

# The Metabolites of Food Microorganisms

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## 1. Introduction

Both medicine and one's daily diet are equally important for health. Ingesting healthy foods every day may reduce the risk of diseases. One of the most important diets in Japan are traditional fermented foods, which possess appetizing flavors, nutritional benefits, and desirable biological activities formed during the fermentation process. Epidemiological studies suggest that the consumption of fermented products may be associated with a lower incidence of certain chronic disease, such as coronary heart diseases, atherosclerosis, and certain types of cancers. In recent years, macrobiotics (i.e. a macrobiotic diet) have been considered to be one of the most popular alternative or complementary comprehensive lifestyle approaches to chronic diseases. The longevity of Japanese people is derived from their diet, which is rich in fermented foods.

Research has found that the physiologically active metabolites of fermentation food starters, specifically long-chain terpenes, such as novel C35-terpenols, were found in a culture of *Bacillus subtilis* KSM 6-10 isolated from pickled vegetables (i.e. tsukemono in Japanese). The biological activities of these foods have been previously studied. In this chapter, we review the active components of fermented foods formed by the starter microorganisms, present our research on these active metabolites, and describe the methods by which to increase the yield of these active components.

## 2. Active components of fermented foods

Fermented foods have been consumed for centuries worldwide. By ingesting these foods, it may be possible to prevent and alleviate certain diseases, such as cancer and hypertension. According to epidemiological studies, many potential benefits have been linked to the intake of fermented foods.

### 2.1 Epidemiological studies

Some case-control studies were conducted to assess the association between breast cancer risk and fermented milk consumption (Pieter et al., 1989; Pryor et al.; 1989, Ronco et al., 2002). High intakes of whole and chocolate milk were associated with a significantly increased risk of breast cancer, whereas ricotta cheese and skim yoghurt were associated with a significantly decreased risk.

To evaluate whether a fermented dairy drink containing a probiotic strain could reduce the incidence of common infectious diseases (CIDs), as well as behavioral changes resulting from illness, in children, a double-blinded clinical trial was conducted in the Washington, DC metropolitan area. Six-hundred and thirty-eight children, aged 3-6 years old, that attended a daycare/school were enrolled in the study. The rate of behavioral changes resulting from illness was similar among the active and control groups. However, the incidence rate for CIDs in the active group was 19% lower than that of the control group. Thus, the daily intake of a fermented dairy drink containing a probiotic strain showed some promise in reducing the overall incidence of illness (Merenstein et al., 2010).

One of the most popular and traditional foods in Japan are fermented soybeans (i.e. natto in Japanese). In a large representative cohort study, the association between habitual natto intake and bone mineral density (BMD) was assessed in 944 healthy Japanese women. It was found that there was a significant positive association between natto intake and the rate of change in BMD assessed at the femoral neck ( $P < 0.0001$ ) (Ikeda et al., 2006). Interestingly, there were no significant associations observed between the intake of tofu (non-fermented soybean curd) or boiled soybeans and the rate of change in BMD in postmenopausal women. It was suggested that natto intake may prevent postmenopausal bone loss via the effects of menaquinone or bioavailable isoflavones, which were more abundant in natto than in other soybean product.

The Rotterdam research group examined whether the dietary intake of menaquinone (vitamin K-2) was related to aortic calcification and coronary heart disease (CHD). Their findings suggested that an adequate intake of menaquinone may be important in the prevention of CHD (Johanna et al., 2004).

Recently, active components with desirable effects, such as antihypertensive, antioxidative, and platelet aggregation inhibiting activities, have been isolated from fermented foods. In the subsequent section, some of the active metabolites of starter microorganisms are reviewed, and our research on fermentation products is introduced.

