identify the source of the increased histidine contents in heated *koji* extracts, we found that histidine in KE30 was partially from the decomposition of arginine through the Maillard reaction. A possible mechanism of histidine production is shown in **Figure 9**. Due to the fact that histidine production was dependent on glucose concentrations in arginine-glucose model system (**Figure 2**), arginine might initially undergo deamination by means of the Maillard reaction followed dehydration with the formation of imidazole moiety as seen in the histidine molecule. Arginine-lysine imidazole, arginine-specific imidazole moiety, is synthesized as a possible advanced glycation

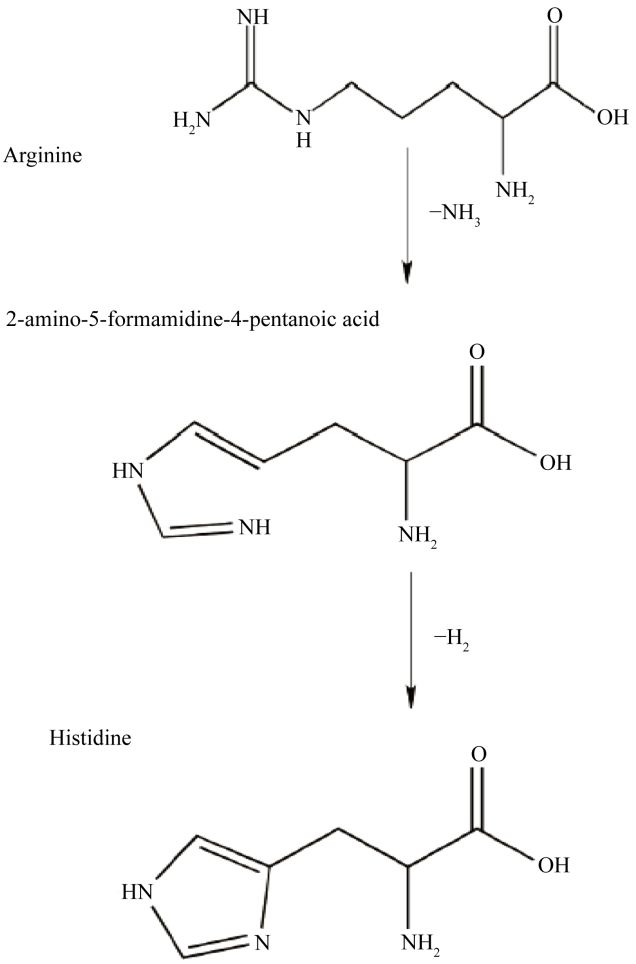

Figure 9. Possible mechanism of conversion from arginine to histidine through heat-treatment of arginine-glucose mixtures.

end-product [19]. On the other hand, imidazole can also be formed from glycine and alanine in model systems [20]. In this work, arginine may be converted to histidine in a similar fashion.

Histidine has the ability of antioxidant through the donation of hydrogen radical from the imidazole ring or the β-carbon by itself [21]. MRP obtained from histidine-glucose possesses peroxyl radical scavenging activity [6]. We have also verified that the heated histidine-glucose mixture was higher than the non-heated mixture in DPPH radical scavenging activity (data not shown). In the viewpoint of oxidative stress in yeast cells, non-heated *koji* extracts took precedence over heated*koji* extracts (**Figure 5**). In other words, nor can it necessary be said that any higher antioxidant activity would result in high tolerance to oxidative stress in yeast cells. Overall, the growth phase and existing environment of yeast cells could be a key factor. For example, despite the higher intracellular ROS levels in the yeasts grown with *koji* extracts, they were resistant to oxidative stress (**Figure 5** and **Figure 8(a)**). This means that yeast cells lived on the rich nutrients such as amino acids from *koji* extracts as to circumvent a stressful environment to oxidation.

Focusing on the significance of growth phase in ethanol tolerance of yeast, there was a distinct difference of the cellular response between heated and non-heated *koji* extracts (**Figure 6**). The reason why the heated *koji* extract was effective for yeast grown in the late stationary phase under ethanol stress is to be interpreted by the result from WST-8 reduction and intracellular ROS levels in the presence of ethanol. Ethanol toxicity is correlated with the production of ROS in the mitochondria [22], while mitochondrial function is required for resistance to oxidative stress [23]. Presumably, the increased NADH and intracellular ROS levels in yeasts grown with KE30 during a shift of 2 days to 5 days were referred to the adaptation response to ethanol stress. Interestingly, our result resembled calorie restriction cells in increased respiration and elevated ROS levels [24]. In addition, ethanol-tolerant strain showed an increased level of reduction capacity of WST-8 in the presence of ethanol compared to the wild type strain [25]. Thus, the acquisition of ethanol resistance in yeast cells could be related to an energy supply through mitochondria necessary for the survivals with ROS detoxification during the late stationary phase.

