Analysis of factors that influence the fermentation profiles of sake yeast

（日本酒酵母の発酵特性に及ぼす影響の解析）

Jannatul Ferdouse

2018

Analysis of factors that influence the fermentation profiles of sake yeast

日本酒酵母の発酵特性に及ぼす影響の解析

BY

JANNATUL FERDOUSE

Bachelor of Science in Microbiology, University of Chittagong, Bangladesh, 2004

Master of Science in Microbiology, University of Chittagong, Bangladesh, 2005

A dissertation submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Bioscience and Biotechnology, The United Graduate School of Agricultural Sciences, Kagoshima University, Japan

September, 2018

Supervisor: Prof. DR. HIROSHI KITAGAKI

The United Graduate School of Agricultural Sciences, Kagoshima
Kagoshima University
Korimoto 1-21-24, Kagoshima 890-8580, Japan

CERTIFICATE OF APPROVAL

This is to certify that the examination committee has read and recommended to the United Graduate School of Agricultural Sciences for acceptance, a PhD Dissertation entitled:

Analysis of factors that influence the fermentation profiles of sake yeast

Submitted by: JANNATUL FERDOUSE

In partial fulfillment of the requirements for the Doctor of Philosophy Degree in Bioscience and Biotechnology on September, 2018.

PROF. DR. HIROSHI KITAGAKI
Chairman, Examination Committee
(Saga University)

PROF. DR. HIROYUKI CHO
Member, Examination Committee
(Saga University)

ASSOC. PROF. DR. SHINJIRO TACHIBANA
Member, Examination Committee
(University of the Ryukyus)

ASSOC. PROF. DR. SHIGEKI INABA
Member, Examination Committee
(Saga University)

ASSOC. PROF. DR. YUMIKO YOSHIZAKI
Member, Examination Committee
(Kagoshima University)

ABSTRACT

In past few decades various factors has been used for developing fermentation profiles of yeasts. Among them, use of additional substances during fermentation affect the several physiological properties of yeast. Hence, this dissertation study was implemented to reveal the effects of the two important additional substances such as glycosylceramide and amino acids on the fermentation profile of sake yeast.

Until date, sake yeast is fermented with koji, which is steamed rice fermented with non-pathogenic fungus *Aspergillus oryzae* for the manufacture of sake. However, *A. oryzae* contain abundant glycosylceramide as cell membrane component. In this study, we first report a novel, simple and efficient method for quantitation of mycelial weight of *A. oryzae* by measuring its glycosylceramide content. Previously reported that *N*-acetylglucosamine is used to quantitate the mycelial weight of *A. oryzae* which is costly, tedious and its quantitative performance is low.

Therefore, in this study the results suggested that the amount of glycosylceramide significantly correlated with both the mycelial weight of *A. oryzae* and the amount of *N*-acetylglucosamine, an established index of the mycelial weight of *A. oryzae* in koji. This new method is simple, easy and highly reproducible.

In this study, we also evaluated that, glycosylceramide, one of the sphingolipids affects yeast fermentation. During fermentation, sake yeast requires unsaturated fatty acids and sterols, in addition to substances provided by koji enzymes. However, the role of sphingolipids on the brewing characteristics of sake yeast has not been studied. The addition of soy, *A. oryzae*, and *G. frondosa* glycosylceramide conferred similar effect on the flavor profiles of sake yeast. In

particular, the addition of *A. oryzae* and *G. frondosa* glycosylceramide were very similar in terms of the decreases in ethyl caprylate and ethyl 9-decenoate esters. Addition of soy glycosylceramide induced metabolic changes to sake yeast such as a decrease in glucose, increases in ethanol and glycerol and changes in several amino acids and organic acids concentrations. Tri carboxylic acid (TCA) cycle, pyruvate metabolism, starch and sucrose metabolism, and glycerolipid metabolism were overrepresented in the cultures incubated with sake yeast and soy glycosylceramide which is first study in this research.

The brewing profiles of yeast are also affected by the addition of amino acids during alcoholic fermentation. For example, addition of methionine decreases hydrogen sulfide production by brewery yeasts. However, a concrete mechanism linking amino acids in fermentation media with mitochondrial activity during fermentation of brewery yeasts is yet unknown. Here, we report that amino acids in fermentation media, especially methionine (Met) and glycine (Gly), stabilize mitochondrial activity during fermentation of sake yeast. By utilizing *atg32Δ* mutant sake yeast, which shows deteriorated mitochondrial activity, we screened candidate amino acids Met and Gly by measuring reactive oxygen species (ROS) levels in sake yeast. Yeast cells supplemented with Met and Gly retained high ROS levels relative to the non-supplemented sake yeast. Moreover, Met-supplemented cells showed a metabolome distinct from that of non-supplemented cells.

In conclusion, we have first determined the altered fermentation characteristics of sake yeast in response to glycosylceramide and amino acids which will enable interpretation on the fermentation characteristics of yeast.

Dedication to

My beloved parents, husband and son

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my PhD supervisor professor Dr. Hiroshi Kitagaki, Department of Environmental Science, Faculty of Agriculture, Saga University, Saga; Deapartment of Biochemistry and Applied Biosciences, United Graduate School of Agricultural Sciences, Kagoshima University, Kagoshima, Japan who trained and allowed me to grow up as a research scientist in the field of microbiology and biotechnology. He was a great mentor and this thesis would not have been possible without his expert guidance, continuous support and motivation. Therefore, first and foremost, I would greatly appreciate and be thankful to Dr. Hiroshi Kitagaki for his unforgettable kindness and support to complete this study successfully.

I would like to convey my special thanks to Professor Hiroyuki Cho and Associate Professor Shinjiro Tachibana my advisory professor of this study for their valuable suggestions and comments.

In recognition of all the help and support, I would like to express my sincere thanks to all staff members of the International Student Center of Saga University and The United Graduate School of Agricultural Sciences, Kagoshima and The government of Japan (MEXT) for providing financial support through Manbukagakusho Research Scholarship to accomplish this research work.

I would like to express my thanks to all of my lab members specially Mr. Kadowaki, Mr Sato, Mr. Taguchi, Mr. Fujimaru, Mr. Yamamoto, Mr. Tagaki, Miss. Sakatani, Miss Fujikawa, Miss Sakamoto, Miss. Miyagawa, Miss Kitajima, Miss Mikako for their cooperation about my study and they also made my lab life happy and productive.

I would like to give thanks to all of my Bangladeshi friends in Saga University for their support and encouragements during my stay in Japan.

Finally, I would like to give my heartiest gratitude to my parents, brothers and sisters for their constant inspiration and encouragement. I would like to express my deepest thanks to my husband Iqbal Ahmed for his wisdom in understanding the situation and tolerate everything and to my lovely son Izaan Ahmed Ayaan who sacrificed his all happiness for his mother.

CONTENTS

ABSTRACT……..iv–v

ACKNOWLEDGEMENTS…….....vii

LIST OF TABLES…….....xii

LIST OF FIGURES…….....xiii-xiv

LIST OF ABBREVIATIONS…….....xv-xvi

CHAPTERS

1. INTRODUCTION…….....1

1.1 General introduction…….....2
1.2 Motivation …….....6
1.3 Aim and objectives of the study …….....9
1.4 Thesis organization…… ….10

2. BACKGROUND…….....11

2.1 Sphingolipids……...12
2.1.1 Structure of sphingolipids…….....13
2.1.2 Scope of sphingolipids……...14
2.1.3 Biosynthesis of sphingolipids……15
2.1.4 Ceramides…….....18
2.1.5 Glycosphingolipids……...19
2.1.6 Mammals glucosylceramide…….....19
2.1.7 Plant glucosylceramide…….....20
2.1.8 Fungal glucosylceramide……...21
2.1.9 Beneficial effects of glycosylceramide ……...……..22
2.2 Amino acids…….....23
2.2.1 Classifications of amino acids……...24

2.2.2 Importance of amino acids…….....26
2.2.3 Biosynthesis of sulfur amino acids……...27
2.2.4 Effects of sulfur amino acids (methionine) in cells…….....28
2.3 Concluding remarks……...29

3. EFFECTS OF GLYCOSYLCERAMIDE ON SAKE YEAST DURING FERMENTATION…….....30

3.1 Introduction…….31
3.2 Material and methods…….....35
3.2.1 Strains, reagents and materials…..…….....35
3.2.2 Culture of *A. oryzae*…….....35
3.2.3 Quantitation of glycosylceramide of the mycelia of *A. oryzae*…….....35
3.2.4 Koji manufacture…….....36
3.2.5 Quantitation of glycosylceramide in koji samples by TLC…….....36
3.2.6 Quantitation of the intensities of spots on TLC using imaging software……..37
3.2.7 Quantitation of *N*-acetylglucosamine in koji samples……...37
3.2.8 Lipid extraction……..38
3.2.9 Separation and detection of lipids using thin layer chromatography…….38
3.2.10 Semi-purification of glycosylceramide using column chromatography…….39
3.2.11 Purification of glycosylceramide using high performance liquid chromatography (HPLC)…….....39
3.2.12 Co culture of sake yeast with glycosylceramide…….....39
3.2.13 Sample collection for metabolites analysis…….....40
3.2.14 Sample preparation for the analysis of volatile compounds…….....40
3.2.15 GC/MS analysis of volatile compounds.....41
3.2.16 Sample preparation for extracellular non-volatile compounds…….....41
3.2.17 GC/FID analysis of non-volatile compounds…….....41
3.2.18 Measurement of ethanol concentrations…….....42
3.2.19 Statistical analysis…….....42
3.3 Results……43
3.3.1 Correlation of glycosylceramide levels of *A. oryzae* with its mycelial weight…….....44
3.3.2 Increase in the glycosylceramide and *N*-acetylglucosamine contents with the growth of *A. oryzae* on koji rice…….....46

3.3.3 Correlation of glycosylceramide levels with *N*-acetylglucosamine content of koji rice……52
3.3.4 Analysis of volatile components with or without glycosylceramide during fermentation…….....54
3.3.5 Analysis of nonvolatile extracellular components with or without glycosylceramide during fermentation…….....58
3.3.6 Analysis of metabolic pathway of extracellular metabolites of sake yeast……...61
3.4 Discussion…….....63
3.5 Concluding remarks……67

4. EFFECTS OF AMINO ACIDS ON SAKE YEAST DURING FERMENTATION…….....68

4.1 Introduction…….....69
4.2 Materials and methods……72
4.2.1 Yeast strains and media…….....72
4.2.2 Fermentation test…….....72
4.2.3 Measurement of ethanol concentration…….....72
4.2.4 Analysis of intracellular ROS content …….....72
4.2.5 Metabolites analysis by GC/FID method……...73
4.2.6 Microscopic observation of mitochondria and vacuoles……74
4.2.7 Measurement of glucose concentrations……74
4.2.8 Statistical analysis…….....74
4.3 Results……75
4.3.1 Fermentation profile of WT and its mutant *atg32⊿* sake yeast with or without amino acids……...75
4.3.2 Interaction effects of amino acids with mitophagy function of WT and its mutant *atg32⊿* sake yeast during fermentation……79
4.3.3 The *atg32⊿* mutant sake yeast shows defective mitochondria by ROS analysis…….....85
4.3.4 Identification of methionine (Met) and glycine (Gly) as mitochondria -activating amino acids…….....87
4.3.5 Metabolome analysis of WT and its mutant *atg32⊿* sake yeast supplemented with Met ……...89
4.3.6 Microscopic observation of mitochondria of WT and its mutant *atg32⊿* sake yeast with or without Met……..91

4.4 Discussion……...93
4.5 Concluding remarks……96

5. DISCUSSION……..97

5.1 Concluding summary…….....98
5.2 Outcome of the research……...101
5.3 Publications list…….102
5.4 Future works…….....103

6. REFERENCES……..104

LIST OF TABLES

Table 1. List of 20 proteinogenic AAs……24

Table 2. The coefficient of variation of glycosylceramide measurement in koji incubated with *A. oryzae*……52

Table 3.Volatile compounds in the culture of sake yeast added with or without glycosylceramide of *A. oryzae* and *G. frondosa*……56

Table 4. Volatile compounds in the culture of sake yeast added with or without glycosylceramide of *A. oryzae* and soy……57

Table 5.Metabolite concentrations in the culture of sake yeast added with or without soy glycosylceramide……60

Table 6. Yeast strains used in this study……75

Table 7. List of amino acids used in this study……75

Table 8. Fermentation characteristics of WT sake yeast……77

Table 9. Fermentation characteristics of the *atg32Δ* mutant sake yeast……78

LIST OF FIGURES

Figure 1. Structures of typical glucosylceramides from mammals, plants, fungi and IPC from *S. cerevisiae*……...14

Figure 2. Outline of sphingolipid metabolism in *S. cerevisiae*……..17

Figure 3. General structure of AAs …….....23

Figure 4. Classification of AAs based on the nature of the R group……25

Figure 5. Sulfur AAs utilization and metabolism in budding yeast……..…..28

Figure 6. Schematic diagram of the experimental steps of the measurement of glycosylceramide levels of koji rice…….....43

Figure 7. Mycelial growth of *A. oryzae*…….....……44

Figure 8. Correlation of the glycosylceramide levels with the mycelial weight of *A. oryzae*…….....45

Figure 9. Four independent trials of koji manufacture using sake rice……47

Figure 10. Correlation between the glycosylceramide and *N*-acetylglucosamine levels during koji production experiment (trial 1)……48

Figure 11. Correlation between the glycosylceramide and *N*-acetylglucosamine levels during koji production experiment (trial 2)……49

Figure 12. Correlation between the glycosylceramide and *N*-acetylglucosamine levels during koji production experiment (trial 3)……50

Figure 13. Correlation between the glycosylceramide and *N*-acetylglucosamine levels during koji production experiment (trial 4)……51

Figure 14. Correlation between the glycosylceramide and *N*-acetylglucosamine levels during four (04) koji production experiments……53

Figure 15. Heatmap of volatile compounds in the culture of sake yeast incubated with or without glycosylceramide…….55

Figure 16. Heatmap of extracellular metabolite concentrations in the culture of yeast incubated with or without soy glycosylceramide.……..59

Figure 17. Ethanol concentrations (% (v/v)) of fermentation broth of yeast with or without soy glycosylceramide…….....61

Figure 18. Pathway analysis of extracellular metabolites of sake yeast incubated with or without soy glycosylceramide……...62

Figure 19. Fermentation test of sake yeast with or without amino acids…….76

Figure 20. The effects of amino acids on final CO_2 evolution (g/liter) with WT and its mutant *atg32 Δ* sake yeast uring fermentation……...80

Figure 21. The effects of amino acids on the maximum fermentation rate (g/liter/day) of WT and its mutant *atg32Δ* sake yeast during fermentation…….82

Figure 22. The effects of amino acids on final ethanol concentration (% (v/v)) of WT and its mutant *atg32Δ* sake yeast during fermentation……83

Figure 23. The effects of amino acids on the growth profile of WT and its mutant *atg32Δ* sake yeast during fermentation…….....84

Figure 24. Evaluation of intracellular ROS content in WT, *petite* and *atg32Δ* mutant sake yeast……..86

Figure 25. Evaluation of intracellular ROS content in the *atg32Δ* mutant sake yeast…....88

Figure 26. The intracellular metabolites of WT and its mutant *atg32Δ* sake yeast supplemented with methionine……90

Figure 27. Mitochondrial and vacuolar morphological features of WT sake yeast strain K7RAKmitGFP (A) and its mutant K7RAK *atg32Δ*mitGFP (B)…….....92

LIST OF ABBREVIATIONS

AA- Amino acid

ANOVA- Analysis of variance

Cer- Ceramide

CSM- Complete supplement media

DMAB- Dimethylaminobenzaldehyde

DCFH-DA- 2, 7-dichlorodihydrofluorescein

EDTA- Ethylenediaminetetraacetic acid

FA- Fatty acid

FDR- False discovery rate

FID- Flame ionization detector

FM4-64-(*N*-(3-Triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl) hexatrienyl) pyridinium dibromide)

GC- Gas chromatography

GFP- Green fluorescent protein

GlcCer- Glucosylceramide

GSH- Glutathione

GSLs- Glycosphingolipids

HPLC- High performance liquid chromatography

LCB- Long-chain base

MQ- MilliQ

MS- Mass spectrometry

MSTFA- *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide

OD- Optical density

OD_{544}- Optical density at 544 nm

OD_{600}- Optical density at 600 nm

PBS- Phosphate buffered saline

PCA- Principle component analysis

PDB- Pentose dextrose broth

ROS- Reactive oxygen species

SEM- Standard error of mean

TCA- Tri carboxylic acid

TLC- Thin layer chromatography

WT- Wild type

YPD- Yeast extract peptone dextrose

CHAPTER 1

INTRODUCTION

1.1 General introduction

Yeasts**,** *Saccharomyces cerevisiae,* are eukaryotic microorganisms which converts carbohydrates to carbon dioxide and alcohols by fermentation process. Fermentation is a metabolic process that converts sugar to acids, gases or alcohol. Sake yeast strains have been selected through a long history of cultivation, ranging from 100 to 400 years. The most frequently used sake yeast at present is Kyokai no. 7 (K7), which was isolated from sake mash in 1946 (Tsukahara et al. 1947, Akao et al. 2011). Glucose is fermented to ethanol by *Saccharomyces cerevisiae* sake yeast strains (Kitagaki and Kitamoto 2013) during sake fermentation. The sake yeast is the most active sake fermenter among brewery yeasts which enhanced fermentation rate (Shiroma et al. 2014). This strain produces a high concentration of ethanol, because it lacks functions of proteins encoded by *MSN4*, *PPT1* and *RIM15*, which are required to mount a stress response (Watanabe et al. 2007, Watanabe et al. 2012, Watanabe et al. 2011).

Sake is a traditional Japanese alcoholic beverage are produced via the fermentation of carbohydrates by the sake yeast *S. cerevisiae*, which can efficiently produce ethanol (Shiroma et al. 2014). However, since *S. cerevisiae* is incapable of hydrolyzing starch to glucose, additional biological catalysts are often added to the fermentation reaction. In East and Southeast Asia, various carbohydrates fermented by fungi are widely used as starch-hydrolyzing catalysts for the production of rice derived alcoholic beverages such as Japanese sake (Shiroma et al. 2014, Kitagaki and Kitamoto 2013) and shochu, Korean Makgeolli and Chinese Huangjiu as well as various other fermented foods such as soy sauce and miso (Hesseltine 1983). During the manufacturing process, glucose is produced (saccharification) from the starch present in rice by the actions of enzymes produced by the koji fungus *Aspergillus oryzae*.

Sake contains the highest ethanol concentration of all the brewed alcoholic beverages worldwide. This high ethanol concentration is generated by technologies that include successive addition of enzymes and nutrients derived from koji during sake brewing (Hirata et al. 2012, Takahashi et al. 2014). Koji is a mixture of steamed cereals or rice cultured with non-pathogenic fungi, such as *Aspergillus oryzae* and *Aspergillus luchuensis*, including *Aspergillus kawachii* and *Aspergillus awamori* (the national fungi of Japan). Therefore, koji serves as a source of the enzymes that degrade cereal-derived high-molecular-weight compounds to simple compounds.

Koji contains abundant glycosylceramide (Sakamoto et al. 2017, Sawada et al. 2015), is a lipid compound of sphingolipids. Glycosylceramide is composed of a sphingoid base moiety, fatty acid and monohexose (glucose or galactose) (Hamajima et al. 2016). Sphingolipids are critical components of the cell membrane, play important roles in fungal mycelial growth (Zhu et al. 2014) and perform various biological functions (Hannun and Obeid 2017, Truman et al. 2014, Russo et al. 2013) such as signaling and regulatory roles in cell. Fatty acids are amide-linked to the sphingoid bases to form ceramides.

In ceramides, either phosphate or carbohydrates are linked to the hydroxyl bond at the 1st position. Phosphate is ester-linked to the 1st hydroxyl bond of ceramides, and further diester-linked to inositol or choline to form acid complex sphingolipids, such as inositol-phosphoceramide (IPC), mannose-inositol-phosphoceramide (MIPC), and mannose-(inositol phosphate)$_2$-ceramide (M(IP) $_2$C) (Dickson et al. 1997) or sphingomyelin (Okazaki et al. 1989). Carbohydrates, including glucose, galactose, sialic acid, and/or their conjugates, are acetal-linked to the 1st hydroxyl bond of ceramide to form glycosphingolipid or glycosylceramides. Sphingolipids form rafts in the lipid bilayer (Simons and Ikonen, 1997) and also function as signalling lipids (Hannun and Obeid 2008).

Recent studies have shown that sphingolipids are synthesized through the membrane-trafficking system and transported across membranes by various mechanisms (Hanada et al. 2003, D' Angelo et al. 2013, Simanshu et al. 2013). Many single-celled eukaryotes such as fungi and protists contain both glycosylceramide and inositolphosphoceramide (IPC) and their more complex metabolites. However, *Saccharomyces kluyveri*, *Candida* and *Cryptococcus* spp., which are phylogenetically closely related to *S. cerevisiae* have GlcCer compound. On the other hand, *S. cerevisiae* does not have glycosylceramide (Takakuwa et al. 2002). It lacks a glycosylceramide-synthesizing enzyme (Saito et al. 2006). Moreover, lipid components such as unsaturated fatty acids (Fujii et al. 1997), sterols (Ohta and Hayashida 1983), and glycosylceramide (Sawada et al. 2015) affect the fermentation profiles of yeast. However, the nutrients available for yeasts have a major impact on the kinetics of alcoholic fermentations (Gikas and Livingston 1997, Gunasekaran and Raj 1999, Holzberg et al. 1967).

Previous report have revealed that one of the sphingolipids, glycosylceramide, abundant in koji increased the alkali tolerance and modified the physiological characteristics of yeast cells by altering the membrane properties (Sawada et al. 2015). Yeast *S. cerevisiae*, which is incapable of synthesizing glycosylceramide, adapted to alkaline condition after exposure to GlcCer from koji cultured with fungus *A. kawachii* and also increased ethanol tolerance.

Most industrial alcoholic fermentations are performed in an environment with a limited concentration of molecular oxygen. Thus, yeast cells are exposed to long and extreme anaerobiosis. Under the condition of low oxygen concentration and high fermentable sugars, yeast cells mainly obtains energy by substrate-level phosphorylation via glycolysis. During stress condition like anaerobic condition and nutrient starvation yeast mitochondria degraded and this degradation process is called mitophagy (Kim et al. 2007, Okamoto et al. 2009, Kanki et al. 2011, Eiyama et al. 2013) and is also induced in yeast cells when the availability of

nutrients are limited which has been shown to play significant roles in the progression of alcoholic fermentation. It should be noted that mitochondrial autophagy is potently regulate by abundant glucose (Wu and Tu 2011) and also by increasing glutathione levels in yeast cells (Sakakibara et al. 2015).

Kitagaki and his colleagues have also found that mitochondrial state of yeast can be manipulated by the production profile of organic acids during sake brewing (Motomura et al. 2012). In addition, mitochondria also play a key role in iron metabolism by synthesizing heme which assemble iron–sulfur (Fe/S) proteins by using sulfur containing substances (Cys, Met, GSH) as a sulfur donor that are essential for DNA synthesis, tRNA modification, protein translation and participate in cellular iron homeostasis (Lill et al. 2012, Lill et al. 2014). Moreover, sulfur containing amino acids such as methionine and cysteine also acts as mitophagy interacting substance (Sutter et al. 2013). Recently, ATG32 (autophagy related gene 32) plays a crucial role in yeast mitophagy during fermentation process (Shiroma et al 2014). During fermentation, yeast mitochondrial activity rapidly loss because of anaerobic condition. Furthermore, previously reported that residual mitochondrial activity plays significant roles in the fermentation characteristics of brewery yeast (Samp 2012, Omura 2008, Verbelen et al. 2009, Motomura et al. 2012). Therefore, addition of substances during fermentation might regulate physiological, metabolic and mitochondrial profiles of yeast.

1.2 Motivation

Yeast *S. cerevisiae* are being extensively used as workhorse for the alcoholic beverages production due to its superior fermentation characteristics, fast growth, high ethanol yield, high product recovery and high ethanol resistant. Many fermenting microbes such as yeast, lactic acid bacteria and acetic acid bacteria secrete acidic substances and grow faster at acidic pH (Sawada et al. 2015). At industrial scale yeast-based fermentation runs with the low pH and high end product alcohol production. Thus, it reduces the risk of contamination in larger extent at an industrial alcoholic fermentation.

In past few decades, various factors have been used for developing fermentation profile of yeast. Several factors such as temperature, pH, oxygen, additional substrate, acidity and availability of nutrient are all important for growth of yeast and also for initiation and progression of the fermentation process (Lin et al. 2012). However, use of additional substances during alcoholic fermentation affect the several physiological properties of yeast including ethanol tolerance and flavor profile by altering the membrane properties (Sawada et al. 2015).

Traditional alcoholic beverages are produced via the fermentation of cereals or rice by the yeast *S. cerevisiae* which can efficiently catabolize glucose to produce ethanol. In addition, recently various cereals or rice fermented by fungi as starch hydrolyzing catalysts for the production of alcoholic beverages (Kitagaki and Kitamoto 2013) and fermented foods (Hesseltime 1983). The koji fungus *A. oryzae* which is abundant sphingolipid compound glycosylceramide (Sakamoto et al. 2017), serve as a source of the enzymes that degrade polysaccharide to sugars.

The non-pathogenic fungus *A. oryzae* is used to produce many fermented foods and alcoholic beverages. Therefore, it is important to know the quantity of biomass of microbes during fermentation which will determine the quality of fermented foods and beverages. Most studies have been performed that quantitation of mycelial weight of the industrially important non-pathogenic fungus *A. oryzae*, which is used for manufacturing fermented foods and koji, by quantitating *N*-acetylglucosamine (Arima and Uozumi. 1967, Sakurai et al. 1977, Sakurai et al. 1985, Gomi et al. 1987, Fujii et al. 1992). These methods are costly, complex and precision are low. Therefore, we have for the first time, developed a simple, easy and efficient method for quantitation of *A. oryzae* mycelial weight by using glycosylceramide (Chapter 3) which can be used in the brewing and food industries.

Vast parts of the use of glycosylceramide and its effects on metabolic profile of sake yeast during fermentation are still unknown. It has been documented that unsaturated fatty acids (Fujii et al. 1997, Fujiwara et al. 1998) and sterols (Belviso et al. 2004) are required for yeast fermentation. Therefore, this study was implemented to unveil the glycosylceramide from *A. oryzae*, soy, *G. frondosa* might affect the physiological and metabolic characteristics of sake yeast when two microbes are co-cultured for the alcoholic fermentation. The yeast *S. cerevisiae* which is incapable of synthesizing glycosylceramide, adapted alkaline condition by the addition of glycosylceramide from *A. kawachii* (Sawada et al. 2015) and also modify aroma profile. Since sphingolipid like ceramide also responsible for mitochondrial dysfunction (Novgorodov et al. 2016, Law et al. 2018), therefore, it might be regulated mitochondrial function of sake yeast. However, the effect of glycosylceramide on yeast mitochondria is still unknown.

In addition, it should be revealed that mitochondrial degradation process or mitophagy affected by several amino acids (Sutter et al. 2013) which plays significant roles in the

progression of alcoholic fermentation (Shiroma et al. 2014). Specially, yeasts supplemented with methionine (Giudici and Kunkee 1994, Boudreau et al. 2017), biotin, pantothenic acids and vitamin B6 (Wainwright 1970, Wang et al. 2003) show decreased production of hydrogen sulfide as an off-flavor (Vos and Gray 1979, Cordente et al. 2009) during fermentation. Therefore, a concrete mechanism underlying the effects of exposure to these amino acids remains to be elucidated. In addition, retaining mitochondrial activity during fermentation is critical to the fermentation profiles of brewery yeasts. However, a existing mechanism linking amino acids in fermentation media with mitochondrial activity or mitophagy function during fermentation of brewery yeasts is yet unknown.

We hypothesized that if intracellular amino acids pool generated by mitophagy process and additional amino acids transported from media are independent, there is no significant interaction. On the other hand, if mitophagy senses the amino acids pool which transported from media and regulate the mitophagy function, there is significant interaction. For all these reasons, there is sufficient evidence to think that there exists a relation between the mitochondrial function and amino acids in yeast.

Therefore, this dissertation extended the study for understanding the effect of additional substances such as simple lipid compound glycosylceramide and amino acids like methionine on the sake yeast during alcoholic fermentation. The ultimate goal of this research is to increase the fermentation profiles of sake yeast by regulating aroma profile, metabolic profile and mitochondrial phenomena.

1.3 Aim and objectives of the study

The aim of this study was to predict or represent the effects of additional substances like lipid components, glycosylceramide and amino acids on the fermentation profile and metabolic profile of sake yeast under anaerobic stress condition.

Specific objectives of the effects of glycosylceramide are the following:

- Observe the correlation of glycosylceramide with the mycelial growth of *A. oryzae*
- Analyze the increase levels of glycosylceramide and *N*-acetylglucosamine contents with the growth of *A. oryzae* on koji rice
- Observe the correlation of glycosylceramide levels with *N*-acetylglucosamine content of koji rice
- Determine the effects of glycosylceramide on the flavor and metabolic profile of sake yeast during fermentation
- Analyse the metabolic pathway of extracellular metabolites of sake yeast

Specific objectives of the effects of amino acids are the following:

- Analyse the fermentation profile of WT sake yeast and its mutant *atg32Δ* with or without amino acids
- Quantitatively and objectively assess the interaction effects of additional amino acids on the mitochondrial phenomena of sake yeast
- Observe the mitochondrial activity of *atg32Δ* mutant sake yeast with or without amino acids by analyzing ROS content
- Analysis of intracellular metabolites of WT and its mutant *atg32Δ* sake yeast with or without methionine
- Microscopic observation of mitochondria in WT sake yeast and its mutant *atg32Δ* with or without methionine

1.4 Thesis organization

Rest of the thesis contains four chapters, the outline of content of the chapters are summarized as follows

Chapter 2: Background

This chapter overviews the basic idea of sphingolipids such as structure, metabolism and function. From this chapter, we are concerned about most common and important ceramide like glycosylceramide lipid compound. Moreover, the structure, classification and function of amino acids and also the metabolism pathway of important and essential amino acids such as methionine are discussed.

Chapter 3: Effects of glycosylceramide on sake yeast during fermentation

This chapter brings the completely novel information about a new method for determining the mycelial weight of koji *A. oryzae* by measuring its glycosylceramide content. Moreover, the effects of glycosylceramide on the flavor and metabolic profile of sake yeast during fermentation are presented.

Chapter 4: Effects of amino acids on sake yeast during fermentation

This chapter explains about the interaction effects of additional substances like amino acids with mitochondrial phenomena of *atg32Δ* mutant sake yeast during alcoholic fermentation. The mitochondrial activity, metabolic profile and microscopic feature of mitochondria of yeast with or without methionine are discussed in details.

Chapter 5: Discussion

This chapter consist of brief discussion on impact of research findings, conclusion and some recommendation of future works.

Finally, the thesis ends with citing references.

CHAPTER 2

BACKGROUND

2.1 Sphingolipids

Sphingolipids, sterols, and glycerophospholipids are the principal lipid components of eukaryotic membranes and are particularly abundant in the plasma membrane. Sphingolipids constitute a class of lipids defined by their carbon amino-alcohol backbones which are synthesized in the endoplasmic reticulm from nonsphingolipid precursors. Modification of this basic structure gives rise to the vast family of sphingolipids that play significant roles in membrane biology and many bioactive metabolites production that regulate cell function (Gault et al. 2010). Sphingolipids are major lipid constituents which have diverse cellular functions include both membrane structural roles and cell signaling (Merrill et al. 1996a). There are three main types of sphingolipids such as ceramides, sphingomyelins and glycosphingolipids which differ in the substituents of head group.

Ceramids are the simplest type of sphingolipid. They consist of a fatty acid chain attached through an amide linkage to sphingosine. The major phosphosphingolipids of mammals are sphingomyelins (ceramide phosphocholines), whereas insects contain mainly ceramide phosphoethanolamines and fungi have phytoceramidephosphoinositols. Sphingomyelins have a phosphorylcholine or phosphoroethanolamine molecule esterified to the 1-hydroxy group of a ceramide. Glycosphingolipids are a diverse family composed of one or more sugar residues linked via a glycosidic bond to the sphingoid base. Glycosphingolipids may be further subdivided into cerebrosides and gangliosides. Cerebrosides have a single glucose or galactose at the 1-hydroxy position, while gangliosides have at least three sugars, one of which must be sialic acid (Merrill et al. 1996a).

2.1.1 Structure of sphingolipids

Sphingolipids are ubiquitous in eukaryotes and their structures are highly diverse (Hanada 2014). Despite the diversity of structure and function of sphingolipids, their creation and destruction are regulated by common synthetic and catabolic pathways. There is considerable structural variation among the sphingolipids of different organisms with respect to the types of polar head groups and ceramide backbones (Merrill et al. 1996b, Merrill and Sweeley 1996). In their simplest forms, sphingosine, phytosphingosine and dihydrosphingosine serve as the backbones for complexity (Gault et al. 2010).