## 2.2 Menaquinones

Vitamin K is one of the fat soluble vitamins, which exists as either vitamin K1 (phylloquinone) in green plants or vitamin K2 (menaquinone) in animals and bacteria. Vitamin K is necessary for the posttranslational modification of certain proteins and blood coagulation. Menaquinones are classified according to the length of their aliphatic side chain, and are designated as MK-n (see Fig. 9). MK-7 is abundant in cheese or fermented soybeans (natto in Japanese). A Natto containing a high amount of MK-7, which is produced by a special starter (i.e. *Bacillus subtilis* 35 OUV 23481), is an approved food product for its specified health use by the National Health and Nutrition Research in Japan.

## 2.3 Lactotriptides

Angiotensin I-converting enzyme (ACE) is an important enzyme in the regulation of blood pressure, as it catalyzes the formation of a potent vasopressor, angiotensin II, from angiotensin I. Two peptides (i.e. Val-Pro-Pro [VPP] and Ile-Pro-Pro [IPP]) that possess ACE inhibiting properties have been isolated from a type of sour milk fermented with

*Lactobacillus helveticus* and *Saccharomyces cerevisiae* (Nakamura et al., 1995). These tripeptides are barely digested by digestive enzymes, suggesting that, despite their oral administration, these tripeptides remain intact within the intestine and retain their antihypertensive activity until absorption (Ohsawa et al., 2008). Indeed, these peptides demonstrated significant antihypertensive effects in clinical studies (Hata et al., 1996). Although these tripeptides were not generated from beta-casein via human gastrointestinal enzymes, they were produced in a fermented soybean paste (miso in Japanese) by adding casein (Inoue et al., 2009). Two proteolytic enzymes, which are capable of releasing the tripeptides from casein, were identified in *Aspergillus oryzae*, one of the starters in fermented foods, such as miso (Gotou et al., 2009).

In sour milk, the production of antihypertensive peptides (VPP and IPP) is limited during *Lactobacillus helveticus* fermentation, as most of the casein remains unprocessed. To improve the production of these peptides, carboxypeptidase was added with *Lactobacillus helveticus* CM4 to process the C-terminal ends of the precursor peptides (i.e. VPP-Xxx and IPP-Xxx) and form VPP and IPP. The amount of tripeptides yielded was 60 mg/L (Ueno et al., 2004).

## 2.4 Isoflavones

Japanese-style fermented soy sauce (shoyu in Japanese) is a typical traditional fermented food, and its functional effects have also been previously studied (Kataoka 2005). Some active components (i.e. metabolites), which were not present in the original raw materials such as boiled soybean and wheat, were isolated from fermentation products.

### 2.4.1 Shoyuflavones

Three tartaric isoflavone derivatives were found in soy sauce (Kinoshita et al., 1997). These isoflavones have not been previously found in any other soy products. They demonstrate inhibitory activities against histidine decarboxylase, resulting in the production of histamine from L-histidine (Kinoshita et al., 1998). Histamine is a mediator of inflammation, allergies, gastric acid secretions, and neurotransmission.

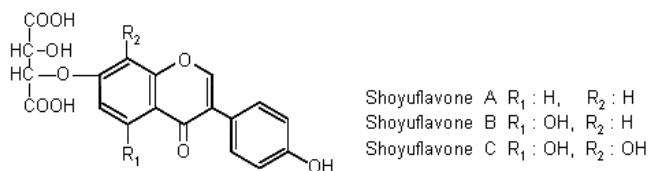


Fig. 1. Structures of shoyuflavones.

### 2.4.2 Orobol (5,7,3',4'-tetrahydroxyisoflavone)

Tempeh is a fermented soybean product originating from Indonesia, which is also popular outside of Indonesia, as it is odorless. European researchers have isolated several isoflavones, including 5,7,4'-trihydroxyisoflavone (genistein), 7,4'-dihydroxyisoflavone (daidzein), and a novel one, 5,7,3',4'-tetrahydroxyisoflavone (orobol), from a tempeh extract

(Kiriakidis et al., 2005). The effects of these isoflavones on angiogenesis were evaluated using a chicken chorioallantoic membrane assay, and it was found that these isoflavones reduced angiogenesis by 49-75% compared to the negative control (6.3%). It was suggested that these isoflavones should be added to the list of low molecular mass therapeutic agents for the inhibition of angiogenesis.