Our prime concern is as to why thermally processed *koji* extracts prolonged chronological life span of yeast. Histidine, which was generated partially from arginine by autoclaving and comprising a large portion in the *koji* extracts, could be a good candidate for bringing about the extended life span in yeast cells (**Figure 3** and **Figure 4**). This can be explained by the fact that the intracellular ROS levels remained relatively unchanged in the yeast cells grown on media containing a histidine-glucose mixture at the late stationary phase in the presence of ethanol compared to those at the exponential phase (**Figure 8(c)**). The free radical theory suggests that aging can be attributed to deleterious effects of free radicals on various cell components [26]. The low ROS levels in yeast cells will alleviate the danger from ROS. However, assuming that the difference in the CLS between heated and non-heated histidine is centered upon the histidine loss by autoclaving, just how are the increased ROS levels in yeast cells grown on media with heated histidine-glucose mixtures explained for the relevance to CLS? It seems to be a bit hasty in concluding that the extension of CLS was depended on the histidine contents in *koji* extracts. The helpful hint which led to understanding the significance was that the yeast cells grown with KE30 had an increased intracellular ROS levels at the late stationary phase in the presence of ethanol and had an extended CLS. Importantly, and in agreement with the previous study indicating that catalase inactivation extends the CLS of yeast by inducing hydrogen peroxide and superoxide dismutase activity [27], our results show that increased ROS levels in yeasts grown with KE30 are required in sustaining the CLS to some extent despite parallel increases in oxidative damage.

A remaining question pertains to the effect of the MRPs on CLS of yeast cells. Due to the fact that the formation of ROS is a function of ambient oxygen concentration [28], yeast cells could adapt to lower the ambient oxygen concentration during the late stationary phase in concurrence with an increase in ROS generation. However, the post-diauxic phase such as the late stationary phase is a hard time when SOD2 activity relevant to eliminating ROS generation remains unchanged [22]. Out of necessity, the yeast cells would favor a decrease in ROS generation from media including the MRPs with the higher antioxidant activity. Thus, the existence of the MRPs is somewhat likely to achieve the prolongation of CLS by augmented antioxidant levels as provided through thermal processing.

Another point worthy of special mention on the effects of thermal processing on yeast physiology was that the heated histidine-glucose mixtures as well as KE30 elicited the increased intracellular ROS levels at the late stationary phase in the presence of ethanol. Presumably, its relevant substances are MRPs. The MRPs are likely to

act positively on the mitochondrial function such as respiration and reduction capacity in yeast cells during the late stationary phase when the yeast cells exist on both ethanol stress and limited nutrients. This is largely related to the previous result that calorie restriction elicits mitohormetic effect, which leads to the prolonged longevity [24].

Lastly, it must not be overlooked that autophagy is induced by nutrient starvation and required for chronological longevity in *S. cerevisiae* [29] [30]. That is exactly what occurred to the yeast grown without *koji* extracts. Moreover, there seems to be something unique in the process of autophagy in the yeasts grown with *koji* extracts. The nutrients in *koji* extracts might be attributed to the different manner of death behavior. Further study of factors affecting the CLS of *S. cerevisiae* will be expected to advance the use of *koji* extracts and promote efficient alcohol fermentation for alcohol beverage and bioethanol production.

5. Conclusions

We have observed that the conversion of arginine to histidine in the autoclaving procedure of *koji* extracts would be helpful of extending the chronological life span of yeast *Saccharomyces cerevisiae* under ethanol stress. The reaction which has not known so far took place at low pH and high glucose concentrations. The thermal processing by autoclaving affected the ethanol tolerance and oxidative response to yeast cells and the effect depended on the culture, especially, the late stationary growth phase under ethanol stress. The Maillard reaction products by the autoclaving procedure triggered the increased NADH and intracellular ROS levels in yeasts grown in the presence of ethanol.

These findings emphasize the important role of the Maillard reaction products on the survival of yeasts under ethanol stress during high-sugar fermentation and the impact on the physiological activities of yeasts that affect aging and chronological life span.

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