The ceramide backbone usually carries several functional groups such as hydroxy groups, (*E*)- or (*Z*)-double bonds, methyl branches at aliphatic or olefinic carbon atoms, or even cyclopropane rings (Ternes et al. 2006). Different patterns of functional groups are characteristic for GIPC *versus* cerebrosides as well as for different organisms (Fig. 1) (Ternes et al. 2006). Mammalian glycosylceramide containing (*E*)-sphing-4 enine as sphingoid base (Sullards et al. 2000) and 2-hydroxystearic acid as fatty acyl residue. In some tissues, the Δ4-double bond is replaced by a C4-hydroxy group. Whereas, plant glucosylceramide contain (*E*, *E*)-sphinga-4, 8-dienine as sphingoid base (Sullards et al. 2000) and 2-hydroxypalmitic acid as fatty acyl residue. The Δ4-double bond may be replaced by a C4-hydroxy group and the Δ8-double bond may be either in the (*E*) or in the (*Z*) configuration. In addition, (*E*, *E*)-9-methyl-4, 8 sphingadienine as sphingoid base and 2-hydroxystearic acid as fatty acyl residue present in fungal GlcCer (Rodrigues et al. 2000, Del Poeta et al. 2014). In some fungi, the fatty acyl residue additionally contains a Δ3-double bond.

On the other hand, IPC from *S. cerevisiae* contain 4-hydroxysphinganine as sphingoid base, 2-hydroxyhexadecanoic acid as fatty acyl residue, and a phosphoinositol head group. Its ceramide backbone differs from that of the glucosylceramides shown in A–C (Fig. 1) in

containing a saturated sphingoid base and a very long chain fatty acyl residue with 24 or 26 carbon atoms (Ejsing et al. 2009, Kitagaki et al. 2007), whereas the length of the fatty acyl residue in GlcCer of plants and fungi is 16 to 20 carbon atoms (Hirata et al. 2012, Takahashi et al. 2014).

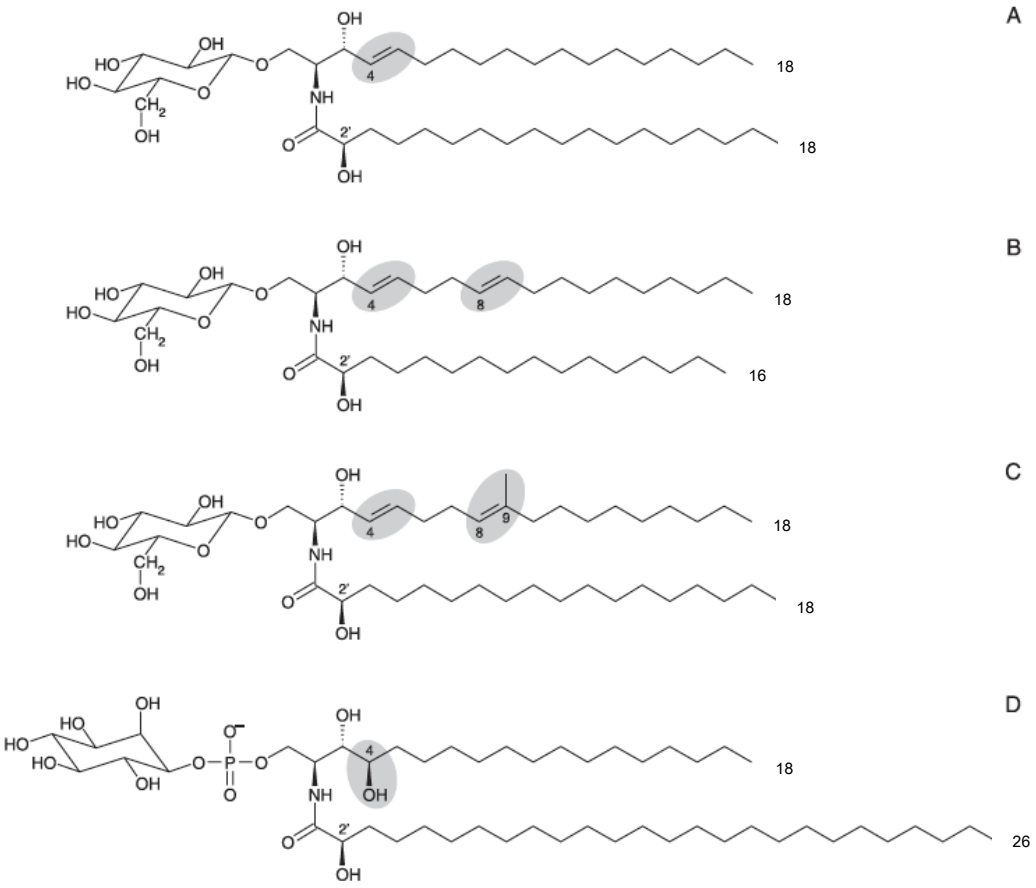

Figure 1. Structures of typical glucosylceramides from mammals (A), plants (B), fungi (C) and of IPC from *S. cerevisiae* (D). In all structures shown in A–D, the C2'-hydroxy group may be missing (Adapted from Ternes et al. 2006)

2.1.2 Scope of sphingolipids

Sphingolipids are a diverse group of lipids which serve a variety of functions in cell biology. Only a few of these functions have been highlighted here, but the scope of sphingolipid biology is vast. Without certain sphingolipids, cells and/or embryos can not survive,

indicating that sphingolipids possess important physiological functions that are not substituted by other lipids. Sphingolipids have been involved in the regulation of cell growth, differentiation and programmed cell death. The current paradigm for their action is that complex sphingolipids such as gangliosides interact with growth factor receptors, the extracellular matrix and neighboring cells (Spiegel and Merrill 1996). The simple sphingolipids serve both as the precursors and the breakdown products of the more complex ones.

Importantly, in recent decades, these simple sphingolipids have gained attention for having significant signaling and regulatory roles within cells. In addition, many tools have emerged to measure the levels of simple sphingolipids and therefore have become the focus of even more intense study in recent years (Gault et al. 2010). Recent studies have revealed that some sphingolipid metabolites, such as long-chain bases (LCBs; sphingosine (Sph) in mammals), long-chain base 1-phosphates (LCBPs; sphingosine 1-phosphate (S1P) in mammals), ceramide (Cer), and ceramide 1-phosphate (C1P), act as signaling molecules (Kihara et al. 2007).

2.1.3 Biosynthesis of sphingolipids

The common baker's yeast *S. cerevisiae* has served in many ways to foster our understanding of sphingolipid metabolism and functions, beginning with the early work of Herbert Carter and his students in the 1950s and early 1960s, who played roles in characterizing sphingolipid LCBs (Carter and Hendrickson. 1963). This was followed by elucidation of the types of complex sphingolipids found in yeast cells and their route of synthesis by Lester and colleagues (Dickson and Lester 1999a, Dickson and Lester 1999b). More recently, yeast has been used to identify nearly all of the genes that encode sphingolipid metabolic enzymes, and many of these were critical in identifying human homologs (Dickson and Lester 2002, Sims

et al. 2004).

Sphingolipids are abundant in *S. cerevisiae* representing about 7% of the mass of the plasma membrane or 30% of phospholipids (Patton and Lester 1991). Like phospholipids, sphingolipids also contain two non-polar tails (sphingosine and fatty acid) and one polar head group (polar group attached to the phosphate moiety). *S. cerevisiae* sphingolipid metabolism including metabolites, enzymes and their relative genes are shown in Fig. 2. Biosynthesis of sphingolipids starts from condensation of serine with a palmitoyl-CoA by serine palmitoyltransferase (SPT) to yield 3-ketodihydrosphingosine (ketosphinganine) and CO_2. 3-ketodihydrosphingosine is reduced to DHS by the Tsc10 protein (Beeler et al. 1998). DHS is then amide-linked to a C26 fatty acid by either of two ceramide synthases (acyl-CoA: sphingosine *N*-acyltransferase), Lag1 and Lac1 (Guillas et al. 2001, Schorling et al. 2001), to yield *N*-acylsphinganine (dihydroceramide), which is hydroxylated at C-4 by Sur2/Syr2 to give phytoceramide.

Ceramides and phytoceramides are transported from the endoplasmic reticulum (ER) to the Golgi apparatus by vesicle trafficking where the C_1 hydroxyl of phytoceramide is decorated with polar head groups by the three sequential reactions diagramed in Fig. 2 which yield three species of complex sphingolipids including inositol phosphoceramide (IPC), mannose inositol phosphoceramide (MIPC) and mannose-(inositol-P) 2-ceramide (M $(IP)_2$ C) (Dickson et al. 1997, Dickson 2008). Additionally, complex sphingolipids serve structural functions in membranes, and they may also undergo dynamic clustering with sterols to form lipid microdomains or ‘rafts’ within the membrane bilayer. This domain structure play a role for signal transduction and/or protein sorting (Futerman and Riezman 2005).

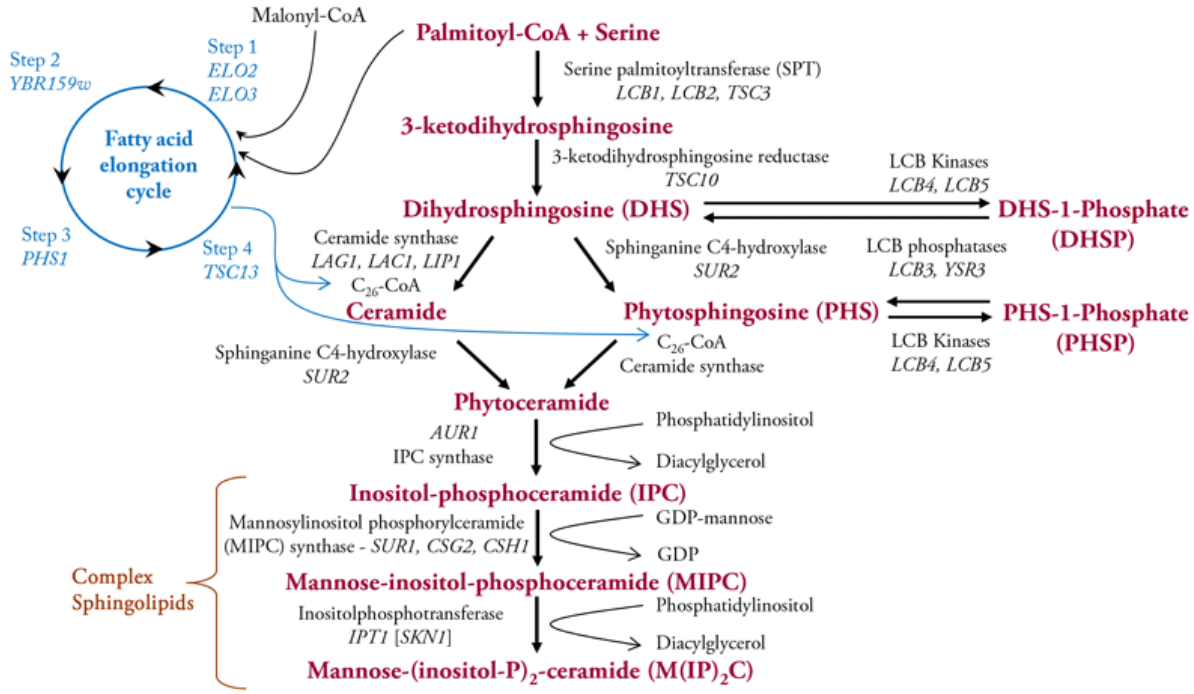

Figure 2. Outline of sphingolipid metabolism in *S. cerevisiae*

Metabolic intermediates and complex sphingolipids are shown in bold red lettering with genes which are indicated by italics, and enzyme names are in regular lettering. More information about genes and other features of yeast sphingolipid metabolism are available as Saccharomyces Genome Database (SGD) (www.yeastgenome.org) (Adapted from Dickson, 2008).

The simple sphingolipids serve as the precursors and the breakdown products of the more complex ones. Importantly, in recent decades these simple sphingolipids have gained attention for having significant signaling and regulatory roles within cells. In addition, many tools have emerged to measure the levels of simple sphingolipids and therefore have become the focus of more intensive study in recent years.

2.1.4 Ceramides

Among the most simple sphingolipids are the ceramides (sphingosine plus a fatty acid), widely distributed in small amounts in plant and animal tissues. The other sphingolipids are derivatives of ceramides. It is well known that ceramides play an essential role in structuring and maintaining the water permeability barrier function of the skin (Coderch et al. 2003) and also regulate signal transduction, cell regulation, transformation and autophagy by activating protein phosphatase 2A (Dobrowsky et al. 1993) in cell. Ceramide (Cer) is a hydrophobic backbone of sphingolipids. Sphingolipids exist in all eukaryotes, but not in prokaryotes, except for a limited number of bacteria species such as *Sphingomonas* (Yamamoto et al. 1978). Cer contains two hydrophobic chains, LCB and a fatty acid (FA), which are connected via an amide bond. Cer is a membrane bound lipid molecule that has very low solubility in an aqueous environment. The cell employs two major mechanisms to mobilize Cer either through vesicular transport or through the protein ceramide transfer protein (CERT). CERT is a cytosolic protein that transfers ceramide from the ER to the Golgi apparatus where it can be modified into sphingomyelins and possibly glycosphingolipids (Gault et al. 2010).

Addition of a polar head group at the C1 hydroxyl group of the LCB portion of Cer, resulting sphingolipid with amphipathic properties. Characteristic *in vivo* head group types differ among organisms (Dickson et al. 2006, Vacaru et al. 2013, Markham et al. 2013, Kihara 2015). In mammals, it is either phosphocholine (in sphingomyelin) or sugar chains (in glycosphingolipids) (Kihara 2015, Kihara et al. 2007). Hundreds of glycosphingolipids differing in sugar classes. The sugar residues are glucose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, fucose and sialic acid (Kihara 2015, Wennekes et al. 2009).

2.1.5 Glycosphingolipids

Glycosphingolipids (GSLs), which are the glycosides of either Cer or *myo* ‐ inositol ‐ (1 ‐ *O*) ‐ phosphoryl ‐ (*O* ‐ 1) ‐ ceramide, are a structurally and functionally diverse sphingolipid subclass. GSLs are ubiquitously distributed among all eukaryotic species and are found in some bacteria (Levery 2005). The simplest GSLs are glucosylceramide (GlcCer) and galactosylceramide (GalCer), which respectively contain a glucose or galactose residue linked to ceramide via β- linkage.

GlcCer is a sugar sphingolipid composed of a sphingoid base, a fatty acid, and a glucose moiety and primarily localized in cell membrane (Gault et al. 2010). Notably, GlcCer is found in plants, fungi, and animals and absent in bacteria and in some eukaryotes such as the yeast *S. cerevisiae* (Ternes et al. 2006). In spite of the presence of GlcCer in most organisms, its synthetic pathway and molecular structure varies significantly (Leipelt et al. 2001, Rhome et al. 2007, Warnecke and Heinz 2003, Nimrichter and Rodrigues 2011), resulting in the occurrence of rather unique GlcCer molecular species in different organisms. GlcCer is synthesized in the *cis*-Golgi from ceramide and uridine 5-diphosphate (UDP)-glucose by the enzyme glucosylceramide synthase (GCS) (Ichikawa et al. 1996). GCS is a transmembrane protein present on the *cis*-Golgi, and it has catalytic site where newly produced glucosylceramide can be recognized by the lipid transport protein FAPP2 (Jeckel et al. 1992, D' Angelo et al. 2007).

2.1.6 Mammals glucosylceramide

The ceramide backbones of most mammalian sphingolipids contain mainly sphingosine (*trans*-4-sphingenine) and lesser amounts of sphinganine and 4-hydroxysphinganine (Sullards et al. 2000). Moreover, whereas in plants and fungi GlcCer is the end product of the synthetic pathway, in mammals GlcCer is used as a substrate to make more complex glycosphingolipids,

such as lactosylceramides and gangliosides (Del Poeta et al. 2014). Unlike galactosylceramide, GlcCer is an absolutely essential sphingolipid for the development of mammals (Yamashita et al. 1999). Mice lacking GCS do not survive to term. The loss of GCS results in embryonic lethality (Yamashita et al. 1999). This specific defect can be rescued by the addition of exogenous GlcCer to the embryos. GlcCer is the precursor for the majority of all glycosphingolipids that can be produced by a mammal and these glycosphingolipids are likely to play an essential role in cell-cell recognition during embryonic and post-natal development (Yamashita et al. 1999).

2.1.7 Plant glucosylceramide

GlcCer is a major sphingolipid of plant tissue and thus, abundant in nature and in dietary food sources. Plants make primarily GlcCer with sphinganine, 4-hydroxysphinganine and *cis* and *trans* isomers of 8- sphingenine, 4, 8-sphingadienine and 4-hydroxy-8-sphingenine. (Sullards et al. 2000). For example, soybean GlcCer was comprised primarily (>95%) of ceramide with 4,8-sphingadiene, a-hydroxypalmitic acid and fatty acids. In addition, wheat GluCer have three major ceramide (Sullards et al. 2000). In plants, GlcCer is considered to be the most abundant glycosphingolipid, and its Cer backbone has the greatest structural variety. Seven different sphingoid bases can be linked to each of ten different a-hydroxy fatty acids, yielding to the production of 70 GlcCer species. This high variety is not present in fungi or mammals, in which only one or two sphingosine backbones can be attached to few different fatty acids (Del Poeta et al. 2014).

2.1.8 Fungal glucosylceramide

GlcCer was first isolated from the yeast *Hansenula ciferri* in 1971 (Kaufman et al. 1971). The biochemical characterization of its structure in a variety of fungi, however, became available during the 1990s. Fungal GlcCer has a very unique chemical structure compared to GlcCers in mammals and plants. Fungal GlcCer is important for fungal cell growth and division, alkaline tolerance (Sawada et al 2015), mycelial growth (Zhu et al. 2014), spore germination and also for the regulation of fungal virulence (Del Poeta et al. 2014). However, in many fungi such as yeasts, molds, and in dimorphic fungi, fungal GlcCer exhibits a characteristic structure which is synthesized by fungal specific enzymes. Thus, it is envisioned as an important fungal target compounds with specific and broad-spectrum activity. The native GlcCer structure in fungi is a 9-methyl-4,8-sphingadienine in an amide linkage to 2-hydroxyoctadecanoic acid (Rodrigues et al. 2000, Del Poeta et al. 2014).

Compared to mammalian GlcCer, fungal GlcCer is unsaturated in position 8 and methylated in position 9 of the sphingosine backbone. Moreover, the methylation of GlcCer in fungi, seems to be essential for allowing cell division compare to plant and mammals. As *C. neoformans* strain lacking the sphingolipid methyltransferase 1 enzyme (Smt1) responsible for this reaction (Ternes et al. 2006), *⊿smt1* is mostly arrested in the stationary phase and cannot proceed through the cell cycle (Rittershaus et al. 2006, Singh et al. 2012. In addition, glycosphingolipid is not present in yeast *S. cerevisiae* contrary to other fungi (Takakuwa et al. 2002), in which cell cycle is very easy to synchronize. The fact that the *⊿gcs1* (GlcCer is absent) and the *⊿smt1* (GlcCer is not methylated), *C. neoformans* strains share the same growth defect and cell cycle arrest phenotypes at neutral/alkaline pH supports that methylation of GlcCer is critical for its function in the regulation of cell cycle progression (Del Poeta et al. 2014).

Notably, *Aspergillus fumigatus* and others fungi produce also GalCer, another type of glycosphingolipid in addition to GlcCer in which galactose is attached to ceramide (Warnecke and Heinz 2003).

2.1.9 Beneficial effects of glycosylceramide

Dietary glycosylceramide has several benefits including improving skin moisture (Coderch et al. 2003) and intestinal microbial flora (Hamajima et al. 2016). Koji, the base for Japanese traditional cuisines, contains abundant glycosylceramide increases the content of several microbes, including *Blautia coccoides* (Hamajima et al. 2016). Since *B. coccoides* have several health benefits, an increase of *B. coccoides* by taking fungal glycosylceramide might be one mechanism explaining Japanese longevity (Hamajima et al. 2016). It was first revealed that glycosylceramide, which has estimated health benefits are abundantly contained in Japanese fermented foods. Especially, amazake (rice drink fermented with fungus *A. oryzae*), contained the most abundant glycosylceramide (up to 4.2 mg per serving) (Sakamoto et al. 2017, Sawada et al. 2015). Thus, the high content of glycosylceramide is now emerging as an unattended health benefit of Japanese fermented foods. However, the koji glycosylceramide constituted the most abundant species (43% of the total glycosylceramide) in the sake lee which will be developed the utilization of sake lee for cosmetics and functional food industry (Takahashi et al. 2014). However, anti-GlcCer antibodies prevent fungal differentiation and growth of fungal pathogens. For instance, these antibodies blocks germ tube formation in *C. albicans*, *Colletotrichum gloeosporioides* and *Pseudallescheria boydii* (Pinto et al. 2002, da Silva et al. 2004). Furthermore, anti-GlcCer antibodies were able to protect mice in a lethal infection by *C. neoformans* (Rodrigues et al. 2007b).

2.2 Amino acids

Amino acids (AAs) are organic nutrients that appear either as building blocks of proteins or as free amino acids. AAs are made of amino group (NH_2), carboxyl group (COOH) and a side chain containing carbon, hydrogen or oxygen. However, two amino acids (cysteine and methionine) also contain sulfur and one (selenocysteine) contains selenium. AAs are crystalline solids which have the capacity to dissolve in water. Amino acids have high melting points up to 200 to 300°C with other properties varying for each particular amino acid. They tends to evolve slower than DNA. However, changes in DNA do not affect amino acid properties or functions. Except for glycine, all AA can have L- and D-isoforms. Because of variations in their side chains, AAs have remarkably different biochemical properties and functions (Brosnan 2001, Suenaga et al. 2008, Wu et al. 2007a, Scot et al. 2006).

Among more than 300 amino acids in nature, 20 amino acids are created to build proteins and therefore termed proteinogen. Besides this, there are approximately 250 amino acids which do not form proteins. These are used to form sugar for example.

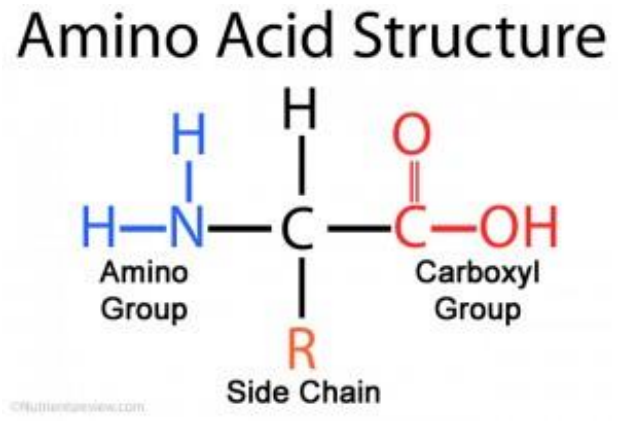

Figure 3. General structure of AAs

All AAs contain the amino and carboxyl group and a side chain that makes amino acids different from each other.

2.2.1 Classification of amino acids

The 20 proteinogen AAs are also called standard AAs, which can be divided into three groups: essential, semi-essential and non-essential.

Table 1. List of 20 proteinogenic AAs

Name of AA with (Abbreviation)	Classification
1. Isoleucine (Ile) 2. Leucine (Leu) 3. Lysine (Lys) 4. Methionine (Met) 5. Phenylalanine (Phe) 6. Threonine (Thr) 7. Tryptophan (Trp) 8. Valine (Val)	Essential AAs: Eight amino acids are essential for humans because the body cannot produce them by themselves and they have to be supplied externally
9. Histidine (His) 10. Arginine (Arg)	Semi-essential AAs: These amino acids can be synthesized in body, but in certain circumstances, like young age, illness or hard exercise, we need to get them in additional amounts from foods to meet the body requirements for them.
11. Alanine (Ala) 12. Asparagine (Asn) 13. Aspartic acid (Asp) 14. Glutamic acid (Glu) 15. Cysteine (Cys) 16. Glutamine (Gln) 17. Glycine (Gly) 18. Proline (Pro) 19. Serine (Ser) 20. Tyrosine (Tyr)	Nonessential AAs: These amino acids can be synthesized in body from other amino acids, glucose and fatty acids, so we do not need to get them from foods.

Ref- https://www.aminoacidsguide.com

An additional AAs classification depends upon the side chain structure, and experts recognize these five as:

- Sulfur-containing AAs include cysteine, homocysteine, methionine and taurine
- Acidic AAs are aspartic and glutamic acid, and basic amino acids are arginine, histidine and lysine
- Neutral AAs include asparagine, serine, threonine, tyrosine, cysteine and glutamine
- Aliphatic AAs include leucine, isoleucine, glycine, valine, methionine, proline and alanine.
- Aromatic AAs include phenylalanine and tryptophan.

Another one final AAs classification is categorized by the side chain structure that divides the list of 20 amino acids into four groups, two of which are the main groups and two that are subgroups. They are: non-polar, polar, acidic and polar, basic and polar

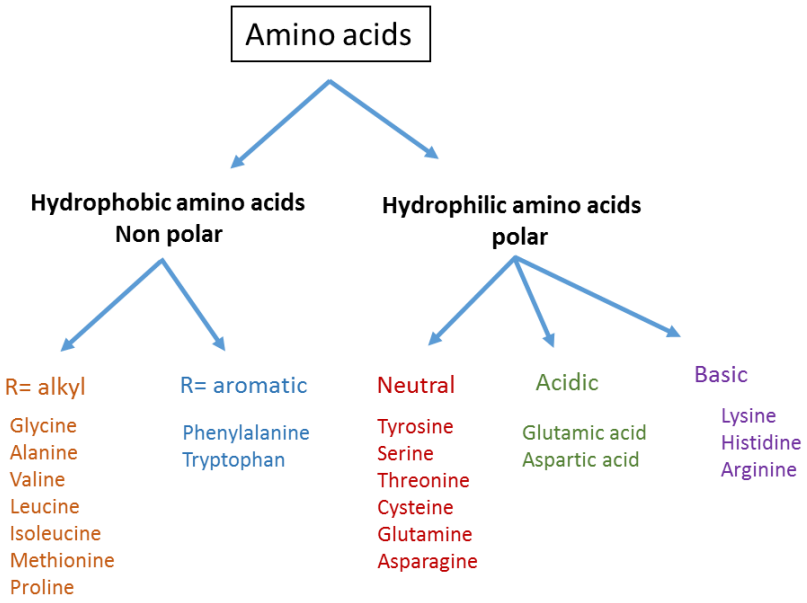

Figure 4. Classification of AAs based on the nature of the R group

2.2.2 Importance of amino acids (Wu 2009)

- Nutrient absorption and metabolism (e.g., nutrient transport, protein turnover, fat synthesis and oxidation, glucose synthesis and oxidation, amino acid synthesis and oxidation, urea and uric synthesis for ammonia detoxification, and efficiency of food utilization)
- Cellular signaling via mTOR, cAMP and cGMP activation pathways (Neklesa and Davis 2009)
- Hormone synthesis and secretion (e.g., insulin, glucagon, growth hormone, glucocorticoids, prolactin, placental lactogen, and epinephrine)
- Endothelial function, blood flow, lymph circulation and relaxation (L-arginine) (Speigel and Merrill 1996)
- Immune function and health (e.g., T-cell proliferation and B-cell maturation, antibody production by B-cells, killing of pathogens, obesity, diabetes and metabolic syndrome)
- Reproduction and lactation (e.g., spermatogenesis, male fertility, ovulation, ovarian steroidogenesis, embryo implantation, placental angiogenesis and growth, fetal growth and development and lactogenesis)
- Acid–base balance, neurotransmission, extracellular and intracellular osmolarity, antioxidative defense and whole body homeostasis
- Fetal and postnatal growth and development, as well as tissue regeneration and remodeling

However, AAs also play an extensive role in gene expression processes, which include the adjustment of protein functions that facilitate messenger RNA (mRNA) translation (Kimball et al. 2006).

2.2.3 Biosynthesis of sulfur amino acids

Methionine and cysteine may be considered to be the principal sulfur-containing AAs because they are 2 of the canonical 20 AAs that are integrated into proteins. However, homocysteine and taurine also play important physiological roles. Sulfur amino acid biosynthesis in *S. cerevisiae* involves a large number of enzymes required for the de novo biosynthesis of methionine and cysteine and the recycling of organic sulfur metabolites.

In yeast cells, the reduced form of sulfate is used in the synthesis of organic sulfur metabolites mostly cysteine, methionine and S-adenosylmethionine (SAM). Methionine metabolism begins with its activation to S-adenosylmethionine (Thomas and Surdin-Kerjan 1997). This is a cofactor of extraordinary versatility, playing roles in methyl group transfer, 59-deoxyadenosyl group transfer, polyamine synthesis, ethylene synthesis in plants and many others. S-adenosylhomocysteine, which is a product of these methyltransferases, gives rise to homocysteine. Homocysteine may be remethylated to methionine by the methionine synthase Met6p or converted to cysteine by the transsulfuration pathway (Thomas and Surdin-Kerjan 1997). Methionine may also be metabolized by a transamination pathway. This pathway, which is significant only at high methionine concentrations, produces a number of toxic end products. Cysteine may be converted to such important products as glutathione and taurine (Fig. 5) (Thomas and Surdin-Kerjan 1997).

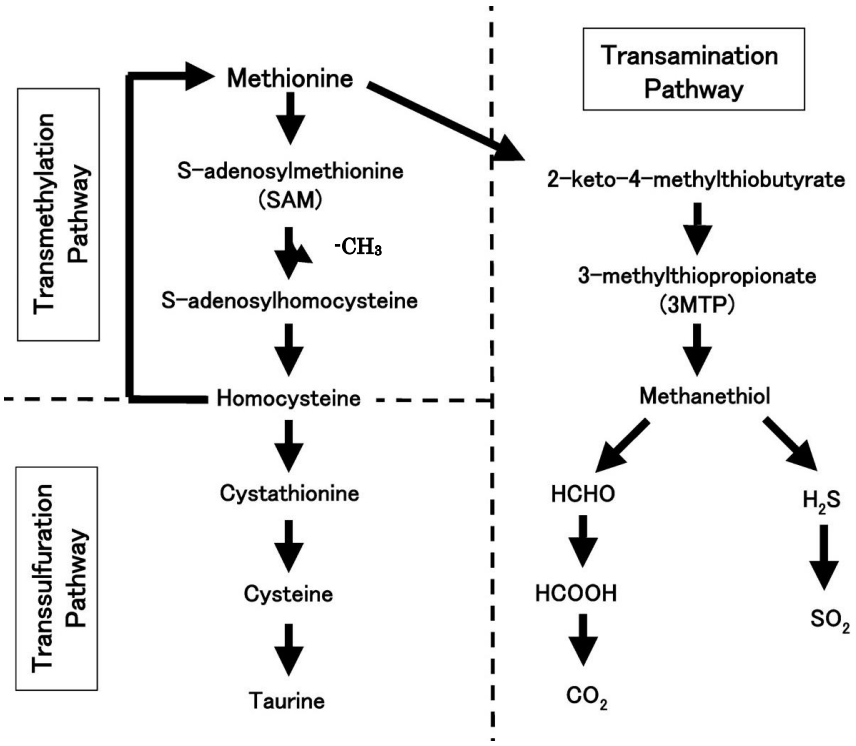

Figure 5. Sulfur AAs utilization and metabolism in budding yeast (adapted from Thomas and Surdin-Kerjan 1997)

2.2.4 Effects of sulfur amino acids (methionine) in cells

The cell requirement for sulfur can be fulfilled by the uptake of sulfur containing amino acids or by assimilation of inorganic sulfur into organic compounds such as methionine, cysteine, homocysteine, glutathione and also some iron-sulfur proteins (Thomas and Surdin-Kerjan 1997). Among cell components, mitochondria plays a key role in iron metabolism in that they assemble iron-sulfur protein by using sulfur containing substances (Cys, Met and GSH) and maintain iron homeostasis (Lill et al. 2012, Lill et al 2014). Since the function of electron transport chain is based on the function of iron, therefore sulfur containing amino acid might be regulated the mitochondrial activity. Addition of methionine also decreased hydrogen sulfide production which leads to off-flavor (Boudreau et al. 2017).

Various organic acids like butyric acid, octanoic acid, hexanoic acid, vanillic acid and decanoic acid are produced by the addition of AAs (Teresa and Carmen 2008). By using transamination pathway methionine produce α-keto-γ-methylthiobutyrate which is also converted to methanethiol, methionol and α-ketobutyrate (Perpete et al.2006) which might be stimulated mitochondria. Moreover, nutrient signaling pathway TORC1 (Target of Rapamycin Complex 1) is regulated in response to the AAs and nutrient status of cells (Kamada et al. 2010, Sutter et al 2013).

2.3 Concluding remarks

This chapter introduced the general background such as structure, types and functional importance of some nutritional substances such as sphingolipids and amino acids. Moreover, glycosylceramide is one of the most important sphingolipid in mammals, plants, fungi but absence in bacteria and yeast. In addition, glycosylceramide which is abundant in *A. oryzae* increases the content of intestinal microflora, develop functional food industry are reviewed. As glycosylceranide is absence in yeast, therefore, the use of glycosylceramide might affect the physiological and metabolic characteristics of yeast which is promising to develop yeast *S. cerevisiae*. In addition, another nutritional substance, sulfur containing amino acids like methionine also have regulatory effects on cell and cell components. As methionine is used to maintain electron transport chain by forming iron/sulfur cluster protein, it might regulate mitochondrial function. Therefore the effects of additional substances such as glycosylceramide (sphingolipid) and methionine (amino acid) on the yeast fermentation are highly encouraged the research in this study.