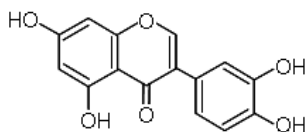


Fig. 2. Structure of orobol (5,7,3',4'-tetrahydroxyisoflavon).

### 2.4.3 6-hydroxydaidzein and 8-hydroxyglycitein

8-Hydroxyglycitein and 6-hydroxydaidzein were isolated from a soybean paste (miso in Japanese), and were found to act as 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavengers (Hirota et al., 2004). These compounds demonstrated DPPH-radical scavenging activity that was as high as that of  $\alpha$ -tocopherol, 8-hydroxygenistein, and 8-hydroxydaidzein (Fig. 3). To our best knowledge, this was the first report on the isolation of 8-hydroxyglycitein from a natural source.

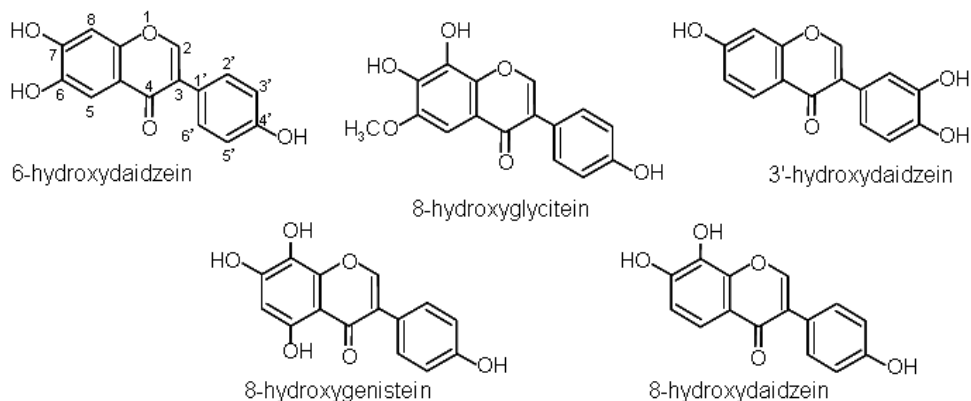


Fig. 3. Structures of hydroxyisoflavones.

### 2.4.4 3'-hydroxydaidzein

Dou-chi, a traditional soybean food fermented with *Aspergillus* sp., is usually used as a seasoning in Chinese food, and has been also used as a folk medicine in both China and Taiwan. Four phenol compounds, one isoflavanone, eight isoflavones, and one 4-pyrone were isolated from dou-chi (Chen et al., 2005). Among these compounds, 3'-hydroxydaidzein, dihydrodaidzein, and the 4-pyrone compound have not yet been isolated from soybean paste (miso). The structure assigned to the novel 4-pyrone compound was 3-((E)-2-carboxyethenyl)-5-(4-hydroxyphenyl)-4-pyrone-2-carboxylic acid.

## 2.5 Beta-carbolines

The diethyl ether extract of soy sauce was found to inhibit platelet aggregation induced by collagen and epinephrine. Specifically, the active components were 1-methyl-1,2,3,4-tetrahydro-beta-carboline (MTBC) and 1-methyl-beta-carboline (MBC) (Tsuchiya et al., 1999). The concentrations of MTBC and MBC in commercially available soy sauces are 28-85 ppm and 0.3-4.2 ppm, respectively. MTBC required mean concentrations of 4.6, 4.2, 28.6, 11.6, and 65.8  $\mu\text{g}/\text{mL}$  to produce a 50% inhibition of the maximal aggregation response induced by epinephrine, platelet-activating factor, collagen, adenosine 5'-diphosphate, and thrombin, respectively. Thus, soy sauce may be a functional seasoning with a potent preventive effect on thrombi formation.

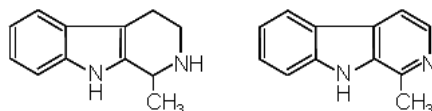


Fig. 4. Structures of beta-carboline derivatives.

## 2.6 Hydroxy-furanones

4-Hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone (HEMF), isolated from the ethyl acetate extract of soy sauce, is a shoyu-like flavor component that is also a potent antioxidant and anticarcinogen (Nagahara et al., 1992). Soy sauce contains other structurally similar flavor components, specifically, 4-hydroxy-5-methyl-3(2H)-furanone (HMF) and 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF) (Fig. 5). HDMF and HMF are thought to form chemically via the Maillard reaction between sugars and amino acids during the heating process. These furanones were investigated for their antioxidative activities. HMF and HDMF, as well as HEMF, were confirmed to have antioxidative properties. The order of potency was as follows: HEMF > HDMF > HMF. Furthermore, HEMF and HDMF are more potent than ascorbic acid.

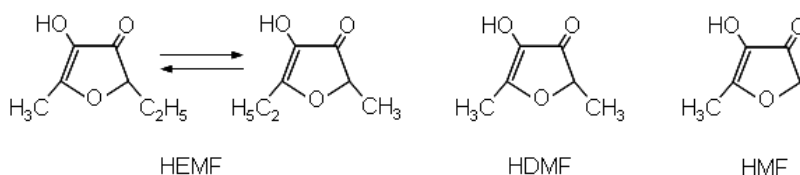


Fig. 5. Structures of 4-Hydroxy-2-ethyl-5-methyl-3-furanone and the related compounds.

## 2.7 Gamma-aminobutyric acid (GABA)

Gamma-aminobutyric acid (GABA) is a non-proteinaceous amino acid formed from the decarboxylation L-glutamate via glutamate decarboxylase (EC 4.1.1.15) (Fig. 6). GABA is a major inhibitory neurotransmitter in the mammalian central nervous system, and has several well-known physiological functions, such as the induction of hypotension and secretion of insulin from the pancreas.

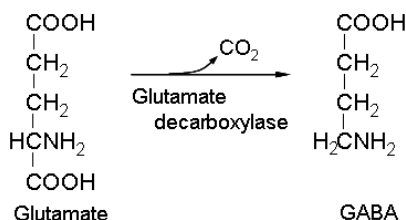


Fig. 6. Structure of  $\gamma$ -aminobutyric acid (GABA).

The blood pressure effects of a fermented milk product containing GABA were evaluated in patients with mild hypertension (Inoue et al., 2003). The study comprised of 39 mildly hypertensive patients (16 women and 23 men), aged 28 to 81 years (mean: 54.2 years). The patients received a daily intake of GABA or placebo for 12 weeks followed by a 2-week treatment-free period (weeks 13 and 14). There was a  $17.4 \pm 4.3$  mmHg decrease in systolic blood pressure and  $7.2 \pm 5.7$  mmHg decrease in diastolic blood pressure in patients receiving GABA. Thus, GABA may be used to lower blood pressure in mildly hypertensive individuals.

Another study evaluated the antihypertensive effects of *Lactobacillus*-fermented milk that was orally administered to spontaneously hypertensive rats (Liu et al., 2011). It was found that eight hours after administering milk fermented with either *Lactobacillus paracasei* subsp. *paracasei* NTU 101 or *Lactobacillus plantarum* NTU 102 containing GABA, there was a significant decrease in the systolic and diastolic blood pressures of these hypertensive rats.

A simple fermentation process was developed to yield a high amount of GABA (Cock et al., 2010). Specifically, cultivating *Lactobacillus sakei* B2-16 in rice bran extract yields a maximum GABA concentration of 660.0 mM, which is 2.4-fold greater than that achieved without the rice bran extract. Furthermore, a simple and effective fed-batch fermentation process was developed to efficiently convert glutamate into GABA (Li et al., 2010). The GABA concentration obtained with this process was  $1005.81 \pm 47.88$  mM, and the residual glucose and glutamate concentration were  $15.28 \pm 0.51$  g/L and  $134.45 \pm 24.22$  mM after 48 h.