CHAPTER 3

EFFECTS OF GLYCOSYLCERAMIDE ON SAKE YEAST DURING FERMENTATION

3.1 Introduction

As a saccharifier of starch, koji which is steamed grains such as rice and barley fermented with the non-pathogenic fungus such as *Aspergillus oryzae* and *Aspergillus luchuensis*, including *Aspergillus kawachii* and *Aspergillus awamori* (the national fungi of Japan) has been used traditionally in Japan. Koji is responsible for the saccharification of starch in crops used in the manufacture of most Japanese fermented foods and corresponds to malt in beer (Kitagaki and Kitamoto, 2013). However, the main function of koji is to facilitate the enzymatic conversion of polysaccharides into sugars. As the mycelial weight of *A. oryzae* is mostly responsible for maintaining the quality of koji, methods for its quantitation have been intensively studied. It has been studied that the quantitation of *N*-acetylglucosamine as an index of the mycelial weight of *A. oryzae*. While this procedure has been applied successfully, it has several technical difficulties. For example, *N*-acetylglucosamine is a component of the fungal cell wall; hence, in order for it to be extracted, cell wall chitin must first be degraded with acid treatment (Arima and Uozumi 1967, Sakurai et al. 1977, Sakurai et al. 1985) or by lytic enzymes (Gomi et al. 1987, Fujii et al. 1992), and the efficiency of this degradation may vary between analyses. Thus, the procedure becomes costly and complex, making quantitation difficulties. In earlier studies, we have shown that the concentration of glycosylceramide is significantly higher in koji than in crops such as rice and barley (Hirata et al. 2012, Takahashi et al. 2014, Sawada et al. 2015).

Koji contain abundant glycosylceramide (Sakamoto et al. 2017, Sawada et al. 2015) which is a component of sphingolipids. Glycosylceramide is composed of a sphingoid base moiety, fatty acid and monohexose (glucose or galactose) (Hamajima et al. 2016).

Sphingolipids are critical components of the cell membrane, where they perform various biological functions (Hunnan and Obeid 2017, Truman et al. 2014, Russo et al. 2013) and also play important roles in fungal mycelial growth (Zhu et al. 2014). In this study, it was demonstrated that glycosylceramide levels significantly correlate with the mycelial weight of *A. oryzae* and increase with incubation time during the koji manufacturing process in a manner similar to that observed with *N*-acetylglucosamine levels. We found that the amount of *N*-acetylglucosamine in koji correlates significantly with that of glycosylceramide which is a good indicator of the mycelial weight of *A. oryzae*.

Koji glycosylceramide not only plays the role in fungal mycelial growth but also perform various biological functions. In addition, other functionally important but unknown, interaction of koji glycosylceramide and yeast might also occur. Japanese traditional alcoholic beverages such as rice wine, sake, are mainly use rice as their raw material. Therefore, rice starch is used as a source of carbohydrates and thus saccharification of starch is necessary for subsequent fermentation by sake yeast *S. cerevisiae*. As *S. cerevisiae* is incapable of hydrolysing starch to glucose, therefore koji is then mixed with steamed rice and sake yeast which serve as a source of enzymes for saccharification and thus simultaneous saccharification and fermentation occurs (Kitagaki and Kitamoto 2013).

Koji contains various enzymes, including glycosidases, proteases and lipases. The role of these koji enzymes on sake brewing has been studied intensively. Indeed, yeast can synthesize most substances, including saturated fatty acids, from substances provided from rice and degraded by koji enzymes, allowing them to proliferate and ferment. However, in addition to these substances, yeast needs lipids other than saturated fatty acids.

Past studies have been limited to unsaturated fatty acids and sterols. For example, the unsaturated fatty acids have been shown to facilitate fermentation and decrease isoamylacetate production (Ohta and Hayashida 1983, Fujii et al. 1997, Mason and Dufour 2000). Unsaturated fatty acids, sinosterol and phospholipids synergistically affect the fusel alcohols in beer and decrease the content of volatile esters and medium chain-length fatty acids (Taylar et al. 1979). In addition, grape phytosterol increases the fermentation rate of yeast (Luparia et al. 2004). However, there has been no report on the effects of sphingolipids on yeast fermentation.

Sphingolipids are ubiquitous in eukaryotes and their structure are highly diverse (Hanada 2014). Sphingolipids is a class of lipids that contain amide bonds of sphingoid bases and fatty acids. Serine and palmitoyl-CoA are conjugated to form sphinganine, which is reduced to sphingoid bases, such as phytosphingosine, sphingosine and dihydrosphingosine. Fatty acids are amide-linked to the sphingoid bases to form ceramides. In ceramides, either phosphate or carbohydrates are linked to the hydroxyl bond at the 1st position. Phosphate is ester-linked to the 1st hydroxyl bond of ceramides, and further diester-linked to inositol or choline to form acid complex sphingolipids such as inositol-phosphoceramide (IPC), mannose-inositol-phosphoceramide (MIPC), and mannose-(inositol phosphate)$_2$-ceramide (M(IP)$_2$C) (Dickson et al. 1997) or sphingomyelin (Okazaki et al. 1989). Carbohydrates, including glucose, galactose, sialic acid and/or their conjugates, are acetal-linked to the 1st hydroxyl bond of ceramide to form glycosylceramide.

Many single cell organisms contain both glycosylceramide and inositol-phosphoceramide (IPC) and their complex metabolites which are synthesized by the membrane-trafficking system and transported across the membrane by different mechanism (Hanada et al. 2003, D' Angelo et al. 2013, Simanshu et al. 2013). Exceptionally, *S. cerevisiae* lacks a

glycosylceramide-synthesizing enzyme (Saito et al. 2006).

Indeed, sphingolipids interact with sterols through van der Waals interactions and form rafts in the lipid bilayer (Simons and Ikonen 1997) and also function as signalling lipids (Hannun and Obeid 2008). In previous studies, it has been reported that one of the sphingolipids, glycosylceramide having galactose (19.2%) or glucose (80.8%) as the monohexosyl moiety, 9-methyl-4,8-sphingadienine as the sphingoid base moiety and 2'-hydroxyoctadecanoic acid as the fatty acid moiety, is contained in shochu and sake koji (Hamajima et al. 2016, Hirata et al. 2012).

Furthermore, other report have revealed that the glycosylceramide contained in shochu koji increased the alkali tolerance, ethanol tolerance and modified the flavor profile of shochu yeast (Sawada et al. 2015). However, the effect of koji glycosylceramide on the fermentation profile of sake yeast remains unknown. Therefore, in the study of this thesis, we investigated the effect of koji glycosylceramide on the brewing characteristics of sake yeast. Although *S cerevisiae* is often used for the production of various foods and beverages without help of other fungi, this organism is available in soil where it forms coexist with other fungi (Xie et al. 2007). Thus it is interesting to hypothesize that requiring glycosylceramide from other microbes might be facilitated the loss of glycosylceramide synthase gene from its genome.

3.2 Materials and methods

3.2.1 Strains, reagents and materials

Koji samples were purchased from Tokushima Seiko Co. Ltd. (Osaka, Japan). Conidia of *A. oryzae* were purchased from Higuchi Moyashi Co. Ltd. (Osaka, Japan). For mycelial growth of *A. oryzae*, potato dextrose agar (0.4 % potato starch, 2 % dextrose, 2 % agar media were used. Cerebroside purchased from Matreva Inc. (USA) was used as a glycosylceramide standard. Glycosylceramide of soy and *Grifola frondosa* were purchased from Funakoshi and that of *A. oryzae* was purified from the mycelia of *A. oryzae*. The *S. cerevisiae* sake yeast K7-4 (isolated and distributed in 2003 from the Brewing Society of Japan,) was obtained from the Brewing Society of Japan (Tokyo, Japan). All chemicals were of analytical grade.

3.2.2 Culture of *A. oryzae*

Conidia of *A. oryzae* (10 mg) were suspended in 1 ml of sterile water and mixed with vortex. Then 100 µl of the solution was inoculated onto potato dextrose agar (0.4% potato starch, 2% dextrose, 2% agar) and incubated at 30°C for 3 to 5 days. An aliquot culture of *A. oryzae* were inoculated into potato dextrose broth (pH 5.1±0.2) (Difco Laboratories, Detroit, USA) and incubated at 30°C and 200 rpm for 3 to 5 days. The mycelia were collected by centrifugation, lyophilized and ground with a mortar.

3.2.3 Quantitation of glycosylceramide of the mycelia of *A. oryzae*

To 100 mg of mycelia, 1 ml methanol and 0.5 ml chloroform were added, and the mixture was vortexed for 3 min. Following this, 0.5 ml chloroform was added to the solution, which was vortexed again for 3 min. The solution was then sonicated for 10 min. Methanol (2 ml) containing 0.8 M KOH was added, and the solution was incubated at 42°C for 30 min. Chloroform (5 ml) and distilled water (2.25 ml) were then added, and the solution was

vortexed until saponification. After centrifugation at 800×*g* for 10 min, the supernatant was recovered, evaporated under vacuum, and dissolved in 200 μl chloroform/methanol (2:1 v/v). An aliquot of the resultant solution (60 μl) was spotted onto a thin layer chromatography (TLC) plate (Silica gel 60 plate, Merck Millipore Inc., Darmstadt, Germany). The dried plate was developed with saturated chloroform:methanol:acetic acid:water (20: 3.5: 2.3: 0.7 v/v). As a standard, 4 μl of 10, 5, 2.5, and 1.25 mg/ml cerebroside (Matreya Inc., PleasantGap, USA) was spotted onto the TLC. To detect glycosylceramide, 2 mg/ml of orcinol (MP Biomedicals, Illkirch, France) in 70% sulphuric acid was sprayed onto theTLC plate and heated at 100°C for 40 min.

3.2.4 Koji manufacture

Koji was manufactured using a rice cultivar (Yamadanishiki) used for sake brewing (whose surface (30%) was polished). Rice was soaked in water until the weight increased by 30% (w/w). Soaked rice was steamed with vapor for 1 h, and then cooled to room temperature. Conidia of *A. oryzae* (10 mg) were inoculated into the steamed rice (28 g). The steamed rice grains were mixed well and incubated in a packed container at 37°C for 72 h.

3.2.5 Quantitation of glycosylceramide in koji samples by TLC

Koji samples were lyophilized and pulverized with a mill. To 1.2 g of the samples, methanol (4 ml) and chloroform (2 ml) were added, and the mixture was vortexed for 1 min. More chloroform (2 ml) was added, and the sample was vortexed again for 1 min. The solution was then sonicated for 5 min; methanol (8 ml) containing 0.8 M KOH was added, and the solution was incubated at 42°C and 160 rpm in a water bath for 30 min. Chloroform (20 ml) and distilled water (9 ml) were then added, and the solution was vortexed until saponification. The tube was centrifuged at 800× *g* for 10 min, and the lower layer was recovered as a

sphingolipid-containing fraction. Sphingolipid-containing fractions obtained as described above were evaporated to dryness and dissolved in 120 µl of chloroform-methanol (2:1 v/v). A 10, 20 or 30µl aliquot of the solution was applied onto a silica gel TLC plate and visualized as described above.

3.2.6 Quantitation of the intensities of spots on TLC using imaging software

Quantitative image analysis was performed using ImageJ software (ij150-win-jre6NIH) obtained from https://imagej.nih.gov/ij/download.html. TLC photo files were opened with ImageJ and converted to a binary format. Spots with an *Rf* value of 0.4 on TLC plates (indicated by arrows in figures) were used for quantitation of glycosylceramide. Spots on each plate were surrounded by a ccompartment, as was a blank space of equal size, and mean intensity values of the compartments were calculated. The intensity of the compartment surrounding the blank space was subtracted from the average intensity of the spots (applied quantity 10 µl, 20 µl, 30 µl) on each plate. The results for each spot across a range of glycosylceramide standards were used to calculate an equation, which was used the quantitate glycosylceramide in TLC spots from koji and *A. oryzae* mycelial extracts. A schematic diagram of the experimental steps is presented in Fig. 6.

3.2.7 Quantitation of *N*-acetylglucosamine in koji samples

Koji (2.0 g) was dried at 100°C for 1 h. Dried koji was pulverized with a mill. Samples were washed 3 times with 50 mM phosphate buffer (pH 7.0), centrifuged, and the supernatant removed. To each pellet, 10 ml of 50 mM phosphate buffer (pH 7.0) containing 1 mg of yatalase (Takara Bio Inc., Kyoto, Japan) was added, following which solutions were incubated at 37°C for 1 h. *N*-acetylglucosamine was quantitated as described previously (Reissig et al. 1955) with minor modifications to the method. Briefly, 0.167 ml of sample was

put into a screw-capped glass tube, and 0.3 ml of saturated potassium tetraborate was added. The reaction mixture was heated at 100°C for 3 min and cooled with water. One milliliter of *p*-dimethylaminobenzaldehyde (DMAB, Nacalai Tesque Inc., Kyoto, Japan) solution (10 g of DMAB was dissolved in 100 ml acetic acid containing 12.5 % (v/v) of 10 N HCl and diluted with water 9-fold) was added to the tube, and incubated at 37°C for 20 min. The absorbance of the final solution was measured at 544 nm.

3.2.8 Lipid extraction

Dried *A. oryzae* mycelia (0.12 g) in a glass tube with a screw cap were added with 2 ml of chloroform/methanol (1:1 v/v) and vortexed for 1 min. To the solution, 2 ml of 0.8 M KOH dissolved in methanol was added and incubated at 42°C and 160 rpm for 30 min. Subsequently, 5 ml of chloroform and 2.25 ml of water was vortexed until saponification, and centrifuged at 700 × *g* for 10 min. The lower organic layer was recovered and evaporated under a vacuum.

3.2.9 Separation and detection of lipids using thin layer chromatography (TLC)

The extracted lipids (500 µl) were dried, dissolved in 50 µl of chloroform/methanol (2:1 v/v), and 20 µl of the solution was spotted onto a TLC plate (Silica gel 60 plate, Merck Millipore Inc., Darmstadt, Germany). The dried plate was developed with saturated chloroform:methanol:acetic acid:water (20: 3.5: 2.3: 0.7 v/v). Cerebroside (Matreya Inc., Pleasant Gap, PA, USA) was used as an internal standard. To detect glycosylceramide, 2 mg/ml of orcinol in 70% sulfuric acid was sprayed onto the TLC plate and heated at 100°C for 40 min. Quantitation of glycosylceramide was performed as described previously (Takahashi et al. 2014).

3.2.10 Semi-purification of glycosylceramide using column chromatography

Crude extracts of sphingolipids were evaporated under a vacuum, solubilized in 3 ml of chloroform, and applied to a silica gel chromatography (diameter 30 mm, column length 300 mm, Silica Gel 60 70–230 mesh, Nacalai Tesque Inc., Japan). First, 600 ml chloroform was used as an eluent to remove contaminants. Then, 10 ml of ethyl acetate:methanol (9:1 v/v) was added 24 times. An aliquot of the samples (800 µl) of each 10 ml fraction was dried, dissolved in 50µl of chloroform/ methanol (2% v/v) and was spotted onto a TLC plates as described above. An *Rf* value of 0.4 was adopted as the glycosylceramide fraction as previously reported (Takakuwa et al. 2005).

3.2.11 Purification of glycosylceramide using high performance liquid chromatography (HPLC)

Fractions that contained glycosylceramides were evaporated under a vacuum, dissolved in 1.5 ml of chloroform, and injected into a 0.5 ml injection loop. Purification was performed as follows; particle size: 5 µm, diameter: 4.6 mm, length: 250 mm (Inertsil SIL 100A, GL science Inc., Japan), column temperature; 30°C, buffer B: chloroform, buffer C: 95% methanol, gradient: 0.01 min B 100%, 60 min B 85%, 80 min B 10%, 90 min B 10%, 90.01 min B 100%, 100 min B 100%, 100.01 min STOP; rate: 0.7 ml/min. Eluents at 40–50 min were sampled at 1 min intervals, and aliquots of the fractions were dried and applied to TLC analysis. The fractions that were confirmed to contain purified glycosylceramide were dried under a vacuum and dissolved in ethanol (4 µg/µl).

3.2.12 Co culture of sake yeast with glycosylceramide

Sake yeast was incubated in 3 ml of YPD medium (1% yeast extract, 2% bactopeptone, 2% glucose) at 30°C for 24 h. Cells were recovered and washed with sterile water. Koji extract

solution (5 ml, pregelatinized koji weighing 5 g was mixed with water and incubated at 55 °C overnight, and the liquid fraction was recovered) was inoculated with nonidet P-40 (final 0.0015% v/v), with or without 50 µl of 4 µg/µl glycosylceramide (either from *A. oryzae*, soy or *G. frondosa*) dissolved in ethanol and 10^6 cells/ml sake yeast (the final glycosylceramide concentration was 40 µg/ml). Sake yeast is exposed to 40 µg/ml koji glycosylceramide during fermentation; therefore this concentration was adopted. Sterile liquid paraffin (1 ml) was overlaid onto the medium, and the samples were incubated at 15°C for 1 week. Control was maintained without glycosylceramide by the same way.

3.2.13 Sample collection for metabolites analysis

After 1 week of fermentation with or without glycosylceramide, the fermented cultures were centrifuged at −9°C, 3200 × *g* for 3 min. The supernatant (5 ml) was collected into two plastic tubes (2.5 ml each) and stored at 4°C for volatile compound analysis or at −27°C for non-volatile compounds analysis. The pellet was resuspended in 3 ml MilliQ water (Millipore Inc., Darmstadt, Germany) and centrifuged again under the same conditions. The pellet was soaked in liquid nitrogen for 3.5 min, freeze-dried for 12 h, and stored at −80°C.

3.2. 14 Sample preparation for the analysis of volatile compounds

Fermented cultures were centrifuged at 4 °C and 3200 × *g* for 3 min, and the supernatant was collected. For target products analysis, 900 µl of fermentation broth and 100 µl of internal standard mixture (methyl hexanoate at 5 mg/L and *n*-amyl alcohol at 200 mg/L) were placed into a 10 ml glass vial on ice. The vial containing the fermentation broth and an internal standard mixture was sealed with a magnet cap and subjected to GC/MS analysis, as previously described (Yoshizaki et al. 2010).

3.2.15 GC/MS analysis of volatile compounds

Aroma compounds of the fermented cultures were analysed using headspace gas chromatography mass spectrometry (GC/MS) (GC-2010, GCMS-QP2010; Shimadzu, Kyoto, Japan) equipped with a DB-WAX column (length, 60 m; internal diameter, 0.25 mm; film thick ness, 0.5 µm; Agilent Technologies, Palo Alto, CA) as previously described (Yoshizaki et al. 2010). Experiments were performed in triplicate from respective independent cultures.

3.2.16 Sample preparation for extracellular non-volatile compounds

Sample preparation for extracellular non-volatile compounds was performed as describe previously (Kadowaki et al. 2017). The fermented cultures (100 µl) or freeze-dried cells (10 mg) were mixed with 1 ml of chloroform: methanol: water (2:5:2 v/v) for extraction. Ribitol solution (0.2 mg/ml, 60 µl) was added to the solution and incubated for 30 min at 30°C with shaking at 1,500 rpm. The supernatant (900 µl) was collected by centrifugation (4°C 16000 × *g* for 3 min) and 400 µl MilliQ water (Millipore Inc., Darmstadt, Germany) was added, mixed, and centrifuged again under the same conditions. The supernatant (800 µl) was evaporated for 3 h and freeze-dried for 12 h. Methoxyamine (20 mg/ml dissolved in pyridine, 100 µl) was mixed with the freeze-dried extract and incubated at 30°C for 90 min with shaking at 1,500 rpm. *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) (50 µl) was added and again incubated at 37°C for 30 min with shaking at 1,500 rpm. The solution (70 µl) was transferred to a vial and subjected to gas chromatography flame ionization detector (GC/FID) analysis.

3.2.17 GC/FID analysis of non-volatile compounds

Non-volatile metabolic compounds, which are produced during fermentation with or without glycosylceramide, were analysed using a GC/FID (GC-2014, Gas Chromatograph, Shimadzu, Kyoto, Japan) with a CP Sil8CB column (length, 30 m; internal diameter, 0.25 mm; film thick ness, 0.25 µm; Agilent Technologies, Palo Alto, CA, USA). The carrier gas was nitrogen,

with a column headspace pressure of 73.9 kPa and a flow rate of 0.97 ml/min. The gas chromatography temperature program was as follows: 60°C for 2 min, raised to 320°C at 13°C /min, and held for 17 min. The split ratios for extracellular metabolites were 10 and 2, respectively. The data were analysed using GC/FID solution software (Labsolution, Shimadzu, Kyoto, Japan). All metabolites concentration were normalized using ribitol as an internal control. Experiments were performed in triplicate from respective independent cultures.

3.2.18 Measurement of ethanol concentration

The ethanol concentrations of the fermented cultures were analysed using a contact combustion system with an alcohol densitometer (Alcohol Checker YSA-200; Yazaki Meter Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions, and as described previously (Katou et al. 2008).

3.2.19 Statistical analysis

Regression analysis was performed using Excel (Microsoft Office Professional Plus 365). Test for no correlation was judged using the *p* value in the *t*-distribution ($t = r\times\sqrt{(n-2)}/\sqrt{(1-r^2)}$). The statistical significances of differences among the averages of volatile flavor and non volatile compounds were judged using Student's *t*-test and the false discovery rate (FDR). The experimental results were expressed as means ± standard error of the means. The results were considered significant when the *p* value was <0.05. For heatmap analysis and integrated pathway analysis, MetaboAnalyst (Xia et al. 2015) was used. Heatmaps were based on interquantile range data filtering, normalization by sum, clustering with Ward's method, distance measure with Euclidean, standardization by autoscale features and without data scaling.

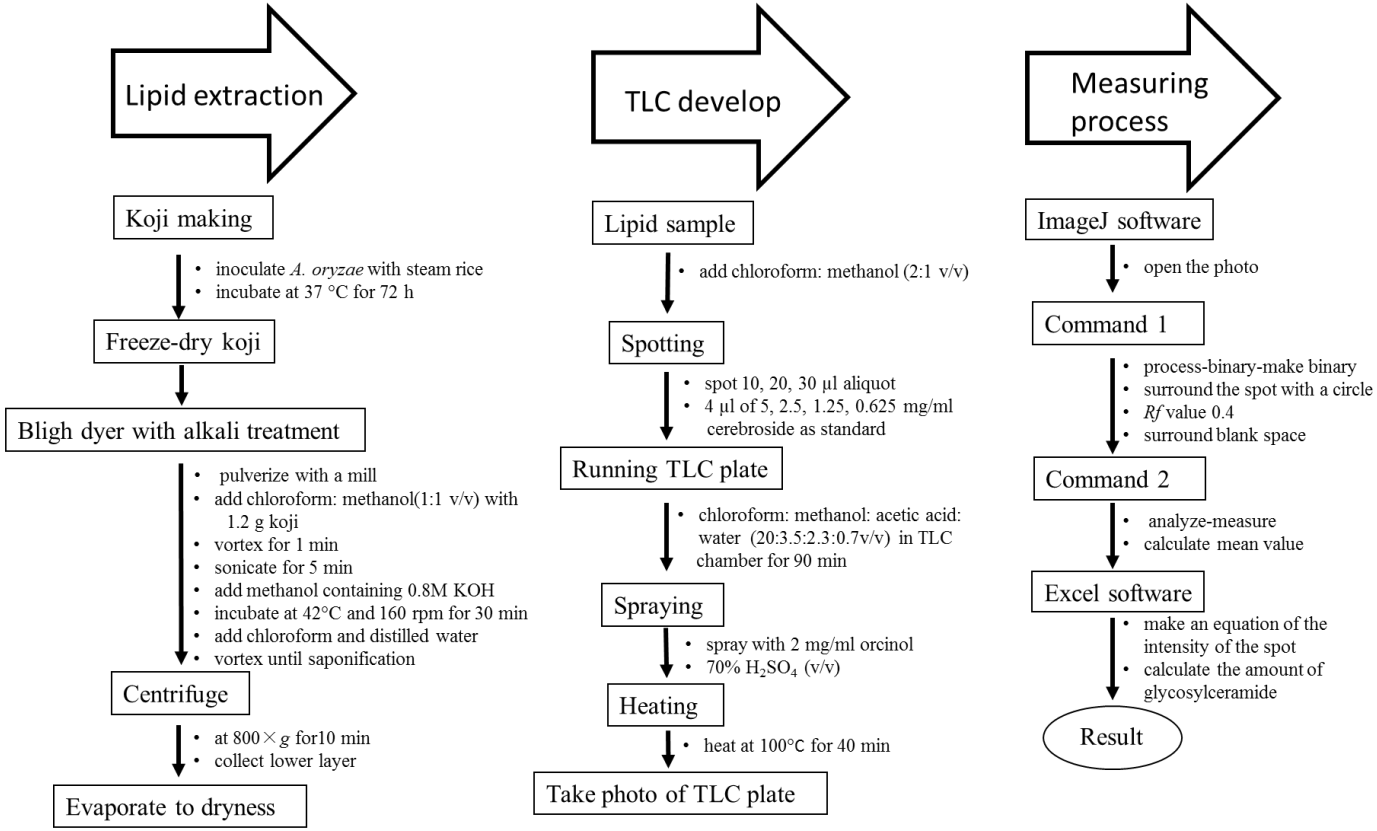

Figure 6. Schematic diagram of the experimental steps of the measurement of glycosylceramide levels of koji rice

3.3.1 Correlation of glycosylceramide levels of *A. oryzae* with its mycelial weight

To determine whether fungus *A. oryzae* mycelial glycosylceramide levels are reflective of mycelial weight, we cultured *A. oryzae* in potato dextrose broth (Fig. 7), collected the mycelia by centrifugation, lyophilized the mycelia, and measured the weight. After that we quantitated glycosylceramide levels within the mycelia (Fig. 8A) by using TLC analysis method and examined the correlation between mycelial weight and glycosylceramide levels (Fig. 8B) of *A. oryzae*. In this study, it was found that glycosylceramide levels highly correlated with the mycelial weight of *A. oryzae* (R^2=0.9735, p<0.01). This result clearly indicates that glycosylceramide content is a good indicator to quantitate the mycelial weight of *A. oryzae*.

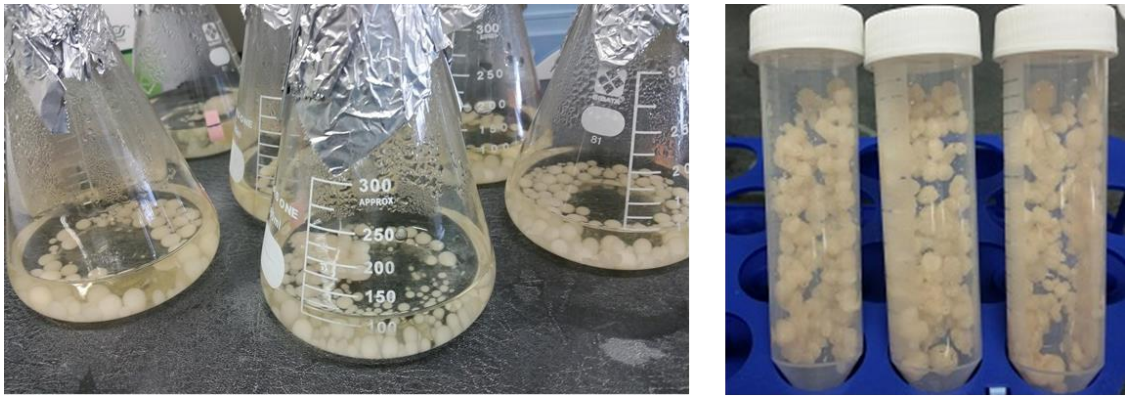

Figure 7. Mycelial growth of *A. oryzae*

Conidia of *A. oryzae* were inoculated into potato dextrose broth (PDB) and incubated at 30°C and 200 rpm for 3 to 5 days and the mycelia were collected by centrifugation.

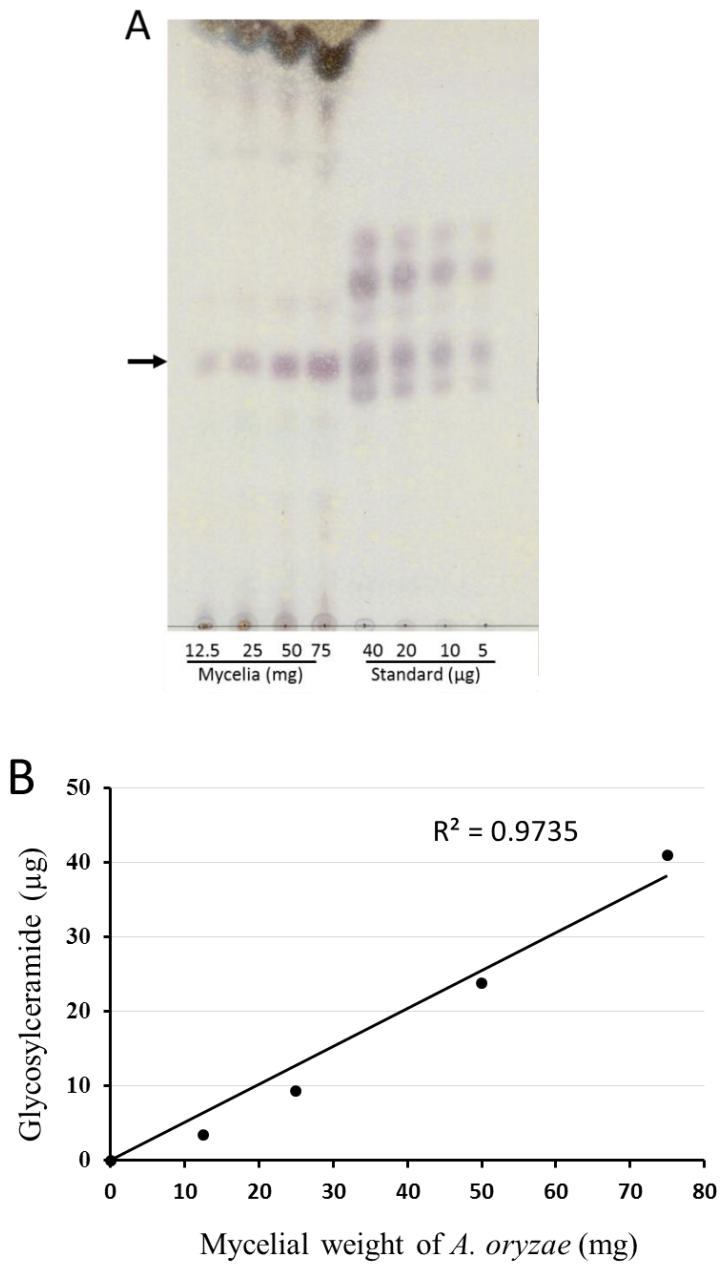

Figure 8. Correlation of the glycosylceramide levels with the mycelial weight of *A. oryzae*

Mycelia of *A. oryzae* were cultured in synthetic liquid media, collected by centrifugation and freeze-dried. Total lipids were extracted from the aliquots of the mycelia with chloroform/methanol (2:1) and separated and detected using TLC analysis. Spots corresponding to glycosylceramide were quantitated with an imaging software. (A) TLC analysis of the mycelia. Arrow indicates the position of glycosylceramide, which was used for quantitation. (B) Correlation of the glycosylceramide levels with the mycelial weight of *A. oryzae*.

3.3.2 Increase in the glycosylceramide and *N*-acetylglucosamine contents with the growth of *A. oryzae* on koji rice

Priviously reported that *N*-acetylglucosamine is used to quantitate the mycelial weight of non-pathogenic fungus *A. oryzae* in koji. Therefore, to confirm that increases in *A. oryzae* mycelial weight in koji can be determined by monitoring its lipid compound glycosylceramide levels and also *N*-acetylglucosamine, we analyzed the time course of glycosylceramide levels and *N*-acetylglucosamine during koji production.

Four independent trials of koji production (Fig. 9) were performed to determine the reproducibility of the experiment. In this study, we found that the content of glycosylceramide of koji was increased with incubation time (Fig. 10A, 10B; 11A, 11B; 12A, 12B; 13A, 13B). The coefficient of variation decreased with incubation time (Table 2) suggesting that the reliability of quantitation using this method is effective for koji incubated for more than 48 h. *N*-acetylglucosamine, which reflects the mycelial weight of *A. oryzae*, was also increased with incubation time (Fig. 10C, 11C, 12C, 13C) as reported previously (Arima and Uozumi 1967).