## 2.8 Polyprenols

Polyprenols are natural long-chain isoprenoid alcohols with a general formula of  $\text{H}-(\text{C}_5\text{H}_8)_n\text{-OH}$ , where  $n$  is the number of isoprene units. Polyprenols serve as sugar carriers in biosynthetic processes that include protein glycosylation and lipopolysaccharide biogenesis, and are found in small quantities in various plant tissues and microorganism cells. Dolichols, which are found in all living organisms, including humans, are their 2,3-dihydro derivatives (Rezanka et al., 2001). Polyprenols are low molecular natural bioregulators, which are physiologically active, and play a significant modulating role in the cellular process of plants, specifically biosynthesis. The dolichol phosphate cycle facilitates the process of cellular membrane glycosylation, that is, the synthesis of glycoproteins that control cell interactions, support the immune system, and stabilize protein molecules. Out of all these glycoproteins, polyglycoprotein has the capacity to kill cancer cells during chemotherapy, while protecting healthy cells within the body. Polyprenols stimulate the immune system, cellular respiration and spermatogenesis, and possess anti-stress,

adaptogenic, anti-ulcerogenic, and wound-healing properties. Dolichols have antioxidative properties, and protect cell membranes from peroxidation. In the subsequent section, our research on polyprenols isolated from food microorganisms will be discussed.

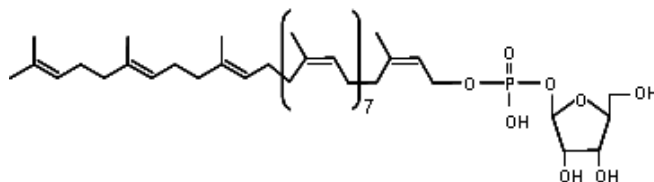


Fig. 7. Structure of a sugar carrier.

### 2.8.1 Isolation of metabolites from *Bacillus subtilis* KSM6-10

*Bacillus subtilis* KSM 6-10 was isolated from a traditional pickled vegetable found in Japan (tsukemono in Japanese). Based on its 16S rDNA sequence, which was compared directly to all known sequences within the GenBank databases via the basic local alignment search tool (BLAST), the strain was identified to belong to the genus *Bacillus*.

The procedure for isolating the metabolites of *Bacillus subtilis* KSM 6-10 is presented in Fig. 8. Briefly, without adjusting the pH of the culture broth (pH 7.5-8.0), the entire broth was