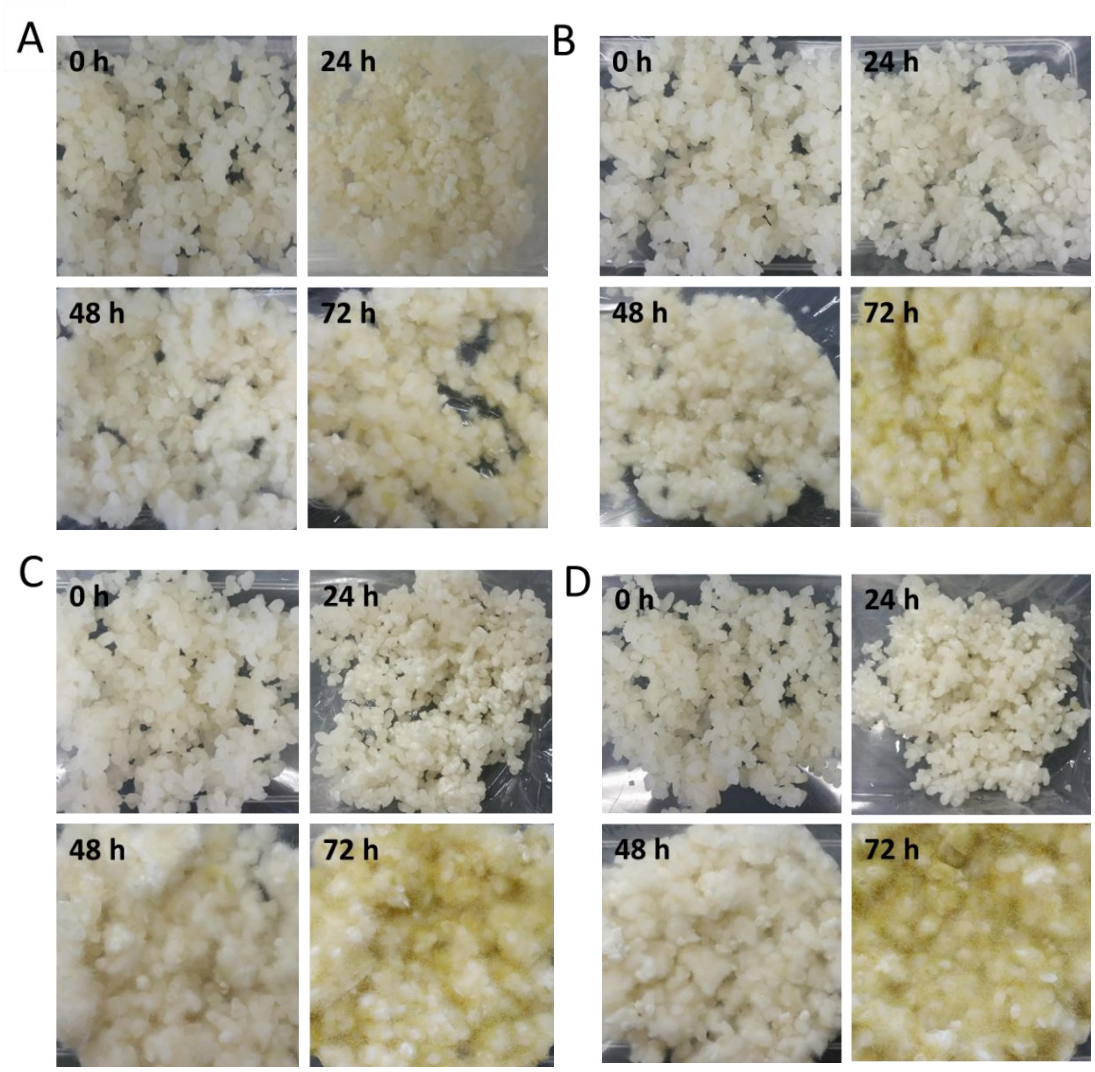

Figure 9. Four independent trials of koji manufacture using sake rice

Steamed rice (28 g) inoculated with conidia of *A. oryzae* (10 mg) and incubated in a packed container at 37^{o}C for 0 h, 24 h, 48 h and 72 h. Here, trial 1(A), trial 2 (B), trial 3 (C) and trial 4 (D) respectively.

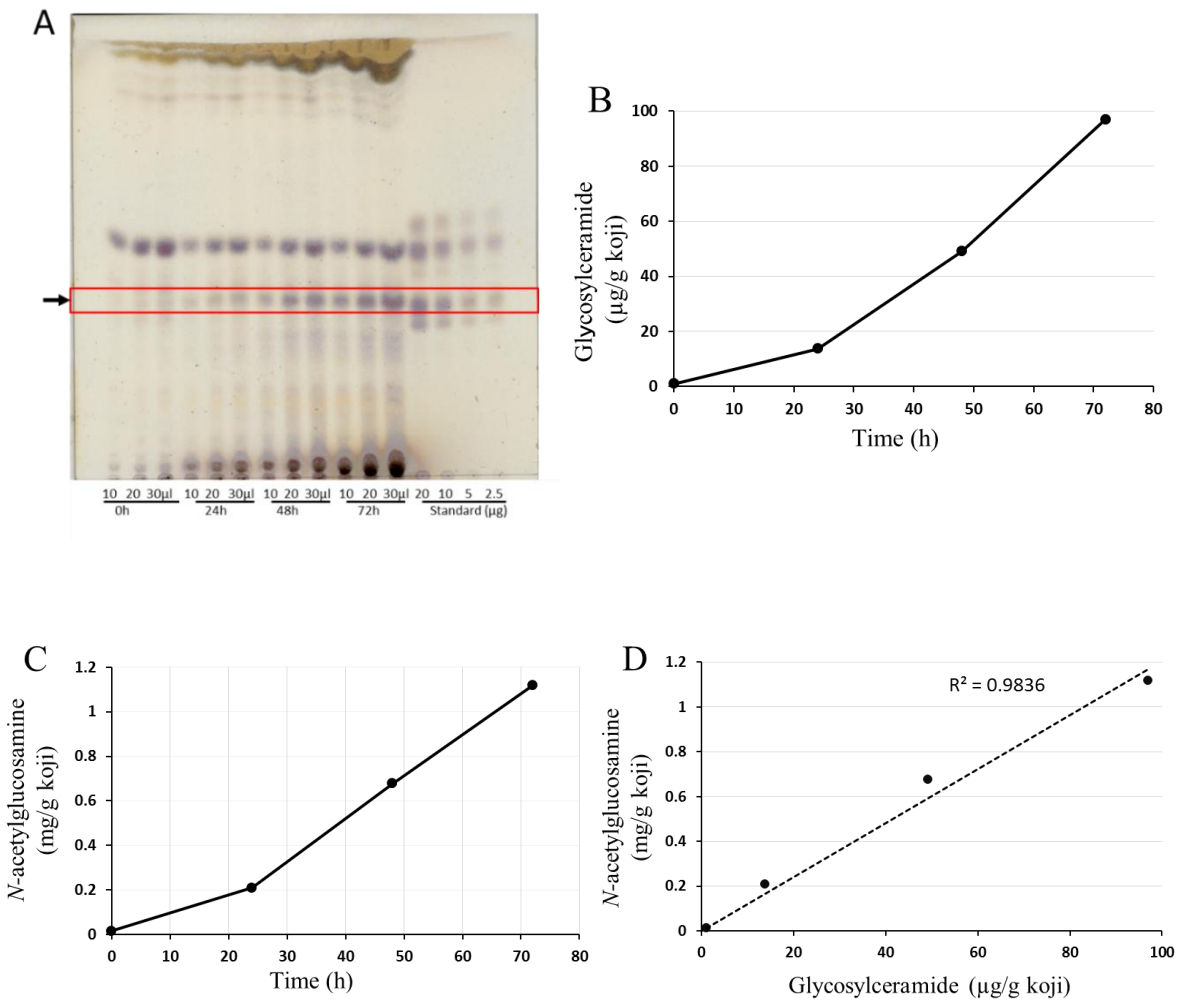

Figure 10. Correlation between the glycosylceramide and *N*-acetylglucosamine levels during koji production experiment (trial 1)

Koji was manufactured using rice used for sake brewing according to the traditional technique. (A) TLC analysis. Arrow indicates the position of glycosylceramide, which was used for quantitation. (B) Time course of glycosylceramide levels during koji production. (C) Time course of *N*-acetylglucosamine levels during koji production. (D) Correlation of glycosylceramide with *N*-acetylglucosamine. Glycosylceramide levels of koji was fitted to the approximation of that of *N*-acetylglucosamine. Correlation coefficients were calculated using Microsoft Excel Software. Glycosylceramide and *N*-acetylglucosamine levels were quantitated using the method described in the text.

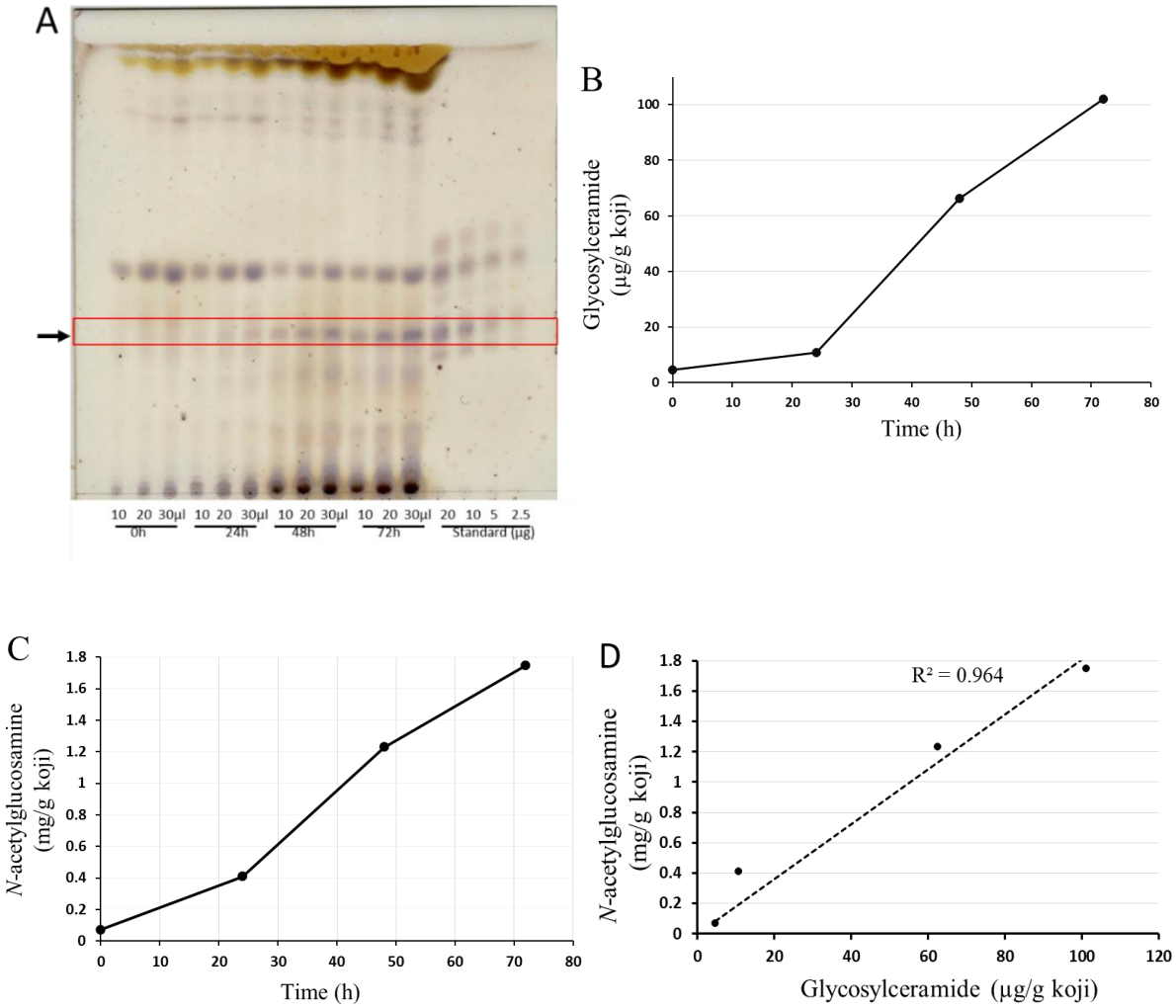

Figure 11. Correlation between the glycosylceramide and *N*-acetylglucosamine levels during koji production experiment (trial 2)

Koji was manufactured using rice used for sake brewing according to the traditional technique. (A) TLC analysis. Arrow indicates the position of glycosylceramide, which was used for quantitation. (B) Time course of glycosylceramide levels during koji production. (C) Time course of *N*-acetylglucosamine levels during *koji* production. (D) Correlation of glycosylceramide with *N*-acetylglucosamine. Glycosylceramide levels of koji was fitted to the approximation of that of *N*-acetylglucosamine. Correlation coefficients were calculated using Microsoft Excel Software. Glycosylceramide and *N*-acetylglucosamine levels were quantitated using the method described in the text.

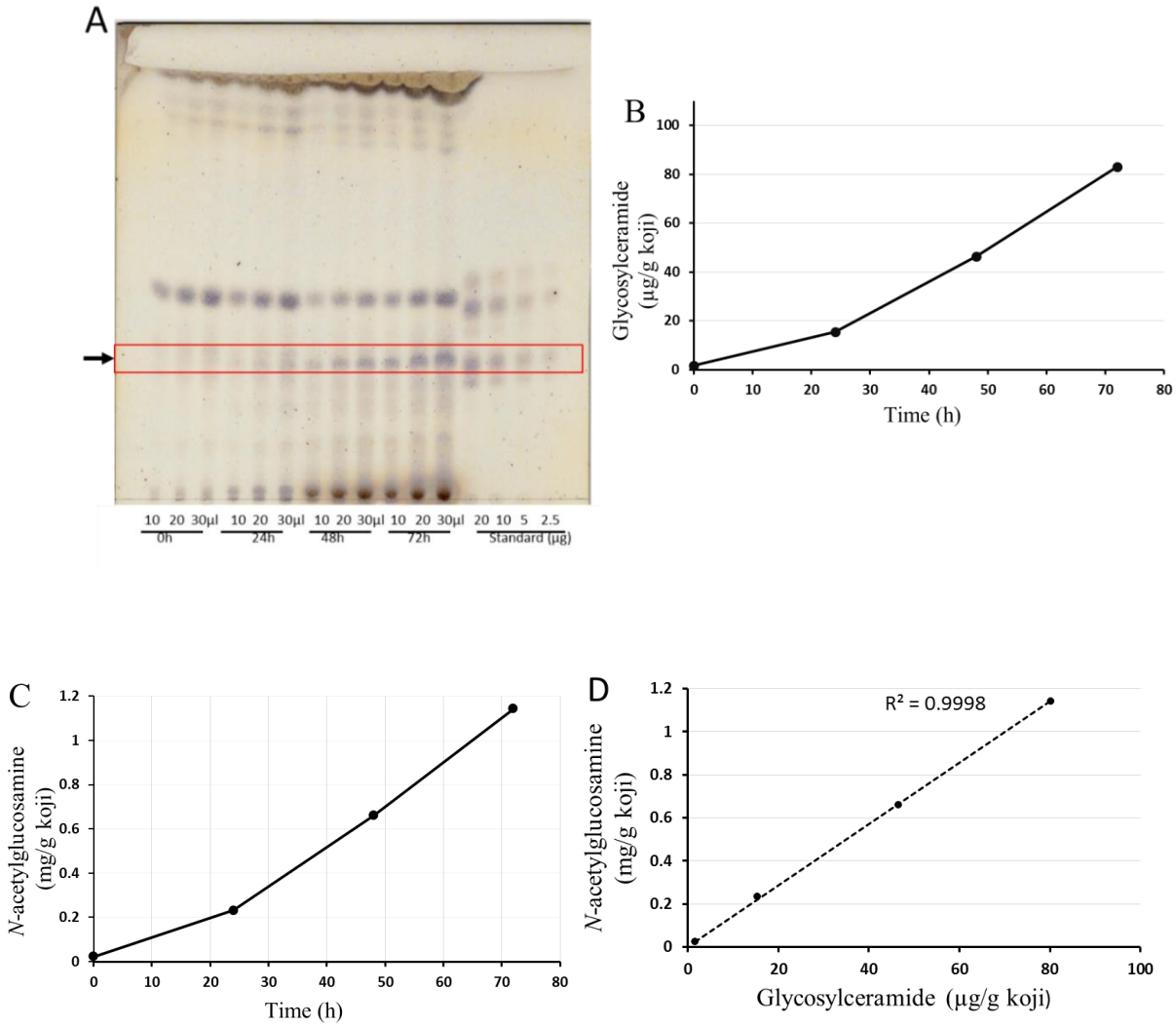

Figure 12. Correlation between the glycosylceramide and *N*-acetylglucosamine levels during koji production experiment (trial 3)

Koji was manufactured using rice used for sake brewing according to the traditional technique. (A) TLC analysis. Arrow indicates the position of glycosylceramide, which was used for quantitation. (B) Time course of glycosylceramide levels during koji production. (C) Time course of *N*-acetylglucosamine levels during koji production. (D) Correlation of glycosylceramide with *N*-acetylglucosamine. Glycosylceramide levels of koji was fitted to the approximation of that of *N*-acetylglucosamine. Correlation coefficients were calculated using Microsoft Excel Software. Glycosylceramide and *N*-acetylglucosamine levels were quantitated using the method described in the text.

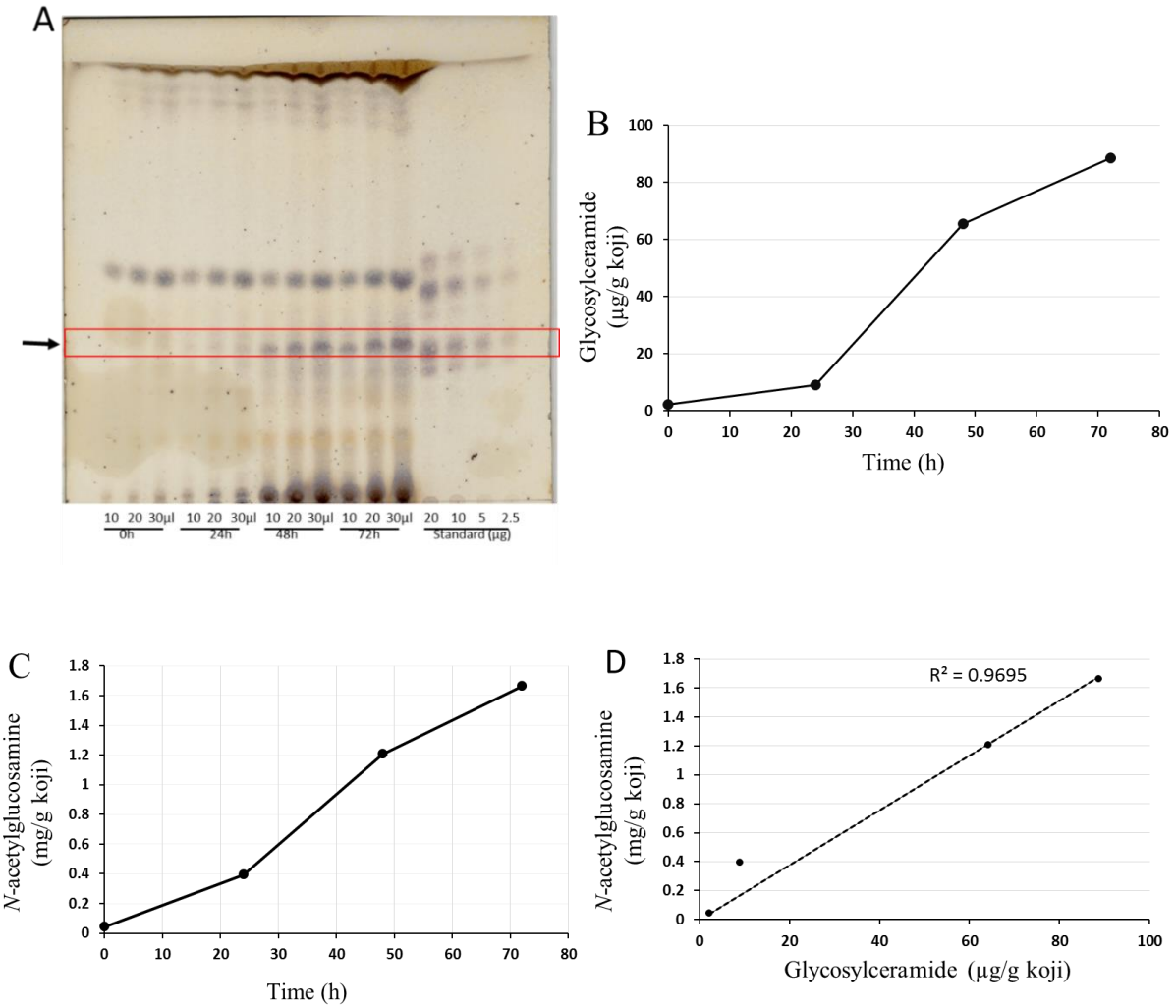

Figure 13. Correlation between the glycosylceramide and *N*-acetylglucosamine and levels during koji production experiment (trial 4)

Koji was manufactured using rice used for sake brewing according to the traditional technique. (A) TLC analysis. Arrow indicates the position of glycosylceramide, which was used for quantitation. (B) Time course of glycosylceramide levels during koji production. (C) Time course of *N*-acetylglucosamine levels during koji production. (D) Correlation of glycosylceramide with *N*-acetylglucosamine. Glycosylceramide levels of koji was fitted to the approximation of that of *N*-acetylglucosamine. Correlation coefficients were calculated using Microsoft Excel Software. Glycosylceramide and *N*-acetylglucosamine levels were quantitated using the method described in the text.

Table 2. The coefficient of variation of glycosylceramide measurement in koji incubated with *A. oryzae*

Time (h)	Trial 1	Trial 2	Trial 3	Trial 4	Average
0	0.81	0.722	0.812	0.43	0.6935
24	0.244	0.243	0.403	0.387	0.3192
48	0.102	0.207	0.044	0.061	0.1035
72	0.058	0.037	0.201	0.096	0.0980

3.3.3 Correlation of glycosylceramide levels with *N*-acetylglucosamine content of koji rice

To investigate the correlation between the glycosylceramide levels and *N*-acetylglucosamine content in koji samples manufactured from rice regression analysis was performed. In this study, the glycosylceramide levels and *N*-acetylglucosamine content in koji samples showed a significant correlation (Fig. 10D, 11D, 12D, 13D). The statistical *p* values of the correlation were less than 0.01 in all trials which indicate a significant correlation between glycosylceramide and *N*-acetylglucosamine in koji. To investigate the variation among the experiments, correlation of glycosylceramide and *N*-acetylglucosamine levels in 4 independent trials were investigated. As a result, *N*-acetylglucosamine and glycosylceramide levels showed a significant correlation (Fig. 14, R^2=0.8942, *p*=0.00150), indicating that glycosylceramide reliably reflects the amount of *N*-acetylglucosamine.

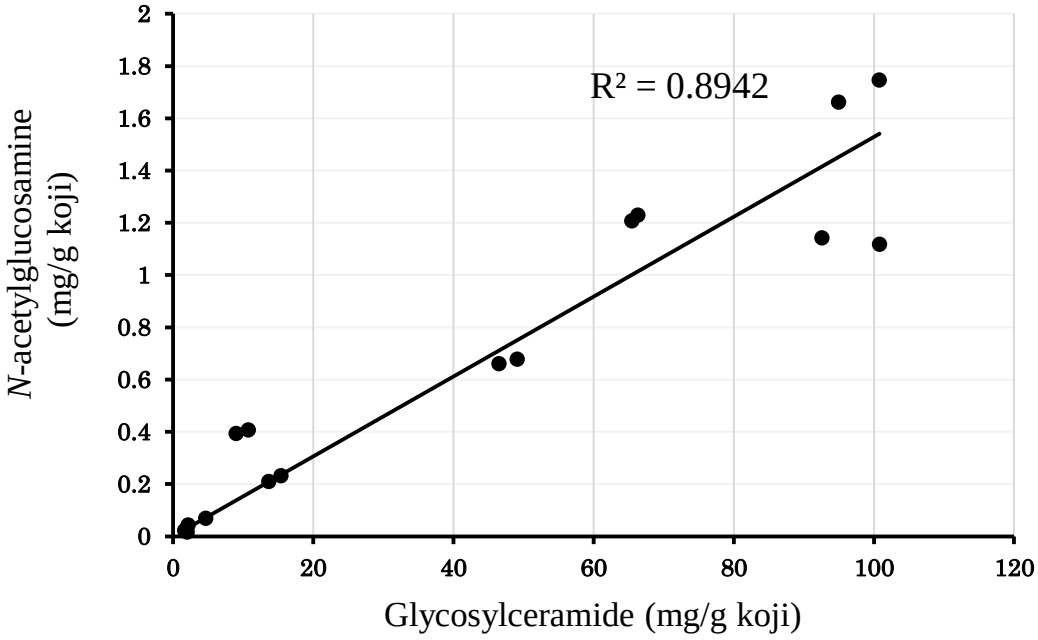

Figure 14. Correlation between the glycosylceramide and *N*-acetylglucosamine levels during four (04) koji production experiments

Though glycosylceramides are critical components of cell membrane, they play significant roles in fungal mycelial growth (Zhu et al. 2014) and perform various biological functions (Hannun and Obeid 2017, Truman et al. 2014, Russo et al. 2013). It has been reported that the shochu yeast *S. cerevisiae* which is incapable of synthesizing glycosylceramide, adapted to alkaline condition and modified flavor profile after exposure to glycosylceramide from fungus *A. kawachii* (Sawada et al. 2015). Therefore, after determination of the mycelial weight of *A. oryzae* by quantitation of the glycosylceramide in koji, we analysed the effects of glycosylceramide from *A. oryzae*, soy, *G. frondosa* on the aroma profile and metabolic profile of sake yeast *S. cerevisiae* under fermentation stress.

During fermentation, sake yeast requires lipids, such as unsaturated fatty acids and sterols, in addition to substances provided by koji enzymes for fermentation (Taylar et al. 1979, Ohta and Hayashida 1983, Fujii et al. 1997, Mason and Dufour 2000). However, the role of sphingolipids on the brewing characteristics of sake yeast has not been studied. In this study, we revealed that glycosylceramide, one of the sphingolipids that is abundant in koji, affects the flavor and metabolic profile of sake yeast during fermentation.

3.3.4 Analysis of volatile compounds with or without glycosylceramide during fermentation

In this study, the flavour components of the cultures fermented with sake yeast added with or without glycosylceramides were analysed. To obtain information on the specificity of the effect of chemical structures of glycosylceramide, glycosylceramides from *A. oryzae*, soy and *G. frondosa* were used. A concentration of 40 μg/ml was adopted, because koji contains approximately 240 μg/g of glycosylceramide (Sawada et al. 2015) and koji is contained at a ratio of 1 to 6 of water and rice in the first step of sake brewing. As a result, addition of *A. oryzae*, soy and *G. frondosa* glycosylceramides showed patterns distinct from vehicle control (ethanol-added culture) (Fig.15). In particular, *A. oryzae* and *G. frondosa* glycosylceramides were clustered in proximity, showing similar decreases in ethyl caprylate and ethyl 9-decenoate and increases in 2-phenylethyl acetate and phenylethyl alcohol (Table 3). Soy glycosylceramide had a similar effect to *A. oryzae* in terms of the increase in 2-phenylethyl acetate and decreases in ethyl 9-decenoate, isoamylalcohol and acetoin (Table 4). Overall, all glycosylceramides from *A. oryzae*, soy and *G. frondosa* showed similar patterns. However, in particular, *A. oryzae* and *G. frondosa* glycosylceramides showed high similarity. By contrast, soy glycosylceramide had a similar but rather weak effect to the decrease of flavor components such as ethyl 9-decenoate.

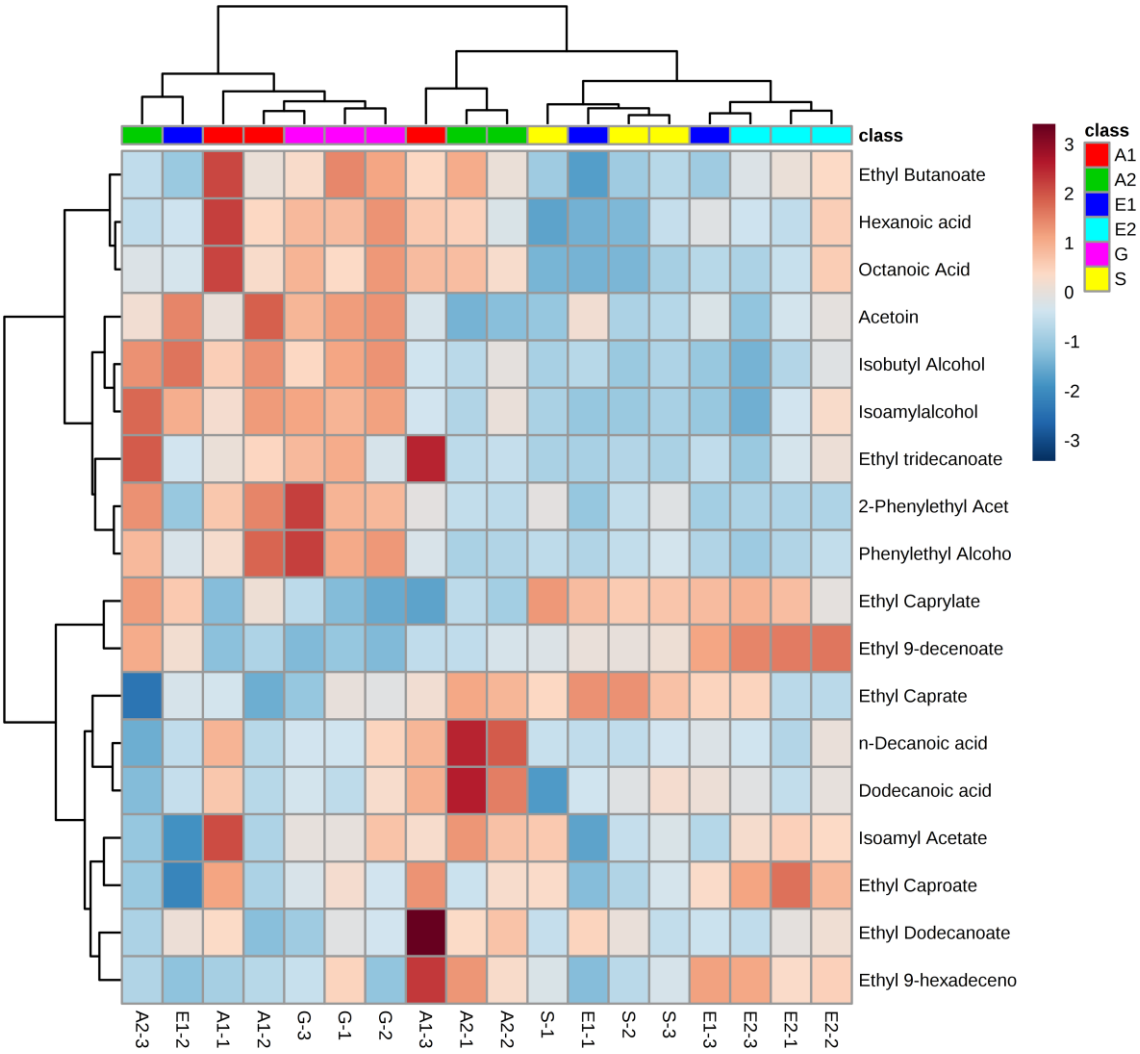

Figure 15. Heatmap of volatile compounds in the culture of sake yeast incubated with or without glycosylceramide

Sake yeasts were incubated in synthetic medium containing 40 μg/ml of *A. oryzae* (A1 and A2), soy (S), *G. frondosa* (G) glycosylceramide or their vehicle control ethanol (E1 and E2) and nonidet P-40 (final concentration 0.0015% v/v) at 15ºC for one week. Volatile compounds were analyzed using headspace gas chromatography mass spectrometry (GC/MS). A heatmap of volatile compounds was created with MetaboAnalyst.

Table 3.Volatile compounds in the culture of sake yeast added with or without glycosylceramide of *A. oryzae* and *G. frondosa*

Volatile compound	Vehicle control	Glycosylceramide of *A. oryzae*		Glycosylceramide of *G. frondosa*	
	Relative percentage (%)	Relative percentage (%)	*p*-value	Relative percentage (%)	*p*-value
Ethyl butanoate	100±13.8	119±19.0	0.2694	110±10.8	0.3268
Isobutyl alcohol	100±7.19	92.9±6.20	0.2879	99.1±11.1	0.4799
Isoamyl acetate	100±24.5	140±32.0	0.2317	116±12.2	0.3263
Isoamyl alcohol	100±3.63	91.7±3.98	0.1457	98.5±6.20	0.4353
Ethyl caproate	100±22.2	95.9±15.7	0.4542	77.7±5.03	0.2344
Acetoin	100±3.06	77.9±8.26	0.0751	94.7±8.67	0.3317
Ethyl caprylate	100±13.1	56.4±1.78	0.0272*	50.4±1.07	0.0185*
Ethyl caprate	100±17.1	59.6±11.0	0.0957	55.8±6.91	0.0607
Ethyl 9-decenoate	100±20.9	38.4±5.49	0.0401*	26.2±2.35	0.0227*
2-Phenylethyl acetate	100±19.5	351±34.6	0.00338*	431±27.7	0.0006*
Hexanoic acid	100±20.1	131±23.4	0.2235	116±10.2	0.2903
Ethyl dodecanoate	100±12.5	110±42.6	0.4314	56.0±8.55	0.0385
Phenylethyl alcohol	100±3.58	159±19.3	0.0342*	216±9.56	0.0003*
Ethyl tridecanoate	100±14.7	262±85.3	0.1293	182±29.7	0.0568
Octanoic acid	100±12.7	136±23.8	0.1725	116±10.5	0.2268
n-Decanoic acid	100±17.1	114±26.7	0.3635	83.2±13.3	0.2809
Ethyl 9-hexadecenoate	100±48.1	100±42.4	0.4968	62.9±15.0	0.2899
Dodecanoic acid	100±20.0	101±23.8	0.4845	70.3±10.9	0.1738

Note: Sake yeasts were incubated in synthetic medium with or without 40 μg/ml glycosylceramide of *A. oryzae* and *G. frondosa* and nonidet P-40 (final 0.0015% v/v) at 15°C for one week. Volatile compounds were analysed using headspace gas chromatography mass spectrometry (GC/MS).The results are the mean values with standard errors (SE) of triplicate independent experiments. The relative percentage as compared to the mean value of the control culture is shown. Volatile compounds which were significantly different between control and glycosylceramide added culture as judged by false discovery rate ($p<0.05$, indicated by *) are described. *P* values were calculated using unpaired one-tailed Student's *t*-test.

Table 4. Volatile compounds in the culture of sake yeast added with or without glycosylceramide of *A. oryzae* and soy

Volatile compound	Vehicle control	Glycosylceramide of *A. oryzae*		Glycosylceramide of soy	
	Relative percentage (%)	Relative percentage (%)	*p*-value	Relative percentage (%)	*p*-value
Ethyl butanoate	100±11.9	67.2±17.0	0.1376	85.5±3.11	0.2146
Isobutyl alcohol	100±4.84	85.8±4.94	0.0843	103±3.60	0.3175
Isoamyl acetate	100±13.1	66.8±19.8	0.1630	93.2±5.85	0.3625
Isoamy lalcohol	100±4.16	75.1±5.18	0.0189*	97.6±1.93	0.347
Ethyl caproate	100±14.1	50.1±9.74	0.0422	84.1±2.66	0.2279
Acetoin	100±7.51	44.7±8.21	0.0077*	84.7±8.58	0.1683
Ethyl caprylate	100±17.2	54.5±7.72	0.0751	107±1.23	0.382
Ethyl caprate	100±22.6	69.4±22.9	0.2404	128±6.94	0.2071
Ethyl 9-decenoate	100±13.4	35.2±2.12	0.0088*	61.5±3.23	0.0427*
2-Phenylethyl acetate	100±12.9	125±24.3	0.249	192±18.9	0.0150*
Hexanoic acid	100±12.4	68.6±18.3	0.1603	70.5±11.0	0.111
Ethyl dodecanoate	100±5.77	76.4±20.3	0.223	99.2±8.00	0.477
Phenylethyl alcohol	100±5.00	90.2±14.0	0.31	143±10.6	0.0200*
Ethyl tridecanoate	100±21.9	100±27.0	0.495	67.1±4.58	0.1722
Octanoic acid	100±8.67	79.1±19.13	0.24	74.5±5.62	0.0623
n-Decanoic acid	100±15.8	126±50.3	0.36	97.8±4.11	0.462
Ethyl 9-hexadecenoate	100±21.6	59.1±21.4	0.167	62.4±4.17	0.1452
Dodecanoic acid	100±17.6	117±47.7	0.4004	87.3±28.3	0.387

Note: Sake yeasts were incubated in synthetic medium with or without 40 μg/ml glycosylceramide of *A. oryzae* and soy and nonidet P-40 (final 0.0015% v/v) at 15°C for 1 week. Volatile compounds were analysed using headspace gas chromatography mass spectrometry (GC/MS).The results are the mean values with standard errors (SE) of triplicate independent experiments. The relative percentage as compared to the mean value of the control culture is shown. Volatile compounds which were significantly different between control and GlcCer-added culture as judged by false discovery rate ($p<0.05$, indicated by *) are described. *P* values were calculated using unpaired one-tailed Student's *t*-test.

3.3.5 Analysis of nonvolatile extracellular compounds with or without glycosylceramide during fermentation

Since all glycosylceramides showed overall similar trends for volatile compounds in this study, the metabolites in the cultures fermented with sake yeast added with or without soy glycosylceramide were analyzed (Fig. 16, Table 5). As a result, independent cultures incubated with or without soy glycosylceramide were divided into respective distinct clusters (control culture and soy glycosylceramide-added culture) except CONT-6-007, SOY-1-004 and SOY-3-003 (Fig. 16). In this study, glucose decreased (28%; $p<0.01$), glycerol increased (11%; $p<0.01$), pyruvate increased (122%; $p< 0.01$), succinic acid increased (16%; $p<0.01$), malate increased (16 %; $p<0.01$), leucine decreased (10%; $p<0.05$), methionine increased (27%; $p<0.05$), threonine increased (46%; $p<0.05$), valine increased (28%; $p<0.01$), glutamate increased (1%; $p<0.05$) (Table 5) and ethanol increased (16%; $p<0.01$) (Fig. 17) upon addition of soy glycosylceramide in the culture media of sake yeast. The increase in ethanol and decrease in glucose indicated an inproved fermentation ability of sake yeast incubated with glycosylceramide, which was consistent with a previous study of shochu yeast (Sawada et al. 2015).

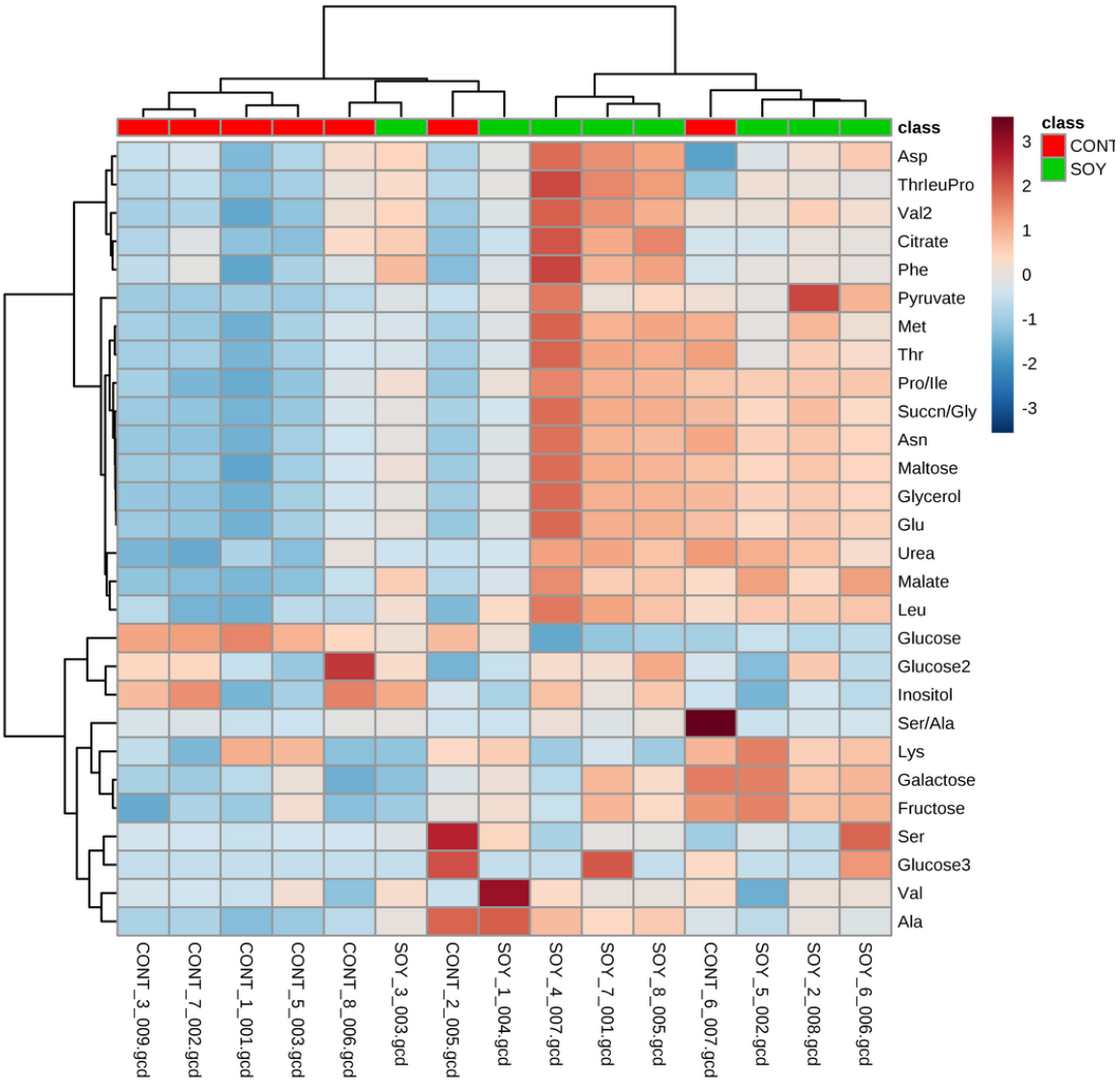

Figure 16. Heatmap of extracellular metabolite concentrations in the culture of yeast incubated with or without soy glycosylceramide

Sake yeasts were incubated in synthetic medium with or without 40 μg/ml of soy glycosylceramide and nonidet P-40 (final concentration 0.0015% v/v) at 15°C for 1 week. Metabolites of the cultures were derivatized with methoxyamine and MSTFA, analysed using GC-FID and normalized using ribitol. A heatmap of metabolites was created with MetaboAnalyst.

Table 5. Metabolite concentrations in the culture of sake yeast added with or without soy glycosylceramide

Metabolic compound	Control	Glycosylceramide treated	
	Relative percentage (%)	Relative percentage (%)	*p*-value
Glycerol	100±2.55	110±1.14	0.0014
Succinate/Glycine	100±4.05	116±2.68	0.00376
Malate	100±2.54	116±3.55	0.00191
Glucose	100±5.17	71.7±3.05	0.000513
Leucine	100±2.73	89.8±1.86	0.0077
Valine	100±5.12	128±4.62	0.00225
Methionine	100±7.23	126±6.12	0.013
Glutamate	100±0.50	101±0.31	0.0392
Pyruvate	100±15.5	221±28.3	0.00235
Threonine	100±13.8	146±9.61	0.0125

Note: Sake yeasts were incubated in synthetic medium with or without 40 μg/ml of soy glycosylceramide and nonidet P-40 (final 0.0015% v/v) at 15°C for 1 week. Metabolites derivatized with methoxyamine and MSTFA, analysed using gas chromatography flame ionization detector (GC/FID) and normalized using ribitol. The results are the mean values with standard errors of 7 (control) or 8 (GlcCer of soy) independent experiments. The relative percentage as compared to the mean value of the control culture is shown. *P*-values were calculated using unpaired one-tailed Student's *t*-test.

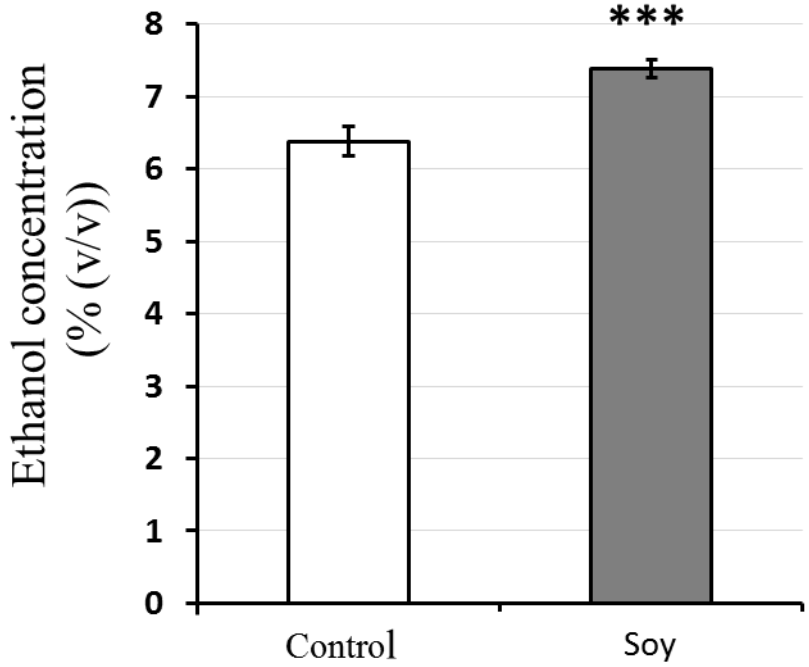

Figure 17. Ethanol concentrations (% (vol/vol)) of fermentation broth of yeast with or without soy glycosylceramide

Sake yeasts were incubated in synthetic medium with or without 40 μg/ml of glycosylceramide and nonidet P-40 (final 0.0015% v/v) at 15°C for 1 week. The ethanol concentration of fermented broth was analyzed using a contact combustion system with an alcohol densitometer. The results are the mean values with standard errors of triplicate independent experiments. The statistical significance of the difference between the averages was assessed using the unpaired one-tailed Student's *t*-test (***, $p<0.001$).

3.3.6 Analysis of metabolic pathway of extracellular metabolites of sake yeast

To extract information about the metabolism occurring in the yeast cells incubated with soy glycosylceramide, the metabolome data of the medium incubated with or without soy glycosylceramide were analyzed using MetaboAnalyst (Xia et al. 2015). Metabolites (glycerol, succinic acid (glycine), malic acid, glucose, leucine, methionine, valine, pyruvate, glutamate and threonine), which were significantly different ($p<0.05$) between medium incubated with or without soy glycosylceramide, were selected and further analyzed. As a

result, pyruvate metabolism, the TCA cycle, starch and sucrose metabolism and glycerolipid metabolism were significantly overrepresented (p<0.01) in the medium incubated with glycosylceramide (Fig. 18). This result indicated that extracellularly added glycosylceramide has effects on the metabolic pathways described above, although the mechanism remains to be determined.

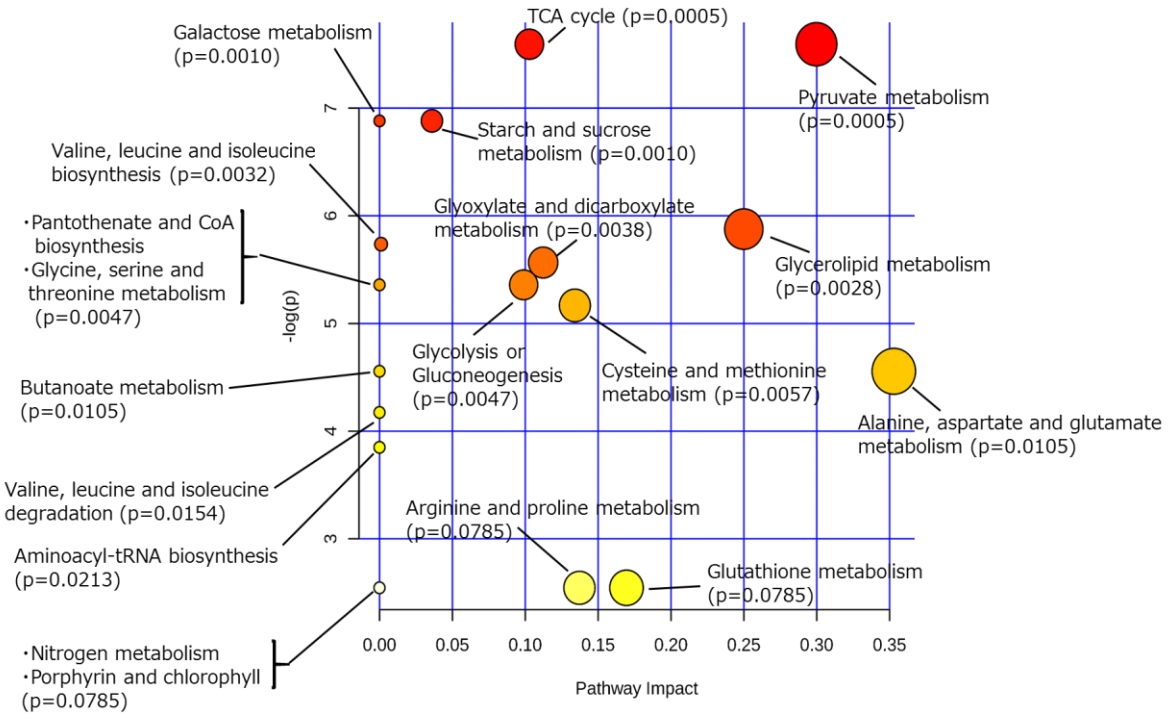

Figure 18. Pathway analysis of extracellular metabolites of sake yeast incubated with or without soy glycosylceramide

The normalized values of metabolites (glycerol, succinate/glycine, malic acid, glucose, leucine, glutamate, valine, methionine, pyruvate and threonine), which were significantly different (p < 0.05) between medium incubated with and without soy glycosylceramide, were used as independent variables for MetaboAnalyst.

3.4 Discussion

In this study, we first revealed the important of koji glycosylceramides to measure fungal mycelial growth and on the fermentation profile of yeast. As glycosylceramide is an important components of cell membrane and play roles in fungal mycelial growth (Zhu et al. 2014). Therefore, glycosylceramide content can serve as a measure of the mycelial weight of *A. oryzae*. Previously reported that glycosylceramide is produced mainly by *A. oryzae* and only a small amount (below 10%) is present in rice (Sawada et al. 2015).

Standard methods that use *N*-acetylglucosamine to determine the content of *A. oryzae* in koji involve complex sample preparation processes, acid or enzymatic treatment, and filtration (Arima and Uozumi 1967, Sakurai et al. 1977, Gomi et al. 1987, Fujii et al. 1992). These methods are costly and tedious, and their quantitativity is low. In contrast, the process developed in this study involves direct extraction of glycosylceramide, an electrophilic lipid, with chloroform and methanol. In this process, there are no need for additional treatment steps and require simplifying sample preparation. The extraction method does not require enzymatic treatment, which has the potential to interfere with quantitation. This method is cost-effective, rapid, highly quantitative and reproducible. As koji contains components from both rice and *A. oryzae*, discrimination of these components was key in this technique.

There is a degree of variability with our method in certain conditions. For example, the residual error of the correlation of *N*-acetylglucosamine with glycosylceramide was large when koji contained approximately 100 μg of glycosylceramide. Considering that the coefficient of variation is low at an incubation time of 72 h, it can be hypothesized that some unknown factor decreases the correlation between *N*-acetylglucosamine and glycosylceramide concentrations by this stage of incubation, which should be considered when using this technique.

However, since most koji production is performed with an incubation time of approximately 48 h, and because the residual error was small in koji samples incubated for 48 h, this technique is appropriate for the koji production system. Recent technical advances have radically decreased the cost of imaging photos, and many imaging programs are now freely available (e.g., NIH ImageJ); thus, quantitation of TLC spot intensities is cost-effective. Therefore, the method developed in this study has several advantages over previous techniques, including cost-effectiveness, simplicity, and ready availability of the equipment required. Therefore, in this study, we have developed a new method for determining the mycelial weight of *A. oryzae* which involves quantitation of glycosylceramide contained in koji. As this method employs a simple extraction procedure, it is a cost-effective quantitative method that can be used in the brewing and food industries.

After that, we also revealed the effect of glycosylceramides on the metabolic and flavor profiles of sake yeast. In this study, *A. oryzae*, soy and *G. frondosa* glycosylceramides conferred similar effects on the flavor profiles of sake yeast; addition of *A. oryzae* and *G. frondosa* glycosylceramides showed especially high similarity, providing a specificity of the 9-methyl base of the sphingoid base. Addition of soy glycosylceramide induced significant changes to the glucose and glycerol concentrations. Several metabolic pathways were altered in sake yeast incubated with soy glycosylceramide. Considering that the content of glycosylceramide differs among koji samples (Sawada et al. 2015), these results suggested that koji exerts its effects on yeast fermentation characteristics through glycosylceramide, and that the effect of koji on sake brewing should be evaluated in terms of the effect of the quantity of glycosylceramide contained in koji.

Alcoholic fermentation is performed without oxygen; therefore, it has been documented that unsaturated fatty acids (Fujii et al. 1997, Fujiwara et al. 1998) and sterols (Belviso et al. 2004), which require molecular oxygen, are required for progression of yeast fermentation. In addition, yeast incubated with or without oxygen (Ishtar Snoek and Yde Steensma 2007) or that incubated after diauxic shift (Kitagaki et al. 2009) have different gene expression profiles or yeast incubated without oxygen change their cell wall proteins (Kitagaki et al. 1997). However, the role of sphingolipids in yeast during fermentation has not been described until our study (Sawada et al. 2015).

Sphingolipids have several characteristics that are different from other lipids. For example, they are amide-linked and have long fatty acid lengths (C20–26) (Kitagaki et al. 2007). The mechanism of glycosylceramide's effect on yeast fermentation characteristics seems to be through its increasing effect on the yeast membrane fluidity. Yeast cells treated with GlcCer collectively disorders the plasma membrane and increase the motion of the fatty acids chain. Indeed, we have shown in a previous report that glycosylceramide shortens the average fluorescence lifetime (τ) of shochu yeast, as measured by trimethylamine-diphenylhexatriene (Sawada et al. 2015). Thus GlcCer seems to be improved membrane properties in a manner similar to unsaturated fatty acids and sterols. Consistent with this hypothesis, we found that volatile esters were decreased in sake yeast incubated with glycosylceramide (Table 3 & 4). Moreover, glycosylceramide containing the 9-methyl-4, 8-sphingadienine base, such as *A. oryzae* and *G. frondosa* glycosylceramide, had a more profound effect to decrease volatile esters as relative to glycosylceramide containing 4, 8-sphingadienine base, suggesting that 9-methyl base in the sphingoid base has a strong volatile ester-decreasing ability. It has been reported that the addition of unsaturated fatty acid, which also increases membrane fluidity, decreases ethyl flavors (Moonjai et al. 2003, Saerens et al. 2008).

Therefore, the effect of glycosylceramide on yeast is believed to be through its effect to increase membrane fluidity. Consistent with this hypothesis, a previous study reported that a 9-methyl base is essential for membrane rigidity (Singh et al. 2012, Takakuwa et al. 2002). However, there are several phenomena that cannot be explained only by the increase in membrane fluidity, such as the increase in glycerol, malate and decrease in leucine because a decrease in mitochondrial activity increases malate productivity in sake yeast (Motomura et al. 2012). Sphingolipids are signal molecules that stimulate protein phosphatase 2A (Dobrowsky et al. 1993) and form rafts in the lipid bilayer (Pralle et al. 2000); therefore, glycosylceramide might act via these mechanisms. It will be intriguing to investigate the effect of *A. oryzae* glycosylceramide on yeasts, which will form the target of our next study.

Koji contains abundant glycosylceramides (Sawada et al. 2015, Sakamoto et al. 2017). Therefore, sake yeast is exposed to a high concentration of glycosylceramide (40 μg/ml) during fermentation. In addition, the impact of glycosylceramide on sake yeast explains, at least in part, the fermentation profile of sake yeast. The amount of koji, or the extent of propagation of *A. oryzae* on the surface of koji (*haze*), has been empirically considered as an important criterion to govern yeast fermentation by brewing technicians in the sake industry. It has been proposed that mycelia of *A. oryzae* increase the complex taste of sake, but the precise mechanism has remained unknown. However, together with the data obtained in this study, it can be hypothesized that the amount of lipids, such as glycosylceramide, unsaturated fatty acids, and sterols, contained in koji, is the key to control yeast fermentation.

Sake yeast cells incubated with glycosylceramide showed significant effects on pyruvate metabolism, the TCA cycle, starch and sucrose metabolism, glyoxylate and decarboxylate and glycerolipid metabolism (Fig. 18).

In addition, lactosylceramide and ceramide cause dysfunction of the mitochondria (Novgorodov et al. 2016, Law et al. 2018); therefore, it might have stimulated the mitochondrial function of sake yeast. Mitochondrial activity has a significant effect on the fermentation profile of sake yeast (Shiroma et al. 2014, Kitagaki and Takagi 2014, Motomura et al. 2012, Sawada and Kitagaki 2016). Therefore, the upregulation of mitochondrial function in sake yeast might be responsible for the altered fermentation profile. The mechanism of the effect of glycosylceramide on yeast mitochondria requires further research.

3.5 Concluding remarks

In conclusion, we have developed a new method for determining the mycelial weight of *A. oryzae* which involves quantitation of glycosylceramide contained in koji. As this method employs a simple extraction procedure, it is a cost-effective, quantitative method that can be used in the brewing and food industries to measure fungal mycelial weight.

However, we have also determined the altered fermentation characteristics of sake yeast in response to koji glycosylceramide, which will enable interpretation of the effect of koji on the fermentation characteristics of yeast. It has been proposed that mycelia of *A. oryzae* also increase the complex taste of sake which is made by steaming rice fermented with *A. oryzae* and yeast.

CHAPTER 4

EFFECTS OF AMINO ACIDS ON SAKE YEAST DURING FERMENTATION

4.1 Introduction

The nutrients in fermentation media affect the characteristics of brewery yeasts. For example, assimilable nitrogen such as ammonium ions and amino acids which impacts the growth rate of yeast (Bely et al. 2003, Martinez-Moreno et al. 2012) and efficiency through the fermentation as much as aroma production. Some amino acids can be used as aromatic precursors to synthetize esters, higher alcohols (Vidal et al. 2015) or sulfur compounds. Furthermore, the response of yeast cells to various amino acids differs (ter Schure et al. 2000, Godard et al. 2007). Sutter et al. 2013, reported that sulfur containing amino acids methionine and cysteine addition was sufficient to potently suppress mitochondrial autophagy and promotes growth of yeast. In addition, different quantities of amino acids also have effects on the formation of volatile compounds during alcoholic fermentation of yeast (Teresa and Carmen 2008).

Most of amino acids are able to suppress the liberation of excess H_2S when used as sole sources of nitrogen in yeast. Specifically, yeasts supplemented with sulfur containing amino acids like methionine (Wainwright 1970, Giudici and Kunkee 1994, Boudreau et al. 2017) inhibited both sulfide and sulfite formation which are the main substrates for hydrogen sulfide production. In addition, cysteine (Jiranek et al. 19950) biotin, pantothenic acid (Wang et al. 2003, Bohlscheid et al. 2011) and vitamin B6 (Wainwright 1970,) show decreased production of hydrogen sulfide, which produces an off-flavor (Vos and Gray 1979, Cordente et al. 2009). The mitochondrion is an organelle essential for oxidative respiration. Molecular oxygen disappears rapidly upon fermentation, leading to rapid loss of mitochondrial activity. However, we and other groups have reported that residual mitochondrial activity plays significant roles in the fermentation characteristics of brewery yeasts, such as hydrogen sulfide formation (Samp 2012), diacetyl formation (Omura 2008), fermentation ability

(Shiroma et al. 2014), volatile ester formation (Verbelen et al. 2009), fatty acid desaturation (Sawada and Kitagaki 2016), and malate and succinate production (Motomura et al. 2012). In addition, mitochondria also play a key role in assemble of iron-sulfur (Fe/S) proteins by using sulfur containing substances (Cys, Met and GSH) which participate in cellular iron homeostasis (Lill et al. 2012, Lill et al. 2014). Furthermore, we have previously shown that mitophagy, the selective degradation of mitochondria by autophagy, contributes to the maintenance of mitochondrial quality by eliminating damaged or excess mitochondria (Kim et al. 2007, Kanki et al. 2011, Eiyama et al.2013, Okamoto et al. 2009) and also plays significant roles in the progression of alcoholic fermentation (Shiroma et al. 2014), as observed by the high fermentation ability of *atg32Δ* sake yeast. It has been proposed that *Atg32* acts as a mitophagy-specific receptor and regulates selective degradation of mitochondria.

Concretely, a mutant disrupted in *atg32* (Okamoto et al. 2009), a gene responsible for mitochondrial degradation, shows elevated fermentation ability compared to its parental strain (Shiroma et al. 2014). This difference can be attributed to the process of mitochondrial degradation or mitophagy process. If some added substances interferes with this process, it should exert a different effect on *atg32* deletant and its parental strain. If the additional substance does not interferes with this process, it should exert a similar effect on *atg32* deletant and its parental strain. To consider the above point, in this study, an analytical system have established to quantitatively and objectively detect the interaction effects of additional substance like amino acids on the mitochondrial degradation process during alcohol fermentation. Until date, oxygenation was the only approach to manipulate the mitochondrial activity of brewery yeasts during fermentation.

In this study, we attempted to develop a novel method of manipulating the mitochondrial activity of brewery yeasts during fermentation by exploring the mechanism underlying the effect of amino acids on mitochondria-related phenomena during fermentation. By adopting a system to detect mitochondria-activating substances, we screened for amino acids that could modify mitochondrial activity during fermentation. As a result, we identified methionine (Met) and glycine (Gly) as candidates to activate mitochondrial activity. Consistent with the hypothesis, these amino acids strengthened mitochondrial activity, as detected by the reactive oxygen species (ROS) reactive dye, 2, 7-Dichlorodihydrofluorescein (DCFH-DA).

Reactive oxygen species (ROS) are by-products of the cell metabolism and the mitochondrial respiratory chain in aerobically growing cells (Cadenas 1989). However, previous studies indicated that ROS production also occur during fermentation (Landolfo et al. 2008). In anaerobic condition, yeast activates cytochrome P450 systems which produce significant level of ROS products such as H_2O_2 (Rosenfeld and Beauvoit 2003). These results indicate that specific amino acids stabilize mitochondrial activity during fermentation, indicating a novel mechanism linking amino acid supplementation and the fermentation profiles of brewery yeast.

4.2 Materials and methods

4.2.1 Yeast strains and media

The yeast strains used in this study are listed in Table 6. To propagate yeast cells, yeast extract-peptone-dextrose (YPD) medium containing 2% (w/v) Bacto peptone,1%(w/v) Bacto yeast extract (Beckton Dickinson), and 2% (w/v) glucose were used. For fermentation tests, minimal synthetic medium containing 0.67% (w/v) yeast nitrogen base without amino acids (Beckton Dickinson), 790 mg $liter^{-1}$ complete supplement mixture (CSM) Dropout: Complete (Formedium), and 15% (w/v) glucose with amino acids were used.

4.2.2 Fermentation test

Yeast cells (1×10^6 cells/ml) were inoculated with 100 ml of minimal synthetic medium containing 15% (w/v) glucose with or without amino acids in a 300-ml Erlenmeyer flask equipped with an air lock on top of the flask. The medium was cultured statically at 30°C for 9 to 11 days, and mass was determined every day.

4.2.3 Measurement of ethanol concentration

The ethanol concentrations of the fermented cultures were analysed using a contact combustion system with an alcohol densitometer (Alcohol Checker YSA-200; Yazaki Meter Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions, and as described previously (Katou et al. 2008).

4.2.4 Analysis of intracellular ROS content

2, 7-Dichlorodihydrofluorescein diacetate (DCFH-DA) stock solution (1 mg) was prepared in 100 µl ethanol and stored at -20°C. Yeast cells (1×10^6 cells/ml) were inoculated in 10 ml minimal synthetic medium containing 15% (w/v) glucose with or without amino acids and incubated for 2−3 days at 30°C with liquid paraffin overlaid on the culture. After fermentation, 10^7 cells/ml yeast cells were recovered and incubated with 13 µl DCFH-DA, 100 µl PBS, and

887 µl sterile water at 30°C for 20 min in a shaker at 200 rpm. After incubation, cells were washed three times with sterile water (centrifuged at 13,000 × *g* for 1 min) and observed under a fluorescence microscope (Olympus BX53, 100 cells were observed per sample).

4.2.5 Metabolite analysis by GC/FID method

After fermentation, the sample pellet was collected by centrifugation at -9°C, 3,200 × *g* for 3 min and then resuspended in 3 ml sterile water and centrifuged again at the same conditions. The pellet was then soaked in liquid nitrogen for 3.5 min, freeze-dried for 12 h, and stored at -80°C. Next, 10 mg of the dried pellet was mixed with a mixture of chloroform:methanol:water (2:5:2 v:v:v) for extraction, followed by addition of 60 µl ribitol (0.2 mg/ml) to the solvent, and incubation for 3 min at 30°C with shaking at 1,500 rpm. Then, 800 µl supernatant was collected after centrifugation (4°C, 16,000 × *g* for 3 min) and 400 µl MQ was added, mixed, and centrifuged again at the same conditions. We then evaporated 800 µl of the supernatant for 3 h and freeze-dried it for 12 h. Next, 100 µl methoxyamine (20 mg/ml: pyridine) was mixed with the freeze-dried extract and incubated at 30°C for 90 min with shaking at 1,500 rpm, followed by addition of 50 µl MSTFA and incubation at 37°C for 30 min at 1,500 rpm with shaking. Then, 70 µl of the mixture was transferred to a vial and the metabolites were analyzed using a gas chromatography frame ionization detector (GC-2014, Gas CHROMATOGRAPH, Shimadzu, Kyoto, Japan) with a CP Sil8CB column (length, 30 m ; internal diameter, 0.25 mm; film thick ness, 0.25 µm; Agilent Technologies, Palo Alto, CA). Nitrogen was used as the carrier gas, with a column headspace pressure of 73.9 kPa, and a flow rate of 0.97 ml/min. The gas chromatography temperature program was as follows: 60°C for 2 min, raised to 320°C at 13°C/min and hold for 17 min. The split ratio for intracellular metabolites was 2. The data were analyzed using GC/FID solution software. Experiments were performed in triplicate with independent cultures.

4.2.6 Microscopic observation of mitochondria and vacuoles

During fermentation, the mitochondria and vacuoles were observed by using fluorescence microscope according to a modified published method (Shiroma et al. 2014). Briefly, samples were obtained from the fermentation broth and 1 ml of log-phase yeast cells were recovered by using centrifugation at 5,000 rpm for 5 min at room temperature. Yeast cells were incubated with the YPD broth and 100 µM complete EDTA free stock solution for 10 min and then 8 µM FM4-64 (Molecular Probes) in a 30°C water bath for 30 min. Then washed with the YPD liquid to remove free complete EDTA free stock solution and FM4-64 and incubated again in the liquid at 30°C for 90 to 120 min with mild shaking, washed with phosphate-buffer saline, resuspended in the same buffer saline and observed yeast cell under a fluorescence microscope (Keyence BZ8000). To observe the three-dimensional structures of mitochondria and vacuoles, z-stack images were acquired.