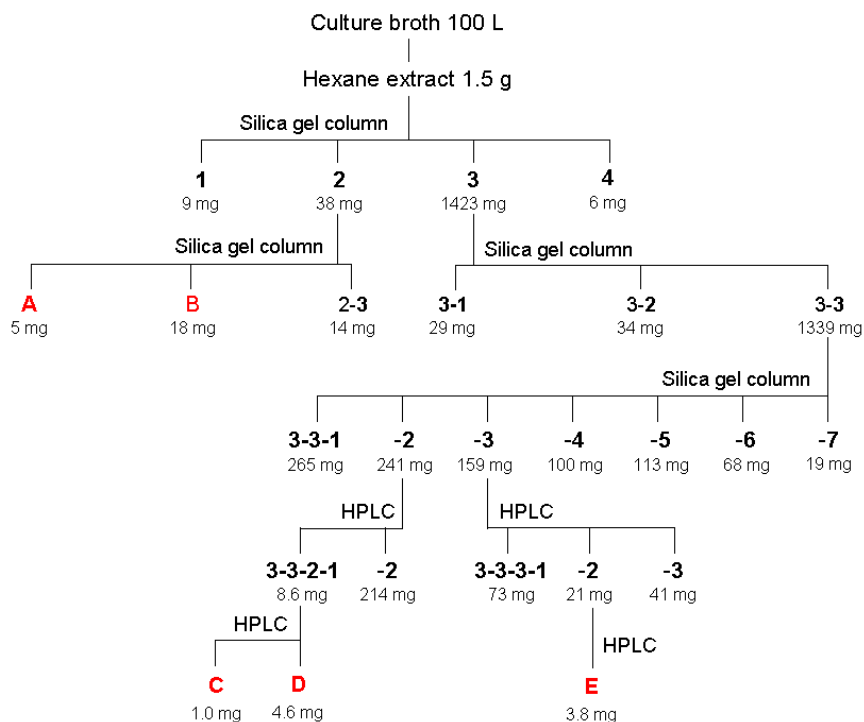


Fig. 8. The procedure for isolating terpenes (A-E).

extracted with EtOAc (2 × 5L) to yield 50 mg of a pale brown material. The EtOAc extract was separated by liquid-liquid extraction (50/50; *n*-hexane/90% aqueous MeOH). Then, the *n*-hexane extract (1.5 g) was fractionated using a High-Flash column (16 × 60 mm, Yamazen, Osaka, Japan) via a gradient elution from *n*-hexane to EtOAc, which yielded 4 fractions (20 mL each). Fraction 2 (38 mg) was further fractionated similarly as described above to yield three fractions. Fraction 2-1 produced 5 mg of pure compound **A** and fraction 2-2 produced 18 mg of pure compound **B**. Fraction 3 (1423 mg) was further fractionated in a similar fashion described above to yield three fractions. Then, fraction 3-3 (1339 mg) was further fractionated to yield seven fractions, and fraction 3-3-2 (241 mg) was further fractionated via gradient preparative HPLC (20% to 100% CH<sub>3</sub>CN in H<sub>2</sub>O) to yield two fractions. Fraction 3-3-2-1 (9 mg) yielded 1 mg of pure compound **C** and 4.6 mg of pure compound **D**. Fraction 3-3-3-2 (21 mg) yielded 3.8 mg of pure compound **E** (Takigawa et al., 2010).

### 2.8.2 Metabolites of *Bacillus subtilis* KSM6-10

Compounds **A** and **B**, tetraprenyl- $\beta$ -curcumene and tetraprenyl- $\alpha$ -curcumene, respectively, were previously isolated from a spore preparation of the same species (Boroczky et al., 2006), whereas compounds **C** and **D** are novel C<sub>35</sub>-terpenoids. Compound **E** was identified as undecaprenol (C<sub>55</sub>; bactoprenol) ((Rezanka et al., 2001)).

The proposed biosynthesis pathway of the five terpenes (**A**-**E**) is presented in Fig. 9. Mevalonic acid is a key precursor in the pathway, which via the mevalonate pathway produces terpenes