4.2.7 Measurement of glucose concentrations

The glucose concentration were measured at OD_{505} by using a UV spectrophotometer (UV-1800; Shimadzu Scientific Instruments, Kyoto, Japan) with a Glucose CII-kit (Waka Diagnostic, Osaka, Japan) as described previously (Jayakody et al. 2012)

4.2.8. Statistical analysis

The statistical significances of differences between the averages of two data groups were evaluated using an unpaired one-sided Student's *t*-test without known deviations. The differences among the averages of three or more groups were evaluated using Tukey's test and Dunnett's test without known deviations. The experimental results were expressed as mean ± standard error of the mean (SEM). The results were considered significant when $p<0.05$. Statistical significance of the interaction was also determined using two-way factorial ANOVA

Table 6. Yeast strains used in this study

Strains	Relevant genotype or description	Source
K7RAK	Sake yeast RAK1536 MATa/œhis3/his3	Hashimoto et al. 2005
K7RAKmitGFP	RAK1536+pRS413GPDmitGFP	Shiroma et al. 2014
K7RAK *atg32⊿*	RAK1536 atg32⊿::kanMX/atg32⊿::NAT1	Shiroma et al. 2014
K7RAK*atg32⊿*mitGFP	K7RAK atg32⊿+ pRS413GPDmitGFP	Shiroma et al. 2014
Petite sake yeast	TCR7haploidpetite	Kitagaki lab, Saga University, Japan

4.3 Results

Table 7. List of amino acids used in this study

List of amino acids with abbreviation	
Methionine (Met)	Tryptophan (Trp)
Cysteine (Cys)	Serine (Ser)
Glycine (Gly)	Threonine (Thr)
Alanine (Ala)	Glutamine (Gln)
Valine(Val)	Lysine (Lys)
Proline (Pro)	Arginine (Arg)
Phenylalanine (Phe)	Histidine (His)
Leucine (Leu)	Aspartic acid (Asp)
Isoleucine (Ile)	Glutamic acid (Glu)
Tyrosine (Tyr)	Asparagine (Asn)

4.3.1 Fermentation profile of wild-type and its mutant *atg32⊿* sake yeast with or without amino acids

First, in order to investigate the effects of amino acids on yeast fermentation (Fig. 19), 1mM concentration of amino acids such as Met, Cys, Gly, Ala, Val, Pro, Phe, Leu, Ile, Tyr, Trp, Ser, Thr, Gln, Lys, Arg, His, Asp, Glu and Asn (Table 7) were added to the culture medium and analyzed different fermentation characteristics of *atg32⊿* mutant sake yeast and its parent strain K7RAK (Table 8 & 9).

A

B

Figure 19. Fermentation test of sake yeast with or without amino acids

For fermentation, wild-type (K7RAK) (A) and its mutant *atg32Δ* (B) sake yeast were cultured in minimal synthetic medium containing 15% glucose with or without amino acids at 30°C for 9 to 11 days respectively.

Table 8. Fermentation characteristics of WT sake yeast

Yeast cells were cultured in minimal synthetic medium containing 15% glucose with or without amino acids such as Met, Cys, Gly, Ala, Val, Pro, Phe, Leu, Ile, Tyr, Trp, Ser, Thr, Gln, Lys, Arg, His, Asp, Glu and Asn.

Sample No.	Final CO_2 evolution (g/liter)		Max. fermentation rate (g/liter/day)		Final ethanol conc. (% (v/v))	
	Average	Fold change	Average	Fold change	Average	Fold change
WT	63.90±0.264	1.00	14.53±0.120	1.00	8.58±0.016	1.00
WT+Met	64.33±0.202	1.01	15.57±0.145*	1.07	8.65±0.028	1.01
WT+Cys	68.33±0.218*	1.07	15.73±0.145*	1.08	8.75±0.028*	1.02
WT+Gly	64.67±0.088	1.01	15.50±0.173*	1.07	8.75±0.00*	1.02
WT+Ala	66.90±0.346*	1.05	15.47±0.120*	1.06	8.72±0.033	1.02
WT+Val	67.90±0.435*	1.06	15.73±0.120*	1.08	8.77±0.033*	1.02
WT+Pro	66.80±0.208*	1.04	15.73±0.202*	1.08	8.73±0.016*	1.02
WT+Phe	65.50±0.360*	1.02	15.13±0.176	1.04	8.57±0.033	1.00
WT+Leu	67.00±0.115*	1.05	15.47±0.120*	1.06	8.73±0.033*	1.02
WT+Ile	65.46±0.176*	1.02	15.00±0.208	1.03	8.55±0.050	1.00
WT+Tyr	66.73±0.185*	1.04	15.50±0.115*	1.07	8.75±0.028*	1.02
WT+Trp	66.03±0.202*	1.03	15.27±0.185*	1.05	8.68±0.016	1.01
WT+Ser	66.97±0.202*	1.05	15.60±0.115*	1.07	8.73±0.033*	1.02
WT+Thr	65.87±0.233*	1.03	15.67±0.145*	1.08	8.75±0.028*	1.02
WT+Gln	65.83±0.185*	1.03	15.50±0.115*	1.07	8.73±0.016*	1.02
WT+lys	63.03±0.202	0.99	15.03±0.145	1.03	8.63±0.016	1.01
WT+Arg	67.10±0.264*	1.05	15.47±0.145*	1.06	8.78±0.033*	1.02
WT+His	67.07±0.185*	1.05	15.43±0.120*	1.06	8.70±0.028	1.01
WT+Asp	67.20±0.152*	1.05	15.70±0.153*	1.08	8.75±0.028*	1.02
WT+Glu	65.93±0.284*	1.03	15.50±0.115*	1.07	8.73±0.033*	1.02
WT+Asn	65.70±0.173*	1.03	15.43±0.120*	1.06	8.71±0.016	1.01

Note: values are means ± SEM of three independent experiments initiated with respective starter cultures. (n=3; *, p<0.05; as determined by Dunnett's test.). Here, WT represent K7RAK sake yeast

Table 9. Fermentation characteristics of the *atg32⊿* mutant sake yeast

Yeast cells were cultured in minimal synthetic medium containing 15% glucose with or without amino acids such as Met, Cys, Gly, Ala, Val, Pro, Phe, Leu, Ile, Tyr, Trp, Ser, Thr, Gln, Lys, Arg, His, Asp, Glu and Asn.

Sample No.	Final CO_2 evolution (g/liter)		Max. fermentation rate (g/liter/day)		Final ethanol conc. (% (v/v))	
	Average	Fold change	Average	Fold change	Average	Fold change
atg32⊿	69.27±0.240	1.00	15.57±0.066	1.00	8.96±0.016	1.00
atg32⊿+Met	68.23±0.176	0.98	15.93±0.120	1.02	8.90±0.028	0.99
atg32⊿+Cys	71.17±0.317*	1.03	16.10±0.152	1.03	9.03±0.016	1.01
atg32⊿+Gly	69.20±0.057	1.00	15.97±0.145	1.02	9.03±0.016	1.01
atg32⊿+Ala	71.03±0.290*	1.02	15.77±0.120	1.01	9.02±0.016	1.00
atg32⊿+Val	71.67±0.480*	1.03	16.00±0.208	1.03	9.05±0.028	1.01
atg32⊿+Pro	68.77±0.120	0.99	15.93±0.120	1.02	8.98±0.016	1.00
atg32⊿+Phe	66.70±0.152	0.96	15.77±0.120	1.01	8.83±0.033	0.98
atg32⊿+Leu	69.20±0.264	1.00	16.16±0.202*	1.04	8.97±0.033	1.00
atg32⊿+Ile	66.63±0.185	0.96	15.43±0.176	0.99	8.80±0.057	0.98
atg32⊿+Tyr	69.00±0.251	0.99	15.90±0.152	1.02	8.98±0.016	1.00
atg32⊿+Trp	67.77±0.176	0.98	15.93±0.120	1.02	8.95±0.028	1.00
atg32⊿+Ser	69.30±0.152	1.00	15.93±0.185	1.02	9.00±0.028	1.00
atg32⊿+Thr	71.17±0.145*	1.03	16.23±0.176*	1.04	9.10±0.028*	1.01
atg32⊿+Gln	68.83±0.088	0.99	16.03±0.145	1.03	8.98±0.016	1.00
atg32⊿+lys	67.83±0.296	0.98	15.53±0.088	0.99	8.90±0.028	0.99
atg32⊿+Arg	70.80±0.251*	1.02	16.00±0.173	1.03	9.02±0.016	1.00
atg32⊿+His	69.83±0.120	1.01	16.00±0.115	1.03	8.98±0.016	1.00
atg32⊿+Asp	70.13±0.176	1.01	16.30±0.208*	1.05	9.08±0.016*	1.01
atg32⊿+Glu	70.80±0.200*	1.02	16.03±0.145	1.03	9.02±0.016	1.00
atg32⊿+Asn	71.00±0.204*	1.02	16.17±0.185*	1.04	9.03±0.016	1.01

Note: values are means ± SEM of three independent experiments initiated with respective starter cultures. (n=3; *, p<0.05; as determined by Dunnett's test).

These results indicate that WT sake yeast significantly ($p< 0.05$, Dunnett's test) increased final CO_2 evolution, maximum fermentation rate and final ethanol concentration when amino acids were added (although additional effects of Met, Ala, Phe, Ile, Trp, Lys, His and Asn on ethanol productivity was not significant) (Table 8). Similarly the addition of Cys, Ala, Val, Thr, Arg, Glu and Asn on CO_2 evolution of the sake mutant *atg32⊿* was increased; the effect of Leu, Thr, Asp and Asn on maximum fermentation rate and the effect of Thr and Asp on the final ethanol concentration of *atg32⊿* was significantly increased ($p<0.05$, Dunnett's test) (Table 9).

4.3.2 Interaction effects of amino acids with mitophagy function of WT and its mutant *atg32⊿* sake yeast during fermentation

The above results do not provide information on the interaction of amino acids addition and mitophagy during fermentation. In wild-type yeast, increased fermentation ability by the addition of amino acids reflects an increase in proteins in the cytosol, leading to the acceleration of glycolysis. This should be the same in *atg32⊿* sake mutant yeast, but its increase ratio should be cancelled by the increase in mitochondrial activity. Based on this scheme, the interaction effect of the amino acid sensing by mitophagy system, a two-way factorial ANOVA test was performed. If two factorial values are parallel, that means intracellular amino acids pool generated by mitophagy and the additional amino acids transported from media are independent, there should be no interaction effect. If two factorial values are not parallel (exponential interaction) or cross (antagonistic interaction) each other that means the additional amino acids pool are transported from media regulate the mitophagy and there should be significant interaction effect. To consider that sensing of amino acid pool by the mitophagy system should be reflected by the statistically significant detection of the interaction by two-way ANOVA. To analyze the interaction of mitophagy and amino acids, a two way factorial ANOVA of amino acids and the existence of mitophagy was designed.

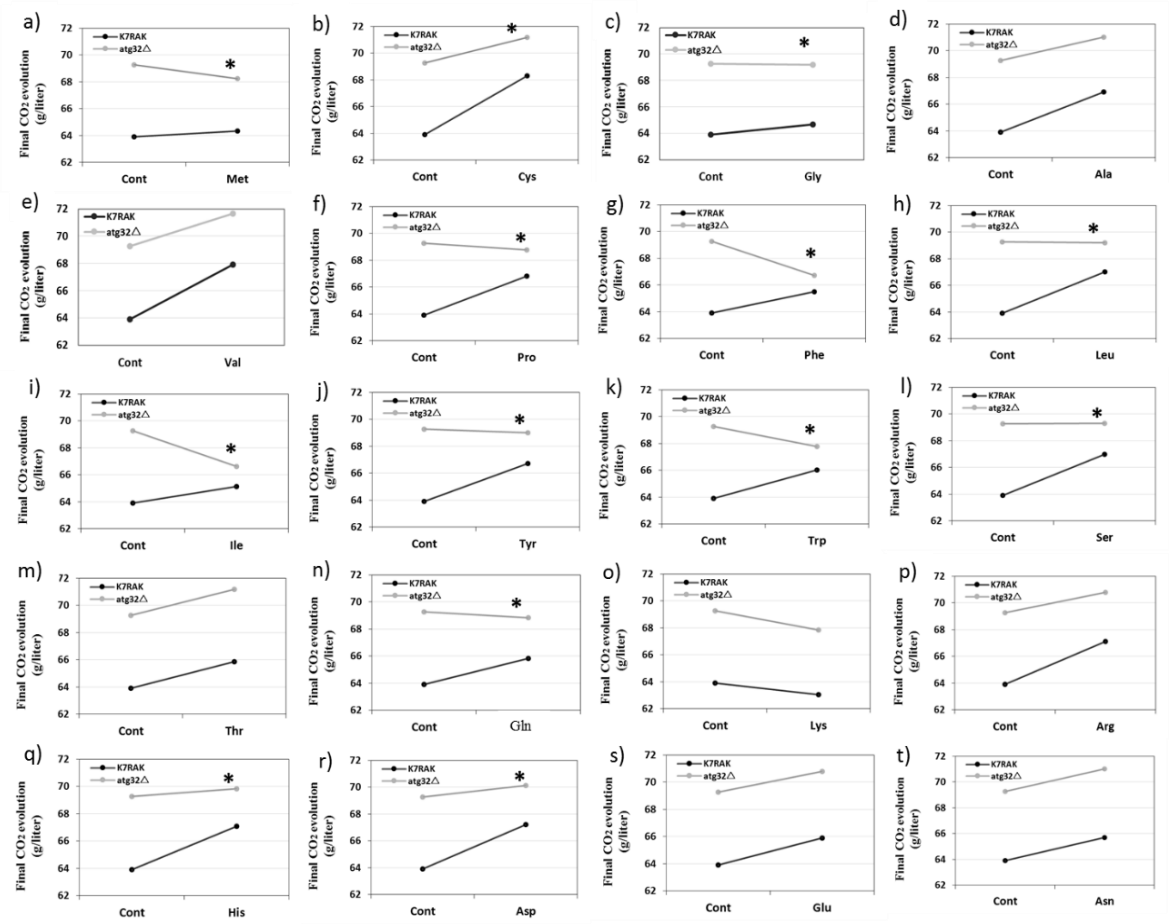

Figure 20. The effects of amino acids on final CO_2 evolution (g/liter) with WT and its mutant *atg32Δ* sake yeast during fermentation

Yeast cells were cultured in minimal synthetic medium containing 15% glucose with or without amino acids and CO_2 evolution was analyzed (a-t). The statistical significance of the interaction was analyzed using two-way factorial ANOVA. *, $p<0.05$ ($n=3$). Here, WT represent K7RAK sake yeast strain.

As a result, amino acids such as Met, Cys, Gly, Pro, Phe, Leu, Ile, Tyr, Trp, Ser, Gln, His and Asp showed the significant (p<0.05) characteristics in CO_2 evolution (g/liter) (Fig. 20). In terms of maximum fermentation rate, amino acids such as Met, Cys, Gly, Ala, Val, Pro, Ile, Tyr, Ser, and Lys were significantly effective (g/liter/day) (Fig. 21). Moreover, in terms of final ethanol concentration (% (v/v)), amino acids such as Met, Cys, Gly, Pro, Leu, Tyr, Trp, Ser, Gln, Lys, Arg, His and Glu showed similar characteristics (Fig. 22). To examine this, we also analyzed the growth profile OD_{600} ($\times 10^7$ cells/ml) of *atg32⊿* mutant sake yeast and its parent strain (K7RAK) with or without amino acids. As for growth profile Met, Cys, Gly, Val, Pro, Tyr, Ser, Gln, Arg and Asp showed similar significant effects in this study (Fig. 23). The above results suggested that Met, Cys, Gly, Pro, Tyr and Ser showed common significant interaction effects for main fermentation characteristics among all amino acids (Fig. 20, 21, 22, 23).

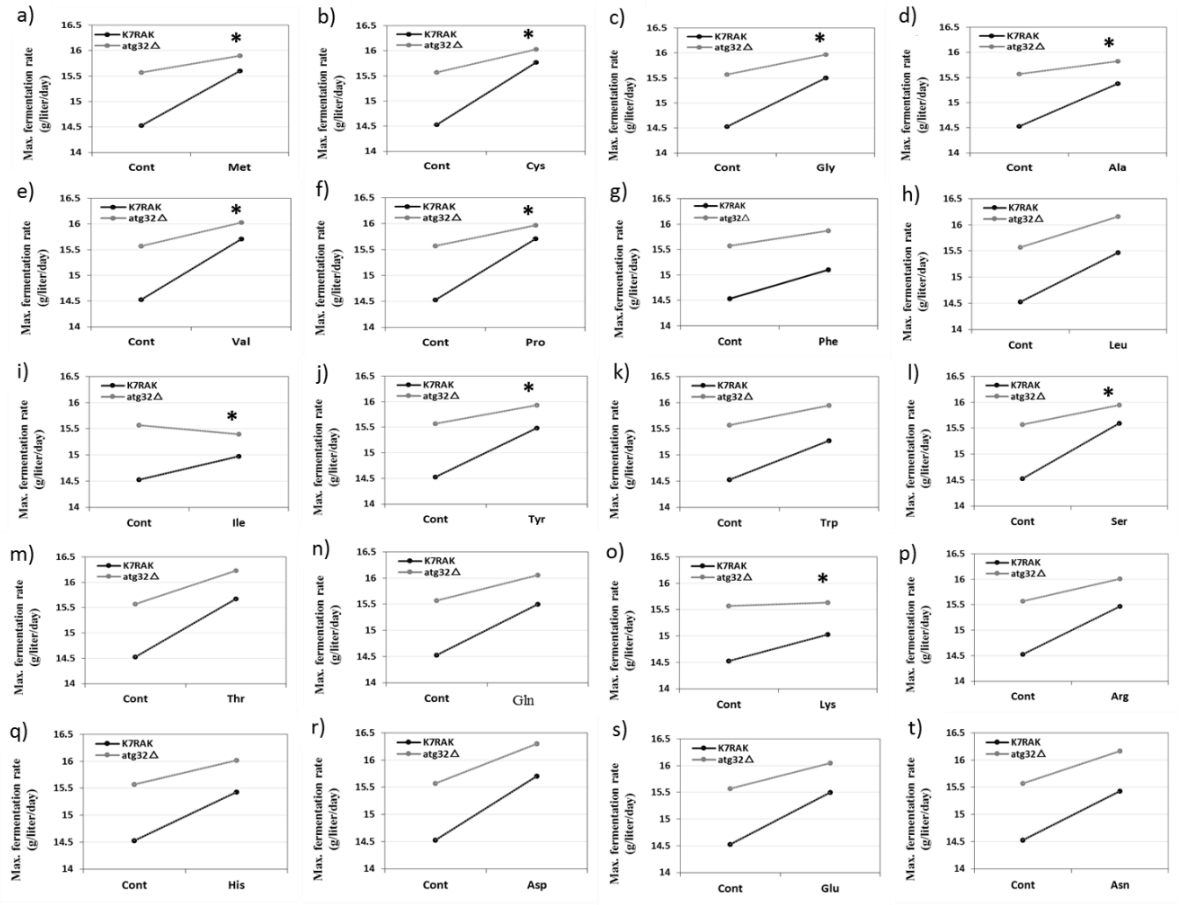

Figure 21. The effects of amino acids on the maximum fermentation rate (g/liter/day) of WT and its mutant *atg32Δ* sake yeast during fermentation

Yeast cells were cultured in minimal synthetic medium containing 15% glucose with or without amino acids and the maximal fermentation rate was analyzed (a-t). The statistical significance of the interaction was analyzed using two-way factorial ANOVA. *, $p<0.05$ ($n=3$). Here, WT represent K7RAK sake yeast strain.

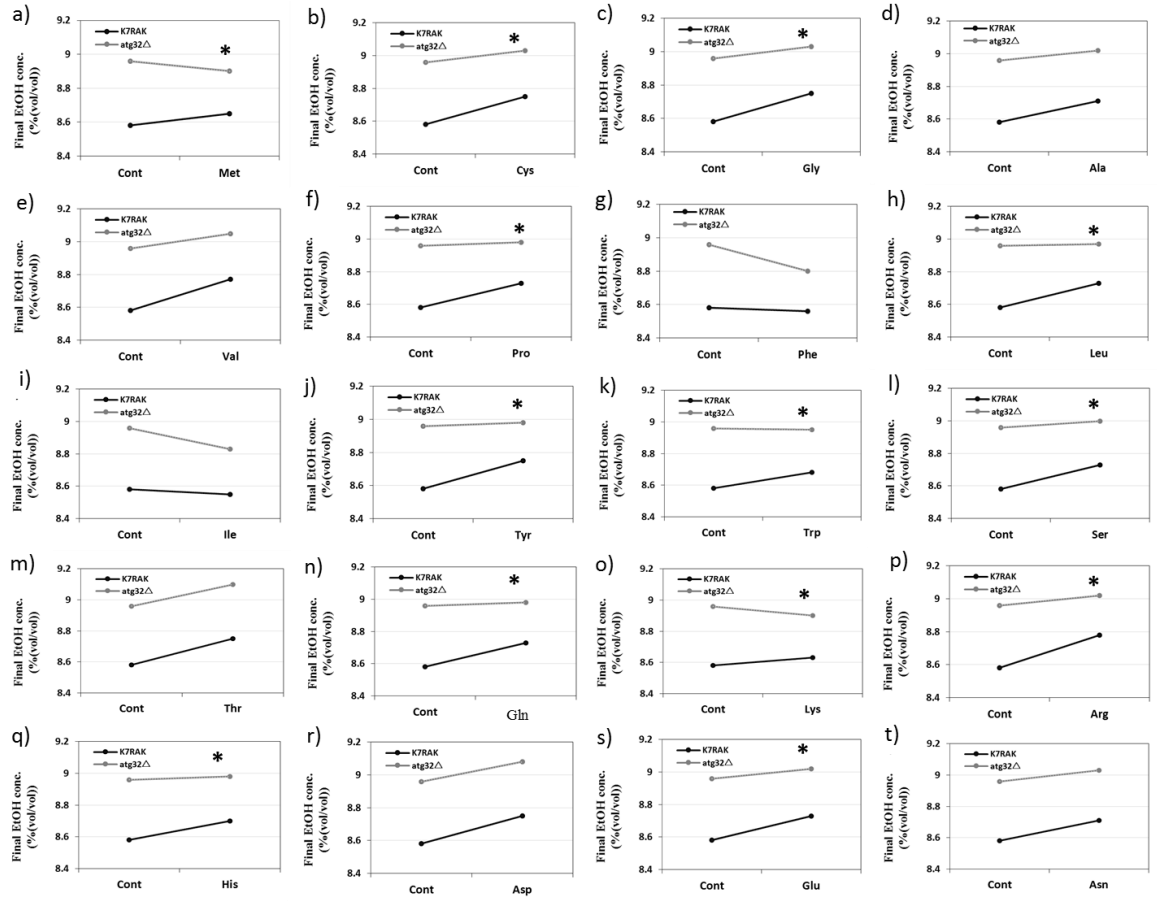

Figure 22. The effects of amino acids on final ethanol concentration (% (v/v)) of WT and its mutant *atg32Δ* sake yeast during fermentation

Yeast cells were cultured in minimal synthetic medium containing 15% glucose with or without amino acids and final ethanol concentration was measured (a-t). The statistical significance of the interaction was analyzed using two-way factorial ANOVA. *, $p<0.05$ ($n=3$). Here, WT represent K7RAK sake yeast strain.

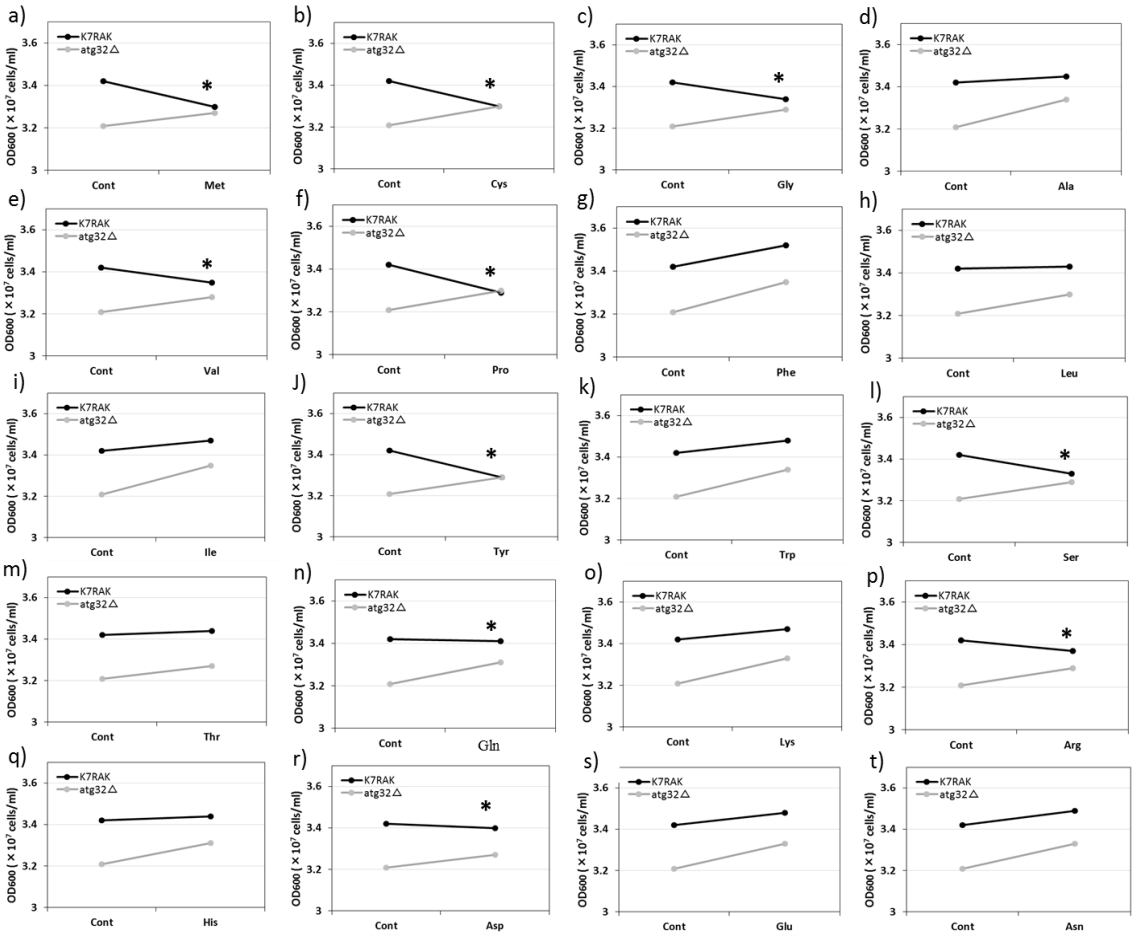

Figure 23. The effects of amino acids on the growth profile of WT and its mutant *atg32Δ* sake yeast during fermentation

Yeast cells were cultured in minimal synthetic medium containing 15% glucose with or without amino acids and final OD_{600} ($\times 10^7$ cells/ml) was measured (a-t). The statistical significance of interaction was analyzed using two-way factorial ANOVA. *, $p<0.05$ ($n=3$). Here, WT represent K7RAK sake yeast strain.

4.3.3. The *atg32⊿* mutant sake yeast shows defective mitochondria by ROS analysis

We hypothesized that augmentation of mitochondrial activity could be detected by comparing the increasing ratio of fermentation ability in wild-type and its mutant *atg32⊿* sake yeast. It was first observed that *atg32⊿* mutant sake yeast showed high fermentation ability (Shiroma et al. 2014) because of defective mitochondria.

To prove this hypothesis, the levels of reactive oxygen species (ROS) which reflects mitochondrial activity in the *atg32⊿* mutant sake yeast were analyzed using DCFH-DA. The *atg32⊿* mutant sake yeast exhibited significantly ($p<0.05$) weak reactive oxygen species activity similarly to *petite* sake yeast compared to the wild-type strain (Fig. 24) which indicating that the *atg32⊿* mutant sake yeast have defective mitochondrial activity. Since *petite* strain do not have mitochondrial DNA, shows deteriorated mitochondrial activity and thus exhibit low ROS activity (Kadowaki et al. 2017).

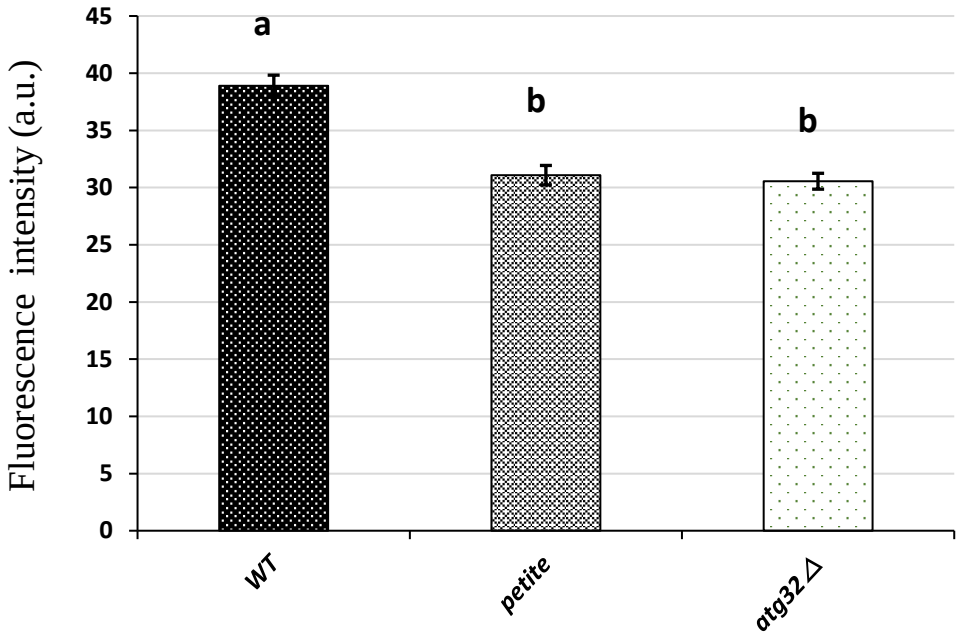

Figure 24. Evaluation of intracellular ROS content in WT, *petite* and *atg32Δ* mutant sake yeast

Yeast cells were incubated in minimal synthetic medium containing 15% glucose. Cells were sampled under fermentation stress, treated with DCFH-DA and analyzed by fluorescence microscopy for determination of ROS content. The *petite* strain was used as positive control. The statistical significance of the difference is indicated by one-way ANOVA followed by Tukey's *post hoc* honest significant difference test.

It was thus considered that the *atg32⊿* mutant sake yeast shows high ethanol fermentation ability (Shiroma et al. 2014), because *atg32⊿* has an elevated cytosolic carbon flux due to its deteriorated mitochondrial function caused by disrupting mitophagy function. This led us to conclude that loss of mitochondrial activity during ethanol fermentation increases pyruvate levels and fermentation ability, namely, augmentation of mitochondrial activity decreases fermentation ability.

4.3.4 Identification of methionine (Met) and glycine (Gly) as mitochondria-activating amino acids

As the fermentation characteristics of the *atg32⊿* mutant sake yeast were not altered by the addition of most amino acids, suggesting that amino acids may be affected the mitochondrial activity of sake mutant strain**.** We hypothesized that these amino acids (Met, Cys, Gly, Pro, Tyr and Ser) might fortify mitochondrial activity of the *atg32⊿* mutant sake yeast during fermentation. To confirm this hypothesis, we measured the mitochondrial activities of *atg32⊿* mutant sake yeast supplemented with these amino acids by measuring the levels of reactive oxygen species (ROS) using DCFH-DA fluorescent dye. Among these amino acids, only addition of Met and Gly significantly ($p<0.05$) increased the fluorescence intensity of *atg32⊿* mutant sake yeast similarly to H_2O_2 treated cells which is used as ROS product. However, ROS product H_2O_2 treated cells also showed significant ($p<0.05$) increases of fluorescence intensity which is used as the positive control (Fig. 25). Together with the above mentioned results, these findings clearly indicate that Met and Gly function to stabilize the mitochondrial activity in the *atg32⊿* mutant sake yeast during alcoholic fermentation.

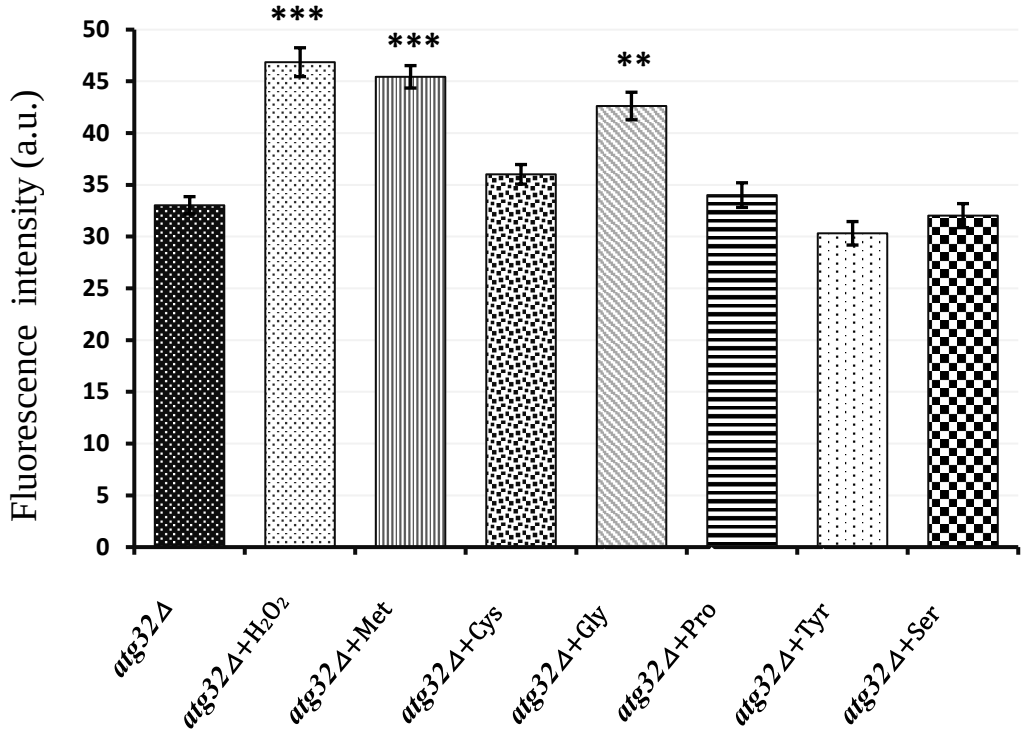

Figure 25. Evaluation of intracellular ROS content in the *atg32Δ* mutant sake yeast

Yeast cells were incubated in a minimal synthetic medium containing 15% glucose with or without amino acids such as methionine (Met), cysteine (Cys), glycine (Gly), proline (Pro), tyrosine (Tyr) and serine (Ser). Cells were collected under fermentation stress, treated with DCFH-DA and analyzed by fluorescence microscopy for ROS content. H_2O_2: cells treated with 1mM hydrogen peroxide (H_2O_2), used as the positive control. The statistical significance of the difference is indicated by Dunnett's test (***, $p<0.001$; **, $p<0.01$).

4.3.5 Metabolome analysis of WT and its mutant *atg32⊿* sake yeast supplemented with methionine

To obtain the physiological information of yeast cells supplemented with amino acids such as methionine (Met), we analyzed the metabolome of *atg32⊿* mutant sake yeast cells supplemented with sulfur containing amino acids Met by principal component analysis (PCA). The contributions of PC1 and PC2 were 70.6% and 8.34% respectively, indicating that these components showed variations in the metabolites of cell. Yeast cells supplemented with Met were clearly separated from non-supplemented cells in score plot of PCA (Fig. 26a) which indicated that addition of Met might regulate metabolites of yeast cell during fermentation stress.

In addition, ethanol contributed to the separation of *atg32⊿* and glucose contributed to the separation of WT in loading plot of PCA (Fig. 26b), consistent with the high fermentation ability of *atg32⊿* mutant sake yeast (Shiroma et al. 2014). Met-supplemented cells were clustered together, consistent with the similar fermentation ability of WT and *atg32⊿* mutant sake yeast. Most of amino acids contributed negatively to the separation of Met-supplemented cells, suggesting that the glycolysis pathway, which generates simple substances, was promoted in Met-supplemented cells.

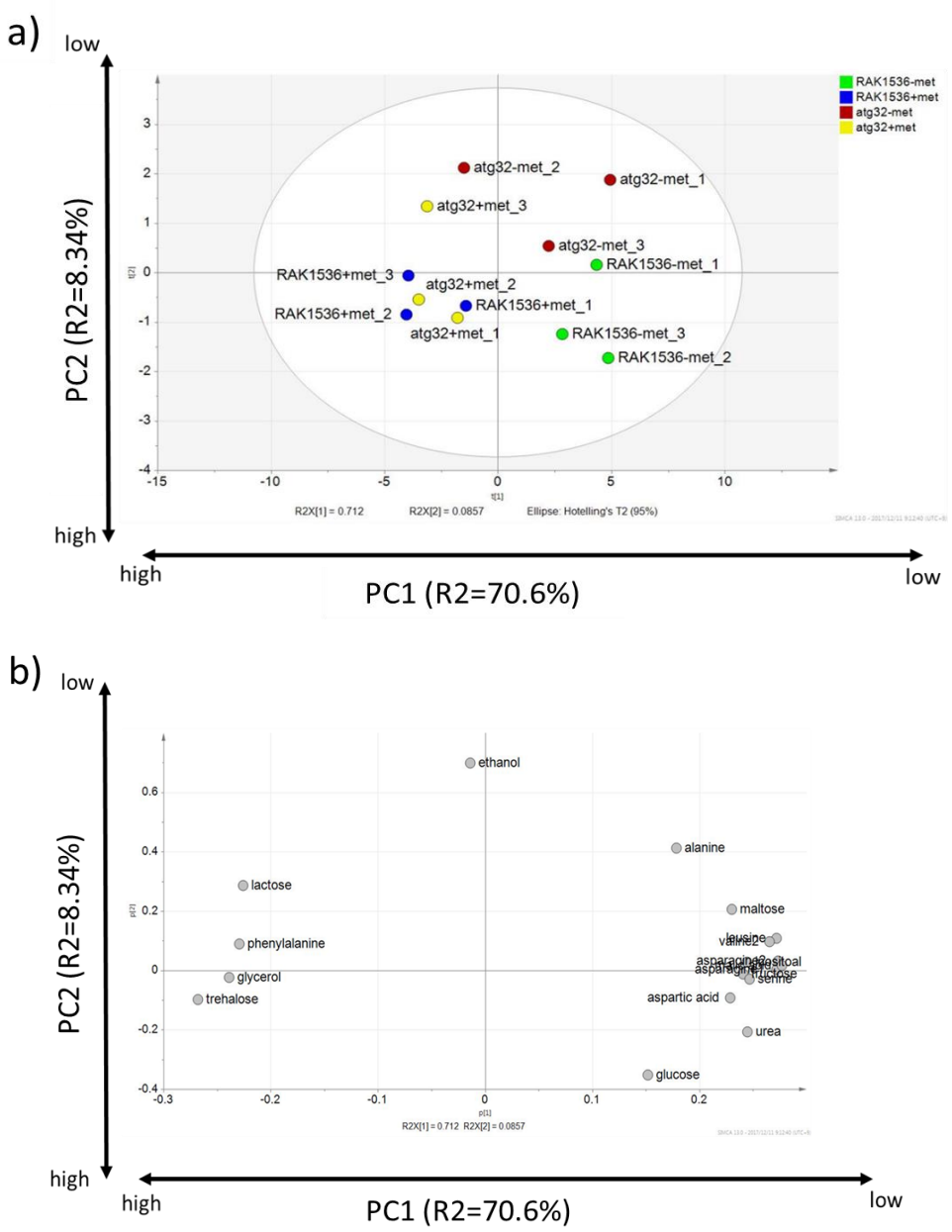

Figure 26. The intracellular metabolites of WT and its mutant *atg32Δ* sake yeast supplemented with methionine

Yeast cells were incubated in minimal synthetic medium containing 15% glucose with or without Met. The metabolites were analyzed using GC/FID and their peak area were divided by the total peak area, and standardized by auto-scaling. These normalized values were used as an independent variable in PCA (Principal component analysis) (p<0.05). (a) Score plot of PCA, (b) Loading plot of PCA.

4.3.6 Microscopic observation of mitochondria of WT and its mutant *atg32⊿* sake yeast with or without methionine

Previously, a study reported that mitophagy occurs in wild-type yeast cells during ethanol fermentation (Shiroma et al. 2014) and mitophagy disrupted by a mutant defective in *Atg32*, a gene responsible for mitochondrial degradation process. Therefore, we used *atg32⊿* mutant sake yeast and its parent strain K7RAK that express GFP targeted to the mitochondria. The mitochondrial and vacuolar structures were observed using a fluorescence microscope (Fig. 27). In this study, we observed that addition of the sulfur-containing amino acids like Met disrupted mitophagy function during alcohol fermentation of wild-type sake yeast (Fig. 27A). During ethanol fermentation in wild-type yeast, some portion of the mitochondria (green) fused with the vacuolar membrane (red) to generate yellow signals in z-stack images (Fig 27A). In contrast, when amino acids like Met used in medium mitochondria (green) were located distant from the vacuolar membrane (red) and virtually no yellow signal was evident in cultures (Fig. 27A). However, we also observed that mitophagy function was disrupted in *atg32⊿* mutant sake yeast strain during fermentation and there was no difference after addition of Met in medium (Fig. 27B). Together, these results indicated that mitochondrial degradation was suppressed either by disrupting a *Atg32* gene or by using mitophagy interacting substances like amino acids.

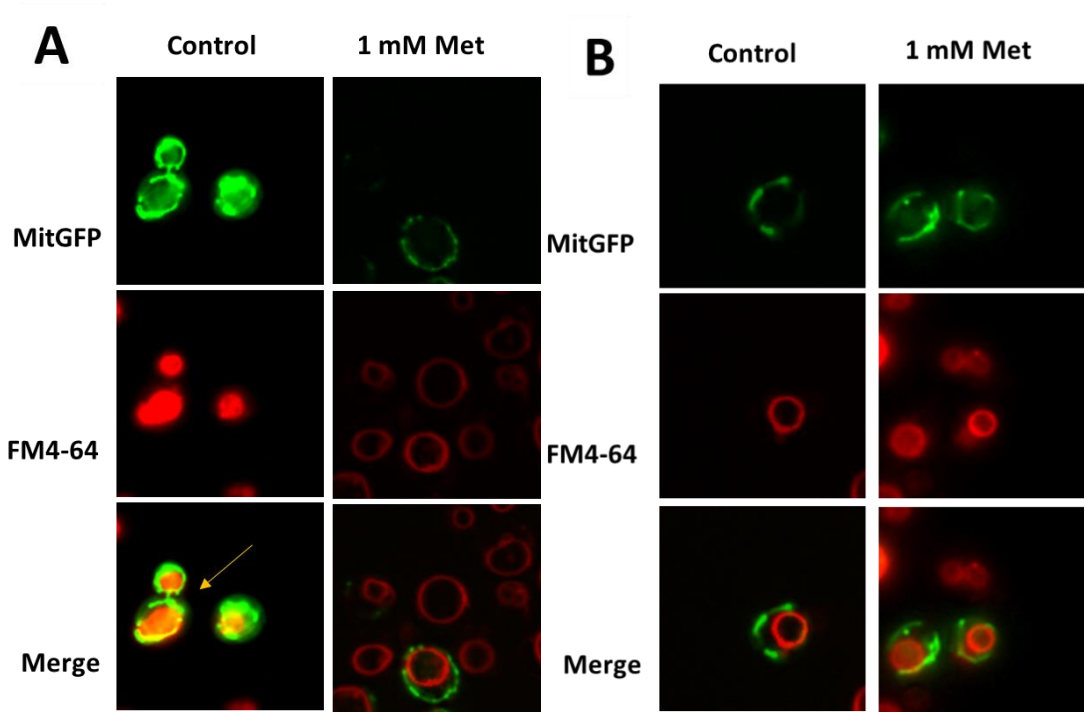

Figure 27. Mitochondrial and vacuolar morphological observation of WT sake yeast strain K7RAKmitGFP (A) and its mutant K7RAK *atg32*⊿mitGFP (B)

Yeast cells were incubated in minimal synthetic medium containing 15% glucose with or without Met. Samples were collected in the second days of fermentation because yeast showed maximum fermentation rate in second day during fermentation. Green signals indicate mitochondrially targeted GFP, Red signals indicate the vacuolar membrane stained with 8 μM FM4-64. Yellow signals indicate colocalization of mitochondria and vacuolar membrane. Z-stack images were obtained using a fluorescence microscope. Mitophagy was suppressed by disrupting the function of mitophagy gene *Atg32* or by using sulfur containing amino acids like Met during fermentation of yeast.

4.3 Discussion

The *atg32⊿* mutant sake yeast has an increased fermentation efficiency because the mitochondrial degradation function is deteriorated (Shiroma et al. 2014) and cytosolic metabolic flux is increased compared with its parents strain. However, there are several studies, which imply that the mitochondria play a role on the fermentation profile of sake yeast (Kitagaki and Takagi 2014, Motomura et al. 2012, Sawada and Kitagaki 2016). It has also been reported that respiratory deficient mutants of wine yeasts improved about 10% and 18% ethanol efficiency compared to respective parent strains (Ooi and Lankford 2009). Moreover, nutrients in fermentation media also affects the fermentation characteristics of brewery yeasts. For example, assimilable nitrogen increases the growth rate of yeast (Bely et al. 2003). Addition of substrate like amino acids have a regulatory effects either on growth or mitochondrial activity of yeast which stimulates the rate of alcohol fermentation (Teresa and Carmen 2008, Tesniere et al. 2013, Lin et al. 2012). Specifically, addition of methionine, decrease hydrogen sulfide production by brewery yeasts (Giudici and Kunkee 1994, Boudreau et al. 2017). Notably mitochondrial degradation process also affected by several amino acids (Sutter et al. 2013). In this study, we demonstrated that the *atg32⊿* mutant sake yeast, which show enhanced fermentation ability, exhibits defective mitochondrial activity. From this finding, we developed a method to screen amino acids that activate the mitochondria which play significant role on the fermentation profile of yeast.

In this study, we found that Met and Gly confer characteristics of mitochondrial activation during fermentation. These findings indicate that addition of amino acids to fermentation media affects the mitochondrial activity of brewery yeasts, providing a new link between the fermentation nutrients and fermentation characteristics of brewery yeasts.

Mitochondria maintain their transmembrane electron potential through the electron transport chain (Zamzami et al. 1995, Waterhouse et al. 2001). Since the function of the electron transport chain is based on the function of iron, formation of iron-sulfur (Fe-S) proteins might be facilitated by specific amino acids like sulfur containing amino substances (Met, Cys, GSH) as a sulfur donor (Lill et al. 2012, Lill et al. 2014). Alternatively, the amino acids used in this research, or organic acids arising from the supplemented amino acids (Teresa and Carmen 2008), might have entered the mitochondria and stimulated the mitochondrial metabolism. The mechanism underlying mitochondrial activity upregulation requires further research.

After Met is incorporated into yeast cells through Mup1 (Kitajima et al. 2010), it is used as a component of proteins and is also transaminated with α-ketoglutarate to α-keto-γ-methylthiobutyrate. It is also converted to methanethiol, methionol, and α-ketobutyrate in a reductive environment (Perpète et al. 2006). These substances are suggested to stimulate the mitochondria. Consistent with this hypothesis, genes encoding mitochondria-localized proteins were highly expressed in yeast cells supplemented with Met (Godard et al. 2007). It is also suggested that one electron pair from the sulfur atom of Met or its reductive form methionol, might scavenge the ROS arising during anaerobiosis (Sideri et al. 2009) and protect iron-sulfur clusters, thus stabilizing the mitochondrial activity. The response of yeast cells supplemented with Met is similar to those supplemented with Leu, Ile, Thr, Trp and Tyr, which confer slow yeast growth (Godard et al. 2007). Together with the data obtained in our study, it is suggested that unfavorable amino acid like Met stimulate mitochondrial activity.

Addition of Met leads to decreased production of hydrogen sulfide, which leads to an undesirable flavor (Boudreau et al. 2017). It can be postulated that iron-sulfur cluster formation in mitochondria (Lill et al. 2012) is enhanced and affects hydrogen sulfite

production from sulfate, as reported previously (Samp 2012). Therefore, mitochondrial stabilization by Met as observed in this study provides a link between Met, mitochondria and hydrogen sulfide production.

After Gly incorporation into yeast cells by the general amino acid permease Gap1 (Jauniaux and Grenson 1990), it is used as a component of proteins and is also cleaved to amino or carboxyl moieties through the glycine-cleavage system (Kikuchi 1973). In this study, Gly increased the mitochondrial activity of *atg32Δ* sake yeast. Glycine is a precursor of heme (iron) and mitochondria play a key role in iron metabolism in that they synthesize heme by using amino acids, assemble iron-sulfur (Fe/S) proteins, and participate in cellular iron regulation (Lill et al. 2012). The degradation products methylene-THF generated by the glycine-cleavage system might have entered the mitochondria and activated their metabolism. The mechanism of the effect of Gly on the mitochondria of sake yeast needs further research. Since the regulation of mitochondria in brewery yeasts affects hydrogen sulfide formation (Samp 2012), diacetyl formation (Omura 2008), fermentation ability (Shiroma et al. 2014), volatile ester formation (Verbelen et al. 2009), fatty acid desaturation (Sawada and Kitagaki 2016), and malate and succinate production (Motomura et al. 2012), many brewers attempt to manipulate the mitochondrial activity of brewery yeasts by oxygenation. The results obtained in this study suggest that amino acid supplementation might act a new approach to manipulate mitochondrial activity in brewery yeasts.

Metabolome analysis revealed the high fermentation ability of WT and *atg32Δ* mutant sake yeast cells supplemented with Met as relative to non-supplemented cells. However, the metabolome did not provide concrete information on the mitochondrial metabolite. This was because the metabolism within mitochondria in the fermentation condition was too low to detect.

The most abundant amino acids in sake are Asp, Arg, Pro, Ala, Gly, Glu and Leu (Tamura et al.1952). It has been reported that pantothenic acid decreases hydrogen sulfide in sake yeast (Kodaira et al. 1958). Increase in Pro confers ethanol resistance in sake yeast (Takagi et al. 2005). The results obtained in this study suggest that the specific amino acids generated during sake brewing which affects the mitochondrial activity of sake yeast and thus its brewing profiles.

4.5 Concluding remarks

In conclusion, we found that the additional substances like amino acids have impact on the fermentation profile, metabolic profile and also mitochondrial activity of sake yeast during fermentation. The regulation of mitophagy function of *atg32⊿* mutant sake yeast strain by additional amino acids could become a completely novel approach to manipulate the fermentation profile and metabolic profile during alcoholic fermentation of sake yeast. These results showed that mitochondrial activity of *atg32⊿* mutant sake yeast is increased by the addition of Met and Gly by ROS analysis approaches. These data can contribute to the development of a new approach to regulate the mitochondrial activity of brewery yeasts during fermentation.

CHAPTER 5

DISCUSSION

In former chapter, we discussed the effects of factors such as additional substances like lipid compounds, glycosylceramide and amino acids on the fermentation profile of yeast. In first phase of the study, as describe in chapter 3, we developed a novel method to quantitate the fungal mycelial growth by determining fungal cell membrane lipid glycosylceramide and also the effects of its on the fermentation profile of sake yeast. In 2nd phase of the study, we analyzed the interaction effects of amino acids with mitochondrial phenomena of *atg32Δ* mutant sake yeast and its parent strain during fermentation as describe in chapter 4. To conclude the findings of this dissertation study, chapter 5 describe as final chapter which includes conclusion of thesis study, outcome of the thesis and future directions of this study.

5.1 Concluding summary

To study the effects of nutritional factors on the fermentation profile of sake yeast, for the first time, we analyzed the effects of lipid compound glycosylceramide which is abundant in koji and one of the most important sphingolipid on the fermentation profile of sake yeast. Koji, which is steamed rice fermented with nonpathogenic fungus *A. oryzae*, contains glycosylceramide. In this thesis, the quantitation of mycelial weight of the industrially important non-pathogenic fungus *A. oryzae*, which is used for manufacturing koji, was performed by quantitating glycosylceramide. This is a novel method for quantitation of *A. oryzae* mycelial weight. The amount of glycosylceramide significantly correlated with both the mycelial weight of *A.oryzae* and the amount of *N*-acetylglucosamine which is used practically as the established index of the mycelial weight of *A. oryzae* in koji. This new method is simple, reproducible and efficient and can be used in the brewing and food industries to determine the mycelial weight or cell biomass of fungi like *A. oryzae*.

Since the quality of koji depends on the mycelial weight of *A. oryzae*, the quantitation of the mycelial weight of *A. oryzae* has been intensively studied.

As a saccharifier of starch, koji has been traditionally used in food industries of Japan as a Japanese version of the malt. In sake brewing, koji is mixed with steamed rice and sake yeast and thus simultaneous saccharification and fermentation process occurs. During fermentation, sake yeast requires lipids, such as unsaturated fatty acids and sterols, in addition to substances provided by koji enzymes for fermentation. In the study of dissertation, we revealed that koji glycosylceramide effects on the metabolic and flavor profiles of sake yeast during fermentation. The addition of glycosylceramide significantly increased 2- phenylethyl acetate, phenylethyl alcohol, glycerol, ethanol, succinate, pyruvate, threonine and malate contents, whereas it significantly decreased the glucose, leucine, ethyl 9-decenoate and ethylcaprylate levels. The effect of 9-methyl base-containing fungal glycosylceramide on the volatile ester-decreasing effect was stronger than that of plant glycosylceramide. The metabolic pathways were analysed by using Metaboanalyst which indicated that pyruvate metabolism, the tri carboxylic acid (TCA) cycle, starch and sucrose metabolism, glyoxylate and decarboxylate metabolism and glycerolipid metabolism were increased ($p<0.05$) by the additional substances like soy glycosylceramide. This is the first study of the effect of glycosylceramide on the flavor and metabolic profile of sake yeast during fermentation which might influence the brewing characteristics of yeast.

However, in the study of thesis, the interaction effect of different amino acids with the mitochondrial activity of sake yeast during ethanol fermentation were evaluated. Amino acids in fermentation media affect the brewing profiles of yeast. For example, addition of Met decreases hydrogen sulfide production by brewery yeasts. In addition, mitochondrial activity during fermentation is critical to the fermentation profiles of brewery yeasts.

However, a concrete mechanism linking amino acids in fermentation media with mitochondrial activity during fermentation of brewery yeasts have yet unknown. In this thesis, we examined the effects of amino acids on the fermentation abilities of wild-type sake yeast and its mutant strain *atg32⊿*. It was found that amino acids in fermentation media, such as methionine (Met), cysteine (Cys), glycine (Gly), proline (Pro), tyrosine (Tyr) and serine (Ser) showed significant interaction effect during main fermentation characteristics of sake yeast.

Among these amino acids, especially Met and Gly, identified as candidate amino acids by measuring reactive oxygen species (ROS) levels which are stabilized mitochondrial activity of sake yeast. Yeast cells supplemented with Met and Gly retained high ROS levels relative to that of the non-supplemented sake yeast. Moreover, Met-supplemented cells showed a metabolome distinct from that of non-supplemented cells. However, addition of Met in wild type yeast also suppressed mitophagy function same as *atg32⊿* mutant sake yeast which has defective mitochondria.

Therefore, these results indicate that additional substances such as glycosylceramide and amino acids are important factors which regulate the metabolic profiles and mitochondrial profile of sake yeast during fermentation, and thus improve fermentation profiles of brewery yeast.

5.2 Outcome of the research

These research works might improve the brewing profiles of sake yeast during sake brewing. Since glycosylceramide is an essential and stable component in cell membrane, the glycosylceramide content seems to be a useful parameter for the estimation of the total sum of the growing mycelium. Therefore, glycosylceramide in koji used to determine fungal mycelial weight, develop a new quantitative method in this research works. Since this method is simple, cost effective and efficient that can be used in the brewing and food industries. In addition, the role of koji glycosylceramide on the metabolic and flavor profiles of sake yeast, suggested that the amount of lipids such as glycosylceramide is the key to control the brewing characteristics of sake yeast and improve fermentation profiles. On the other hand, since the regulation of mitochondria in brewery yeast has a significant effects on the fermentation ability, this results suggested that amino acids supplementation might act a new approach to manipulate mitochondrial activity in brewery yeasts and improve fermentation profiles.

The key original findings

- Developed a novel method for quantitation of mycelial weight of *A. oryzae* by measuring its glycosylceramide content in koji.
- Determined the effect of koji glycosylceramide on the flavor and metabolic profiles of sake yeast during fermentation. Therefore, the effects of koji on sake brewing should be evaluated in terms of the effect of the glycosylceramide in koji.
- Developed a concrete mechanism linking amino acids in fermentation media with mitochondrial activity of sake yeast during fermentation.
- Identified methionine (Met) and glycine (Gly) as candidate amino acids fortifying mitochondrial activity in sake yeast during fermentation and improve fermentation profiles.

5.3 Publications list

International peer-reviewed Journal

1. Jayakody LN, **Ferdouse J**, Hayashi N, Kitagaki H. (2017) Identification and detoxification of glycolaldehyde, an unattended bioethanol fermentation inhibitor. *Critcal Reviews in Biotechnology* **37(2):** 177-189

2. Kadowaki M, Fujimaru Y, Taguchi S, **Ferdouse J**, Sawada K, Kimura Y, Terasawa Y, Agrimi G, Anai T, Noguchi H, Toyoda A, Fujiyama A, Akao T, Kitagaki H (2017) Chromosomal aneuploidy improves the brewing characteristics of sake yeast. *Applied and Environmental Microbiology* **83(24):** e01620-17

3. Sakamoto M, Sakatani M, **Ferdouse J**, Hamajima H, Tsuge K, Nishimukai M, Yanagita T, Nagao K, Mitsutake S, Kitagaki H. (2017) Development of quantitative method for the contents of glycosylceramide contained in Japanese foods brewed with koji and its application. *Journal of the Brewing Society of Japan* **112(9):** 655-662 (in Japanese)

4. **Ferdouse J,** Yuki Yamamoto Y, Taguchi S, Yoshizaki Y, Takamine K , Kitagaki H (2018) Glycosylceramide modifies the flavour and metabolic characteristics of sake yeast. *PeerJ*. **6:** e4768

5. **Ferdouse J,** Miyagawa M, Hirano M, Kitajima Y, Inaba S, Kitagaki H (2018) A new method to determine the mycelial weight of Koji *Aspergillus oryzae* by measuring its glycosylceramide content. *The Journal of General and Applied microbiology* (in press).

6. **Ferdouse J,** Fujimaru Y, Yamamoto Y, Kitagaki H. (2018) Methionine and glycine stabilize mitochondrial activity in sake yeast during ethanol fermentation. *Food Technology and Biotechnology* (in review)

5.4 Future works

The novel research works revealed in this study opens several new areas in the field of biotechnology and microbiology, which could be targeted in future works.

- Analysis of the effects of amino acids, glycosylceramide or other nutritional factors on the fermentation profile, metabolic profile and mitochondrial activity by using other industrially important yeast because sake yeast mainly used in this study.
- Analysis of the effects of additional nutrient substances on the nutritional signaling pathway and aging process of yeast under fermentation stress.
- As glycosylceramide in koji is the key to control yeast fermentation and mitochondrial activity showed significant effect on the fermentation profile of yeast therefore, the mechanism of the effect of glycosylceramide on yeast mitochondria requires further and details study.
- In this study, only extracellular metabolites of yeast were analyzed with glycosylceramide. Therefore, analysis of intracellular metabolites of sake yeast by using glycosylceramide should require to obtain detail information about metabolomics.
- Use of other nutritional substances such as glutathione and other sulfur containing compounds which could regulate mitochondrial activity during fermentation and enhance fermentation process.
- As Met and Gly regulated yeast mitochondrial activity in this study, therefore the mechanism of regulation require pilot-level study.
- In this study, the metabolome analysis with methionine did not provide sufficient information about mitochondrial metabolites. Therefore, this hypothesis needs further and detail study.

REFERENCES

1. Akao T, Yashiro I, Hosoyama A, Kitagaki H, Horikawa H, Watanabe D, Akada R, Ando Y, Harashima S, Inoue T, Inoue Y, Kajiwara S, Kitamoto K, Kitamoto N, Kobayashi O, Kuhara S, Masubuchi T, Mizoguchi H, Nakao Y, Nakazato A, Namise M, Oba T, Ogata T, Ohta A, Sato M, Shibasaki S, Takatsume Y, Tanimoto S, Tsuboi H, Nishimura A, Yoda K, Ishikawa T, Iwashita K, Fujita N, Shimoi H. (2011) Whole-genome sequencing of sake yeast *Saccharomyces cerevisiae* Kyokai no. 7. *DNA Res.*, **18:** 423–434
2. Arima K, Uozumi T. (1967) A new method for estimation of the mycelial weight in koji. *Agri. Biol. Chem.*, **31**: 119–123
3. Beeler T, Bacikova D, Gable K, Hopkins L, Johnson C, Slife H, Dunn T. (1998) The *Saccharomyces cerevisiae* TSC10/YBR265w gene encoding 3-ketosphinganine reductase is identified in a screen for temperature-sensitive suppressors of the Ca21-sensitive *csg2Δ* mutant. *J. Biol. Chem.*, **273:** 30688–30694
4. Belviso S, Bardi L, Bartolini AB, Marzona M. (2004) Lipid nutrition of *Saccharomyces cerevisiae* in winemaking. *Can. J. Microbiol.*, **50(9):** 669–674
5. Bely M, Rinaldi A, Dubourdieu D. (2003) Influence of assimilable nitrogen on volatile acidity production by *Saccharomyces cerevisiae* during high sugar fermentation. *J. Biosci. Bioeng.*, **96:** 507–512
6. Bohlscheid JC, Osborne JP, Ross CF, Edwards CG. (2011) Interactive effects of selected nutrients and fermentation temperature on H_2S production by wine strains of *Saccharomyces*. *J. Food Qual.*, **34(1):** 51–55
7. Boudreau TF, Peck GM, Ma S, Patrick N, Duncan S, O'Keefe SF, et al. (2017) Hydrogen sulphide production during cider fermentation is moderated by pre-fermentation methionine addition. *J. Inst. Brew.*, **123:** 553–561

8. Brosnan JT. (2001) Amino acids, then and now—a reflection on Sir Hans Kreb's contribution to nitrogen metabolism. *IUBMB Life* , **52:** 265–270
9. Cadenas E. (1989) Biochemistry of oxygen toxicity. *Annu. Rev. Biochem*., **58:** 79–110.
10. Carter H E, Hendrickson H S. (1963) Biochemistry of the sphingolipids. XV. Structure of phytosphingosine and dehydrophytosphingosine. *Biochemistry*., **2:** 389–393
11. Coderch L, López O, de la Maza A, Parra JL (2003) Ceramides and skin function. *Am. J. Clin. Dermatol*., **4(2):** 107–29
12. Cordente AG, Heinrich A, Pretorius IS, Swiegers JH. (2009) Isolation of sulfite reductase variants of a commercial wine yeast with significantly reduced hydrogen sulfide production. *FEMS Yeast Res.,* **9(3):** 446–459
13. D'Angelo G, Polishchuk E, Di Tullio G , Santoro M, Di Campli A, Godi A, West G, Bielawski J, Chuang CC, van der Spoel AC, Platt FM, Hannun YA, Polishchuk R, Mattjus P, De Matteis AM. (2007) Glycosphingolipid synthesis requires FAPP2 transfer of glucosylceramide. *Nature,* **449(7158):** 62–7
14. D'Angelo G, Uemura T, Chuang CC, Polishchuk E, Santoro M, Ohvo- Rekilä H, Sato T, Di Tullio G, Varriale A, D'Auria S, Daniele T, Capuani F, Johannes L, Mattjus P, Monti M, Pucci P, Williams RL, Burke JE, Platt FM, Harada A, De Matteis MA. (2013) Vesicular and non-vesicular transport feed distinct glycosylation pathways in the Golgi. *Nature,* **501:**116–120
15. da Silva A F, Rodrigues ML, Farias S E, Almeida I C, Pinto M R, Barreto-Bergter E. (2004) Glucosylceramides in *Colletotrichum gloeosporioides* are involved in the differentiation of conidia into mycelial cells. *FEBS Lett.,* **561:** 137–143
16. del Poeta M, Nimrichter L, Rodrigues ML, Luberto C. (2014) Synthesis and biological properties of fungal glucosylceramide. *PLoS Pathog.* **10(1):** e1003832
17. Dickson R. C. (2008) Thematic review series: sphingolipids. New insights into

sphingolipid metabolism and function in budding yeast. *J. Lipid Res.*, **49:** 909–921

18. Dickson RC, Lester R L. (1999a) Metabolism and selected functions of sphingolipids in the yeast *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta.,*, **1438:** 305–321
19. Dickson R C, Lester RL. (1999b) Yeast sphingolipids. *Biochim. Biophys. Acta*., **1426:** 347–357
20. Dickson R C, Lester RL. (2002) Sphingolipid functions in *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta.*, **1583:** 13–25.
21. Dickson R C, Nagiec EE, Wells GB, Nagiec MM, Lester RL. (1997) Synthesis of mannose- (inositol-P)2-ceramide, the major sphingolipid in *Saccharomyces cerevisiae*, requires the *IPT1 (YDR072c)* gene. *J. Biol. Chem.*, **272(47):** 29620–29625
22. Dickson R C, Sumanasekera C, Lester R L. (2006) Functions and metabolism of sphingolipids in *Saccharomyces cerevisiae*. *Prog. Lipid Res.*, **45:**447–465
23. Dobrowsky RT, Kamibayashi C, Mumby MC, Hannun YA. (1993) Ceramide activates heterotrimeric protein phosphatase 2A. *J. Biol. Chem.*, **268(21):** 15523–30
24. Eiyama A, Kondo-Okamoto N, Okamoto K. (2013) Mitochondrial degradation during starvation is selective and temporally distinct from bulk autophagy in yeast. *FEBS Lett.*, **587(12):** 1787–1792
25. Ejsing CS, Sampaio JL, Surendranath V, Duchoslav E, Ekroos K, Klemm RW, Simons K, Shevchenko A. (2009) Global analysis of the yeast lipidome by quantitative shotgun mass spectrometry. *Proc. Natl. Acad. Sci. U S A*, **106:** 2136–2141
26. Fujii F, Ozeki K, Kanda A, Hamachi M, Nunokawa Y. (1992) A simple method for the determination of grown mycelial content in rice *koji* using commercial cell wall lytic enzyme, yatalase. *J. Brew. Soc. Japan.*, **87:**757–759
27. Fujii T, Kobayashi O, Yoshimoto H, Furukawa S, Tamai Y. (1997) Effect of aeration and unsaturated fatty acids on expression of the *Saccharomyces cerevisiae* alcohol

acetyltransferase gene. *Appl. Environ. Microbiol.,* **63(3):** 910–915

28. Fujiwara D, Yoshimoto H, Sone H, Harashima S, Tamai Y. (1998) Transcriptional co-regulation of *Saccharomyces cerevisiae* alcohol acetyltransferase gene, *ATF1* and delta-9 fatty acid desaturase gene, *OLE1* by unsaturated fatty acids. *Yeast,* **14(8):** 711–721
29. Futerman AH, Riezman H. (2005) The ins and outs of sphingolipid synthesis. *Trends Cell Biol*., **15:** 312–318
30. Gault CR, Obeid LM, Hannun YA. (2010) An overview of sphingolipid metabolism: from synthesis to breakdown. *Adv. Exp. Med. Biol.*, **688:** 1–23
31. Gikas P, Livingston, AG. (1997) Specific ATP and specific oxygen uptake rate in immobilized cell aggregates: experimental results and theoretical analysis using a structured model of immobilized cell growth. *Biotechnol. Bioeng*., **55(4):** 660–72
32. Giudici P, Kunkee RE. (1994) The effect of nitrogen deficiency and sulfur-containing amino acids on the reduction of sulfate to hydrogen sulfide by wine yeasts. *Am. J. Enol. Vitic.*, **45:** 107–112
33. Godard P, Urrestarazu A, Vissers S, Kontos K, Bontempi G, Helden J, et al. (2007) Effect of 21 different nitrogen sources on global gene expression in the yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biol.,* **27(8):** 3065–3086
34. Gomi K, Okazaki N, Tanaka T, Kumagai C, Inoue H. et al. (1987) Estimation of mycelial weight in rice-*koji* with use of fungal cell wall lytic enzyme Studies on application of fungal cell wall lytic enzyme produced by *Oerskcvia* sp. CK. (part II). *J. Brew. Soc. Japan.,* **82:**130–133.
35. Guillas I, Kirchman PA, Chuard R, Pfefferli M, Jiang JC, Jazwinski SM, Conzelmann A. (2001) C26-CoA-dependent ceramide synthesis of Saccharomyces cerevisiae is operated by Lag1p and Lac1p. *EMBO J.*, **20:** 2655–2665
36. Gunasekaran P, Raj KC. (1999) Ethanol fermentation technologyee *Zymomonas mobilis*.

Curr Sci., **77:** 56–68

37. Hamajima H, Fujikawa A, Yamashiro M, Ogami T, Kitamura S, Tsubata M, Tan S, Matsunaga H, Sawada K, Kumagai S, Hayashi N, Nagao K, Yanagita T, Oka T, Mitsutake S, Kitagaki H. (2016) Chemical analysis of the sugar moiety of monohexosylceramide contained in koji, Japanese traditional rice fermented with *Aspergillus*. *Fermentation,* **2:** 2
38. Hamajima H, Matsunaga H, Fujikawa A, Sato T, Mitsutake S,Yanagita T, Nagao K, Nakayama J, Kitagaki H. (2016) Japanese traditional dietary fungus koji *Aspergillus oryzae* functions as a prebiotic for *Blautia coccoides* through glycosylceramide: Japanese dietary fungus koji is a new prebiotic. *Springer Plus,* **5:** 1321
39. Hanada K. (2014) Co-evolution of sphingomyelin and the ceramide transport protein CERT. *Biochem. Biophys Acta.,* **1841:** 704–719
40. Hanada K, Kumagai K, Yasuda S, Miura Y, Kawano M, Fukasawa M, Nishijima M. **(**2003) Molecular machinery for non-vesicular trafficking of ceramide. *Nature,* **426:** 803–809
41. Hannun, Y. A. Obeid, L. M. (2017) Sphingolipids and their metabolism in physiology and disease. *Nat Rev Mol Cell Biol.,* doi: 10.1038/nrm.2017.107
42. Hannun YA, Obeid LM. (2008) Principles of bioactive lipid signaling: lessons from sphingolipids. *Nat. Rev. Mol. Cell Biol.,* **9(2):** 139–150
43. Hashimoto S, Ogura M, Aritomi K, Hoshida H, Nishizawa Y, Akada R. (2005) Isolation of auxotrophic mutants of diploid industrial yeast strains after UV mutagenesis. *Appl. Environ. Microbiol.,* **71:** 312–319
44. Hesseltine CW. (1983) Microbiology of oriental fermented foods. *Annu Rev Microbiol*., **37:** 575–601
45. Hirata M, Tsuge K, Jayakody L, Urano Y, Sawada K, Inaba S, Nagao K, Kitagaki H.

(2012) Structural determination of glucosylceramides in the distillation remnants of shochu, the Japanese traditional liquor, and its production by *Aspergillus kawachii*. *J. Agric. Food Chem.,* **60(46):** 11473–11482

46. Holzberg I, Finn RF, Steinkraus KH. (1967) A kinetic study of the alcoholic fermentation of grape juice. *Biotechnol. Bioeng.,* **9(3):** 413–423
47. Ichikawa S, Sskiyama H, Suzuki G, Hidari KI, Hirabayashi Y. (1996) Expression cloning of a cDNA for human ceramide glucosyltransferase that catalyzes the first glycosylation step of glycosphingolipid synthesis. *Proc Natl Acad Sci U S A.,* **93(10):** 4638–43
48. Ishtar Snoek IS, Yde Steensma H. (2007) Factors involved in anaerobic growth of *Saccharomyces cerevisiae*. *Yeast,* **24(1):** 1–10.
49. Jauniaux JC, Grenson M. (1990) *GAP1*, the general amino acid permease gene of *Saccharomyces cerevisiae*. Nucleotide sequence, protein similarity with the other baker's yeast amino acid permeases, and nitrogen catabolite repression. *Eur. J. Biochem.,* **190(1):** 39–44
50. Jayakody LN, Horie K, Hayashi N, Kitagaki H. (2012) Improvement of *Saccharomyces cerevisiae* to hot-compressed water treated cellulose by expression of *ADH1*. *Appl. Microbiol. Biotechnol.,* **94(1):** 273–283
51. Jeckel D, Karrenbauer A, Burger KN, van Meer G, Wieland F. (1992) Glucosylceramide is synthesized at the cytosolic surface of various Golgi subfractions. *J Cell Biol.,* **117(2):** 259–267
52. Jiranek V, Langridge P, Henschke PA. (1995) Regulation of hydrogen sulfide liberation in wine-producing *Saccharomyces cerevisiae* strains by assimilable nitrogen. *Appl. Environ. Microbiol*., **61(2):** 461–467
53. Kadowaki M, Fujimaru Y, Taguchi S, Ferdouse J, Sawada K, Kimura Y, Terasawa Y, Agrimi G, Anai T, Noguchi H, Toyoda A, Fujiyama A, Akao T, Kitagaki H. (2017)

Chromosomal aneuploidy improves brewing characteristics of sake yeast. *Appl. Environ. Microbiol.*, **83(24):** e01620–17

54. Kamada Y, Yoshino K, Kondo C, Kawamata T, Oshiro N, Yonezawa K, Ohsumi Y. (2010) Tor directly controls the Atg1 kinase complex to regulate autophagy. *Mol. Cell. Biol.*, **30:** 1049–1058
55. Kanki T, Klionsky DJ, Okamoto K. (2011) Mitochondria autophagy in yeast. *Antioxid. Redox Signal.*, **14(10):** 1989–2001
56. Katou T, Kitagaki H, Akao T, Shimoi H. (2008) Brewing characteristics of haploid strains isolated from sake yeast Kyokai no.7. *Yeast*, **25(11):** 799–807
57. Kaufman B, Basu S, Roseman S. (1971) Isolation of glucosylceramides from yeast (*Hansenula ciferri*). *J Biol Chem.*, **246:** 4266–4271
58. Kihara A. (2015) Sphingolipid metabolism via sphingosine 1-phosphate. In: Yokomizo T, Murakami M, editors. Bioactive lipid mediators: *current reviews and protocols.* Tokyo: Springer Japan, p. 127–38
59. Kihara A, Mitsutake S, Mizutani Y, Igarashi Y.(2007) Metabolism and biological functions of two phosphorylated sphingolipids, sphingosine 1-phosphate and ceramide 1- phosphate. *Prog Lipid Res.*, **46:** 126–44
60. Kikuchi G. (1973) The glycine cleavage system: composition, reaction mechanism, and physiological significance. *Mol. Cell. Biochem.*, **1(2):** 169–187
61. Kimball SR, Jefferson LS. (2006) New functions for amino acids: effects on gene transcription and translation. *Am. J. Clin. Nutr.*, **83(2):** 500S–507S
62. Kim I, Rodriguez-Enriquez S, Lemasters JJ. (2007) Selective degradation of mitochondria by mitophagy. *Arch. Biochem. Biophys.*, **462(2):** 245–253
63. Kitagaki H, Cowart LA, Matmati N, Montefusco D, Gandy J, Vaena de Avalos S,Novgorodov S, Zheng J, Obeid LM, Hannun YA. (2009) *ISC1*-dependent metabolic

adaptation reveals an indispensable role for mitochondria in induction of nuclear genes during the diauxic shift in *S. cerevisiae*. *J. Biol. Chem.,* **284(16):** 10818–10830

64. Kitagaki H, Cowart LA, Matmati N, Vaena de Avalos S, Novgorodov SA, Zeidan YH, Bielawski J, Obeid LM, Hannun YA. (2007) Isc1 regulates sphingolipid metabolism in yeast mitochondria. *Biochem. Biophys. Acta.*, **1768(11):** 2849–2861
65. Kitagaki H, Kitamoto K. (2013) Breeding researches of sake yeasts in Japan: History, recent technological advances, and future perspectives. *Annu. Rev. Food Sci. Technol*., **4:** 215-235
66. Kitagaki H, Shimoi H, Itoh K. (1997) Identification and analysis of a static culture-specific cell wall protein, Tir1p/Srp1p in *Saccharomyces cerevisiae*. *Eur. J. Biochem.*, **249(1):** 343–349
67. Kitagaki H, Takagi H. (2014) Mitochondrial metabolism and stress response of yeast: Applications in fermentation technologies. *J. Biosci. Bioeng.*, **117(4):** 383–393
68. Kitajima T, Chiba Y, Jigami Y. (2010) Mutation of high-affinity methionine permease contributes to selenomethionyl protein production in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.*, **76(19):** 6351–6359
69. Kodaira R, Ito Y, Uemura T. (1958) On the production of hydrogen sulfide by sake yeast. I. Pantothenic acid deficiency and the production of hydrogen sulphide. *Nihonnougeikagakukaishi,* **32(1):** 49–54 (in Japanese)
70. Landolfo S, Politi H, Angelozzi D, Mannazzu I. (2008) ROS accumulation and oxidative damage to cell structures in *Saccharomyces cerevisiae* wine strains during fermentation of high-sugar-containing medium. *Biochim. Biophys. Acta.*, **1780(6):** 892–898
71. Law BA, Liao X, Moore KS, Southard A, Roddy P, Ji R, Sculz Z, Bielawska A, Schulze PC, Cowart LA. (2018) Lipotoxic very-long-chain ceramides cause mitochondrial dysfunction, oxidative stress, and cell death in cardiomyocytes. *The FASEB Journal,*

32(3): 1403–1416

72. Leipelt M, Warnecke D, Zähringer U, Ott C, Müller F, Hube B, Heinz E. **(**2001) Glucosylceramide synthases, a gene family responsible for the biosynthesis of glucosphingolipids in animals, plants, and fungi. *J Biol Chem.,* **276:** 33621–33629
73. Levery S B. (2005) Glycosphingolipid Structure Analysis and Glycosphingolipidomics. *Methods in Enzymology.,* **405:** 300–369
74. Lill R, Hoffmann B, Molik S, Pierik AJ, Rietzschel N, Stehling O, et al. (2012) The role of mitochondria in cellular iron-sulfur protein biogenesis and iron metabolism. *Biochim. Biophys. Acta.,* **1823(9):** 1491–1508
75. Lill R, Srinivasan V, Muhlenhoff U. (2014) The role of mitochondria in cytosolic-nuclear iron-sulfur protein biogenesis and in cellular iron regulation. *Curr. Opin. Microbiol.,* **22**: 111–119
76. Lin Y, Zhang W, Li C, Sakakibara K, Tanaka S, Kong H. (2012) Factors affecting ethanol fermentation using *Saccharomyces cerevisiae* BY4742. *Biomass Bioenergy.,* **47:** 395–401
77. Luparia V, Soubeyrand V, Berges T, Julien,A, Salmon JM. (2004) Assimilation of grape phytosterols by *Saccharomyces cerevisiae* and their impact on enological fermentations. *Appl. Microbiol. Biotechnol.,* **65(1):** 25–32
78. Markham JE, Lynch DV, Napier JA, Dunn TM, Cahoon EB. (2013) Plant sphingolipids: function follows form. *Curr Opin Plant Biol.,* **16:** 350–357
79. Martınez-Moreno R, Morales P, Gonzalez R, Mas A, Beltran G. (2012) Biomass production and alcoholic fermentation performance of *Saccharomyces cerevisiae* as a function of nitrogen source. *FEMS Yeast Res.,* **12:** 477–485
80. Mason AB, Dufour JP. (2000) Alcohol acetyltransferases and the significance of ester synthesis in yeast. *Yeast,* **16(14):** 1287–1298

81. Merrill AH Jr, Liotta DC, Riley RT. (1996a) Fumonisins: fungal toxins that shed light on sphingolipid function. *Trends Cell Biol.*, **6a**: 218

82. Merrill AH Jr, Liotta DC, Riley RT. (1996b) In Handbook of Lipid Research Volume 8: Lipid Second Messengers, Bell RM (ed.) Willy New York

83. Merrill AH Jr, Sweeley CC (1996) In Biochemistry of Lipids, Lipiproteins and Membranes, Vol.31. Vance DE, Vance J (eds). Elsevier: Amsterdam

84. Moonjai N, Verstrepen KJ, Shen HY, Derdelinckx G, Verachtert H, Delvaux FR. (2003) Linoleic acid supplementation of a cropped brewing lager strain: effects on subsequent fermentation performance with serial repitching. *J. Inst. Brew.*,**109(3):** 262–272

85. Motomura S, Horie K, Kitagaki H. 2012) Mitochondrial activity of sake brewery yeast affects malic and succinic acid production during alcoholic fermentation. *J. Inst. Brew.*, **118(1):** 22–26

86. Neklesa TK, Davis RW. (2009) A genome-wide screen for regulators of TORC1 in response to amino acid starvation reveals a conserved Npr2/3 complex. *PLoS Genet.*, **5:** e1000515

87. Nimrichter L, Rodrigues ML. (2011) Fungal glucosylceramides: from structural components to biologically active targets of new antimicrobials. *Front Microbiol* **2:** 212

88. Novgorodov SA, Riley CL, Yu J, Keffler JA, Clarke CJ, Van Laer AO, Baicu CF, Zile MR,Gudz TI. (2016) Lactosylceramide contributes to mitochondrial dysfunction in diabetes. *J. Lipid Res.*, **57(4):** 546–562

89. Ohta K, Hayashida S. (1983) Role of tween 80 and monoolein in a lipid-sterol-protein complex, which enhances ethanol tolerance of sake yeasts. *Appl. Environ. Microbiol.*, **46(4):** 821–825.

90. Okamoto K, Kondo-Okamoto N, Ohsumi Y. (2009) Mitochondria anchored receptor Atg32 mediates degradation of mitochondria via selective autophagy. *Dev. Cell.*, **17(1):**

87–97

91. Okazaki T, Bell RM, Hannun YA. (1989) Sphingomyelin turnover induced by vitamin D3 in HL-60 cells. Role in cell differentiation. *J. Biol. Chem.,* **264(32):** 19076–19080.
92. Omura F. (2008) Targeting of mitochondrial *Saccharomyces cerevisiae* Ilv5p to the cytosol and its effect on vicinal diketone formation in brewing. *Appl. Microbiol. Biotechnol*., **78(3):** 503–513
93. Ooi BG, Lankford KR. 2009) Strategy for Adapting Wine Yeasts for Bioethanol Production. *Int J Mol Sci.*, **10(1):** 387–384
94. Patton JR, Lester RL. (1991) The phosphoinositol sphingolipids of Saccharomyces cerevisiae are highly localized in the plasma membrane. *J Bacteriol.,* **173 (10):** 3101–3108
95. Perpète P, Duthoit O, De Maeyer S, Imray L, Lawton AI, Stavropoulos KE, et al. (2006) Methionine catabolism in *Saccharomyces cerevisiae*. *FEMS Yeast Res.*, **6(1):** 48–56
96. Pinto M R, Rodrigues M L, Travassos L R, Haido R M, Wait R, Barreto-Bergter E. (2002) Characterization of glucosylceramides in *Pseudallescheria boydii* and their involvement in fungal differentiation. *Glycobiology,* **12:** 251–260
97. Pralle A, Keller P, Florin EL, Simons K, Horber JK. (2000) Sphingolipid-cholesterol rafts diffuse as small entities in the plasma membrane of mammalian cells. *J. Cell Biol.,* **148(5):** 997–1008
98. Reissig JL, Strominger JL, Leloir LF. (1955) A modified colorimetric method for the estimation of *N*-acetylamino sugars. *J. Biol. Chem.,* **217:**959–966
99. Rhome R, McQuiston T, Kechichian T, Bielawska A, Hennig M, et al. (2007) Biosynthesis and immunogenicity of glucosylceramide in *Cryptococcus neoformans* and other human pathogens. *Eukaryot. Cell.,* **6:** 1715–1726
100. Rittershaus PC, Kechichian TB, Allegood J, Merrill AHJ, Hennig M, et al. (2006)

Glucosylceramide is an essential regulator of pathogenicity of *Cryptococcus neoformans*. *J. Clin. Invest.*, **116:** 1651–1659

101. Rodrigues ML , Travassos LR, Miranda KR, Franzen AJ, Rozental S , de Souza W, Alviano CS, Barreto-Bergter E. (2000) Human antibodies against a purified glucosylceramide from Cryptococcus neoformans inhibit cell budding and fungal growth. *Infect. Immun.*, **68:** 7049–7060.
102. Rosenfeld E, Beauvoit B. (2003) Role of the non-respiratory pathways in the utilization of molecular oxygen by *Saccharomyces cerevisiae*. *Yeast*, **20:** 1115–1144
103. Russo, S.B., Ross, J.S. and Cowart, L.A. (2013) Sphingolipids in obesity, type 2 diabetes, and metabolic disease. *Handb. Exp. Pharmacol.*, **216:** 373–401
104. Saerens SM, Delvaux F, Verstrepen KJ, Van Dijck P, Thevelein JM, Delvaux FR. (2008) Parameters affecting ethyl ester production by *Saccharomyces cerevisiae* during fermentation. *Appl. Environ. Microbiol.*, **74(2):** 454–461
105. Saito K, Takakuwa N, Ohnishi M, Oda Y. (2006) Presence of glucosylceramide in yeast and its relation to alkali tolerance of yeast. *Appl. Microbiol. Biotechnol.*, **71(4):** 515–521
106. Sakakibara K, Eiyama1 A, Suzuki1 SW, Sakoh-Nakatogawa M, Okumura N, Tani M, Hashimoto A, Nagumo1 S, Kondo-Okamoto N, Kondo-Kakuta C, Asai E, Kirisako H, Nakatogawa H, Kuge O, Takao T, Ohsumi Y, Okamoto 1K. (2015) Phospholipid methylation controls Atg32-mediated mitophagy and Atg8 recycling. *EMBO J*., **34(21):** 2703–2719
107. Sakamoto M, Sakatani M, Ferdouse J, Hamajima H, Tsuge K, Nishimukai M, Yanagita T, Nagao K, Mitsutake S, Kitagaki H. (2017) Development of quantitative method for the contents of glycosylceramide contained in Japanese foods brewed with koji and its application. *J. Brew. Soc. Japan.*, **112(9):** 655-662 (in Japanese)
108. Sakurai Y, Lee T H, Shiota H. (1977) On the convenient method for glucosamine

estimation in *Koji*. *Agric. Biol. Chem.,* **41:** 619–624

109. Sakurai Y, Misawa S, Shiota H. (1985) Growth and respiratory activity of *Aspergillus oryzae* grown on solid state medium. *Agric. Biol. Chem.,* **49:** 745–750

110. Samp EJ. (2012) Possible roles of the mitochondria in sulfur dioxide production by lager yeast. *J. Am. Soc. Brew. Chem.,* **70(4):** 219–229

111. Sawada K, Kitagaki H. (2016) Residual mitochondrial transmembrane potential decreases unsaturated fatty acid level in sake yeast during alcoholic fermentation. *PeerJ,* **4:** e1552

112. Sawada K, Sato T, Hamajima H, Jayakody LN, Hirata M, Yamashiro M, Tajima M, Mitsutake S, Nagao K, Tsuge K, Abe F, Hanada K, Kitagaki H. (2015) Glucosylceramide contained in *koji* mold-cultured cereal confers membrane and flavor modification and stress tolerance to *Saccharomyces cerevisiae* during co-culture fermentation. *Appl. Environ. Microbiol.,* **81:** 3688–3698

113. Schorling S, Vallee B, Barz WP, Riezman H, Oesterhelt D. (2001) Lag1p and Lac1p are essential for the acyl-CoA-dependent ceramide synthase reaction in *Saccharomyces cerevisiae*. *Mol. Biol. Cell.,* **12:** 3417–3427

114. Scot R, Leonard S. (2006) New functions for amino acids: effects on gene transcription and translation. *Am. J. Clin. Nut.,* **83(2):** 500–507

115. Shiroma S, Jayakody LN, Horie K, Okamoto K, Kitagaki H. (2014) Enhancement of ethanol fermentation in *Saccharomyces cerevisiae* sake yeast by disrupting mitophagy function. *Appl. Environ. Microbiol.,* **80(3):** 1002–1012

116. Sideri TC, Willetts SA, Avery SV. (2009) Methionine sulphoxide reductases protect iron-sulphur clusters from oxidative inactivation in yeast. *Microbiology,* **155:** 612–623

117. Simanshu DK, Kamlekar RK, Wijesinghe DS, Zou X, Zhai X, Mishra SK, Molotkovsky JG, Malinina L, Hinchcliffe EH, Chalfant CE, Brown RE, Patel DJ. (2013) Non-vesicular

trafficking by a ceramide-1-phosphate transfer protein regulates eicosanoids. *Nature*, **500:** 463–467

118. Simons K, Ikonen E. (1997) Functional rafts in cell membranes. *Nature*, **387 (6633):** 569–572

119. Sims K J, Spassieva S D, Voit EO, Obeid L M. (2004) Yeast sphingolipid metabolism: clues and connections. *Biochem. Cell Biol.*, **82:** 45–61

120. Singh A, Na C, Silva LC, Prieto M, Futerman AH, et al. (2012) Membrane lipid topography controlled by sphingolipids regulates pathogenicity of *Cryptococcus neoformans*. *Cell Microbiol.*, **14:** 500–516

121. Singh A, Wang H, Silva LC, Na C, Prieto M, Futerman AH, Luberto C, Del Poeta M. (2012) Methylation of glycosylated sphingolipid modulates membrane lipid topography and pathogenicity of *Cryptococcus neoformans*. *Cell. Microbiol.*, **14(4):** 500–516

122. Speigel S, Merrill AH Jr. (1996) Sphingolipid metabolism and cell growth regulation. *FASEB J.*, **10(12):** 1388–97

123. Suenaga R, Yamane H, Tomonaga S, Asechi M, Adachi N, Tsuneyoshi Y, Kurauchi I, Sato H, Denbow DM, Furuse M. (2008) Central L-arginine reduced stress responses are mediated by L-ornithine in neonatal chicks. *Amino acids*, **35(1):** 107–113

124. Sullards M C, Lynch D V, Merrill Jr A H, Adams J. (2000) Structure determination of soybean and wheat glucosylceramides by tandem mass spectrometry. *J. Mass Spectrom.*, **35:** 347–353

125. Sutter BM, Xi Wu, Laxman S, Tu BP. (2013) Methionine inhibits autophagy and promotes growth by inducing the SAM-responsive methylation of PP2A. *Cell*, **154(2):** 403–415

126. Takagi H, Takaoka M, Kawaguchi A, Kubo Y. (2005) Effect of L-proline on sake brewing and ethanol stress in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.*

71(12): 8656–8662

127. Takahashi K, Izumi K, Nakahata E, Hirata M, Sawada K, Tsuge K, Nagao K, Kitagaki H. (2014) Quantitation and structural determination of glucosylceramides contained in sake lees. *J. Oleo Sci.,* **63(1):**15–23

128. Takakuwa N, Kinoshita M, Oda Y, Ohnishi M. (2002) Existence of cerebroside in *Saccharomyces kluyveri* and its related species. *FEMS Yeast Res.,* **2:** 533–53

129. Takakuwa N, Saito K, Ohnishi M, Oda Y. (2005) Determination of glucosylceramide contents in crop tissues and by-products from their processing. *Bioresour Technol*., **96(9):** 1089–92

130. Takakuwa N, Tanji M, Oda Y, Ohnishi M. (2002) Distribution of 9-methyl sphingoid base in mushrooms and its effects on the fluidity of phospholipid liposomes. *J. Oleo Sci.,* **51(11):**741–747

131. Tamura G, Tsunoda T, Kirimura J, Milyazawa S. (1952) The microbiological determination of amino acids in various rice proteins and sake. *Nihonnougeikagakukaishi,* **26(9):** 480–483 (in Japanese)

132. Taylor GT, Thurston PA, Kirsop BH. (1979) The influence of lipids derived from malt spent grains on yeast metabolism and fermentation. *J. Inst. Brew.,* **85:**219–227

133. Teresa GC, Carmen AA. (2008) Effect of the addition of different quantities of amino acids to nitrogen-deficient must on the formation of esters, alcohols and acids during wine alcoholic fermentation. *LWT-Food Sci. Technol.,* **41(3):** 501–508

134. Ternes P, Sperling P, Albrecht S, Franke S, Cregg JM, et al. (2006) Identification of fungal sphingolipid C9-methyltransferases by phylogenetic profiling. *J Biol Chem.,* **281:** 5582–5592

135. ter Schure EG, van Riel NA, Verrips WCT. (2000) The role of ammonia metabolism in nitrogen catabolite repression in *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.,*

24(1): 67–83

136. Tesniere C, Delobel P, Pradal M, Blondin B. (2013) Impact of nutrient imbalance on wine alcoholic fermentations: nitrogen excess enhances yeast cell death in lipid-limited must. *PLoS One,* **8 (4):** e61645

137. Thomas D, Surdin-Kerjan Y. (1997) Metabolism of sulfur amino acids in *Saccharomyces cerevisiae. Microbiol. Mol. Biol. Rev*., **61:** 503–532

138. Truman, J.P., Garcia-Barros, M., Obeid, L.M. and Hannun, Y.A. (2014) Evolving concept in cancer therapy through targeting sphingolipid metabolism. *Biochim. Biophys. Acta.,* **1841:** 1174–1188

139. Tsukahara T, Nakamura Y, Sato S, Tanaka Y. **(**1947) Brewing characteristics of Kyokai no. 6 and no. 7. *J. Brew. Soc. Jpn.,* **42:** 26–27

140. Vacaru AM, van den Dikkenberg J, Ternes P, Holthuis JC. (2013) Ceramide phosphoethanolamine biosynthesis in Drosophila is mediated by a unique ethanolamine phosphotransferase in the Golgi lumen. *J Biol Chem.,* **288:**11520–30

141. Verbelen PJ, Saerens SM, Van Mulders SE, Delvaux F, Delvaux FR. (2009) The role of oxygen in yeast metabolism during high cell density brewery fermentations. *Appl. Microbiol. Biotechnol.,* **82(6):**1143–1156

142. Vidal E E, de Morais Jr MA, François JM, de Billerbeck GM. (2015) Biosynthesis of higher alcohol flavour compounds by the yeast Saccharomyces cerevisiae: impact of oxygen availability and responses to glucose pulse in minimal growth medium with leucine as sole nitrogen source. *Yeast,* **32:** 47–56

143. Vos PJA, Gray RS. (1979) The origin and control of hydrogen sulfide during fermentation of grape must. *Am. J. Enol. Vitic.,* **30(3):** 187–197

144. Wainwright T. (1970) Hydrogen sulphide production by yeast under conditions of methionine, pantothenate or vitamin B6 deficiency. *J. Gen. Microbiol.,* **61(1):** 107–119

145. Wang XD, Bohlscheid JC, Edwards CG. (2003) Fermentative activity and production of volatile compounds by *Saccharomyces* grown in synthetic grape juice media deficient in assimilable nitrogen and/or pantothenic acid. *J. Appl. Microbiol.,* **94(3):** 349–359

146. Warnecke D, Heinz E. (2003) Recently discovered functions of glucosylceramides in plants and fungi. *Cell. Mol. Life Sci.,* **60:** 919–941

147. Watanabe D, Araki Y, Zhou Y, Maeya N, Akao T, Shimoi H. (2012) A loss-of-function mutation in the PAS kinase Rim15p is related to defective quiescence entry and high fermentation rates of *Saccharomyces cerevisiae* sake yeast strains. A*ppl. Environ. Microbiol.* **78:** 4008–4016

148. Watanabe D, Wu H, Noguchi C, Zhou Y, Akao T, Shimoi H. (2011) Enhancement of the initial rate of ethanol fermentation due to dysfunction of yeast stress response components Msn2p and/or Msn4p. *Appl. Environ. Microbiol.,* **77:** 934–941

149. Watanabe M, Tamura K, Magbanua JP, Takano K, Kitamoto K, Kitagaki H, Akao T, Shimoi H. **(**2007) Elevated expression of genes under the control of stress response element (STRE) and Msn2p in an ethanol tolerance sake yeast Kyokai no. 11. *J. Biosci. Bioeng.,* **104:**163–170

150. Waterhouse NJ, Goldstein JC, Ahsen von O, Schuler M, Newmeyer DD, Green DR. (2001) Cytochrome c maintains mitochondrial transmembrane potential and ATP generation after outer mitochondrial membrane permeabilization during the apoptotic process. *J. Cell Biol.,* **153(2)**: 319–328

151. Wennekes T, van den Berg RJ, Boot RG, van der Marel GA, Overkleeft HS, Aerts JM. (2009) Glycosphingolipids—nature, function, and pharmacological modulation. *Angew. Chem. Int. Ed. Engl*., **48:** 8848–69

152. Wu G. (2009) Amino acids: metabolism, functions, and nutrition. *Amino acids*, **37:** 1–

17.

153. Wu G, Bazer FW, Davis TA et al. (2007a) Important roles for the arginine family of amino acids in swine nutrition and production. *Livest. Sci.,* **112:** 8–22

154. Wu X, Tu B P. (2011) Selective regulation of autophagy by the lml1-Npr2-Npr3 complex in the absence of nitrogen starvation. *Mol. Biol. Cell*., **22:** 4124–4133

155. Xia J, Sinelnikov IV, Han B, Wishart DS. (2015) MetaboAnalyst 3.0--making metabolomics more meaningful. *Nucleic Acids Research.,* **43(W1):** 251–257

156. Xie G, Li W, Lu J, Cao Y, Fang H, Zou H, Hu Z. (2007) Isolation and identification of representative fungi from Shaoxing rice wine wheat Qu using a polyphasic approach of culture-based and molecular based methods. *J. Inst. Brew.,* **113:** 272–279

157. Yamamoto A, Yano I, Masui M, Yabuuchi E. (1978) Isolation of a novel sphingoglycolipid containing glucuronic acid and 2-hydroxy fatty acid from *Flavobacterium devorans* ATCC 10829. *J Biochem.,* **83:** 1213–6

158. Yamashita T, Wada R, Sasaki T, Deng C, Bierfreund U, Sandhoff K, Proia RL. (1999) A vital role for glycosphingolipid synthesis during development and differentiation. *Proc. Natl. Acad. Sci. U S A.,* **96(16):** 9142–9147

159. Yoshizaki Y, Yamato H, Takamine K, Tamaki H, Ito K, Sameshima YJ. (2010) Analysis of volatile compounds in shochu koji, sake koji, and steamed rice by gas chromatography-mass spectrometry. *J. Inst. Brew.,* **116(1):** 49–55

160. Zamzami N, Marchetti P, Castedo M, Decaudin D, Macho A, Hirsch T, et al.(1995) Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. *J. Exp. Med*., **182(2):** 367–377

161. Zhu, C., Wang, M., Wang, W., Ruan, R., Ma, H. et al. (2014) Glucosylceramides are required for mycelial growth and full virulence in *Penicillium digitatum*. *Biochem. Biophys. Res. Commun.,* **455:** 165–171