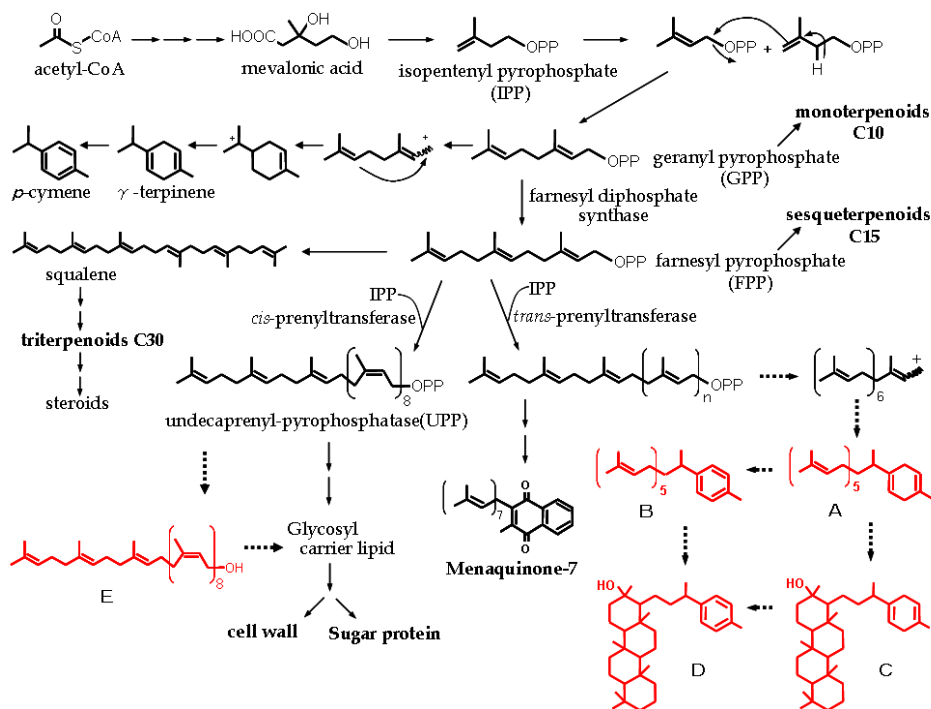


Fig. 9. The proposed biosynthesis pathway of terpenoids.



and steroids. Since the C35-terpenols (C and D) possess polycyclic skeletons, they are thought to be formed via the cyclization of acyclic C35-terpenes (A and B).

Recently, a new terpene cyclase, which is capable of forming pentacyclic C35 terpenols (C and D) from acyclic C35-terpenes (A and B), was purified from a standard strain of *Bacillus subtilis* (Sato et al., 2011). Since our isolate, *Bacillus subtilis* KSM 6-10, showed a 99.8% sequence homology to the standard strain of *Bacillus subtilis*, we are currently studying whether the terpene cyclase of *Bacillus subtilis* KSM 6-10 is homologous to the reported enzyme (Sato et al., 2011).

### 2.8.2.1 Cell proliferation activity

The cell proliferation activity of the five terpenes (A-E) were assayed in co-cultured hair follicle dermal papilla cells (HFDPC: Cell Applications Inc. USA) and human epidermal keratinocytes (HKC: Invitrogen Corp. USA) (Yuspa et al., 1993). The total amount of bromodeoxyuridine (BrdU) incorporated into the DNA of the monolayer of co-cultured cells was measured with an enzyme-linked immunosorbent assay (ELISA) (Muir et al., 1990). Following a dilution step, compounds A-E (1 mg/ml) were assayed (Fig. 10). Compound E (undecaprenol) was found to be the most active in the co-cultured cells.

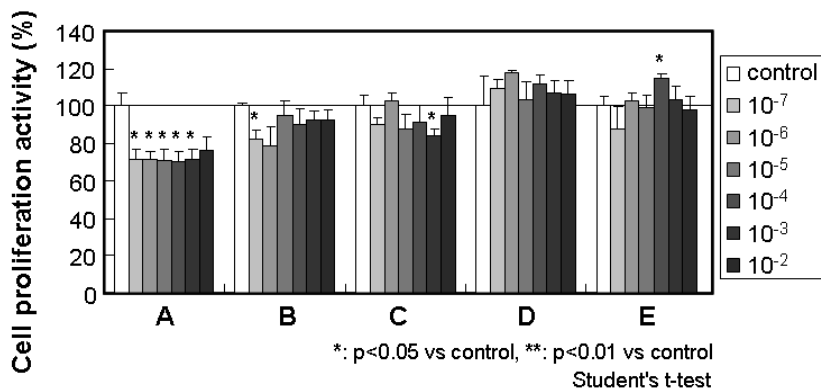


Fig. 10. Cell proliferation activities of various concentrations of compounds A-E.

### 2.8.2.2 Tyrosinase inhibition

A mushroom tyrosinase inhibition assay was previously conducted using L-dopamine (L-DOPA) as the substrate, and ascorbic acid as the positive control (Lee et al., 2002). Dopacrome formation was measured at 490 nm using a 96-well reader. The assay results are presented in Fig. 11. It was found that, although the C35 terpenes (A, B) did not inhibit tyrosinase, the polyprenols (C, D, E) demonstrated a mild inhibitory effect (Fig. 11).

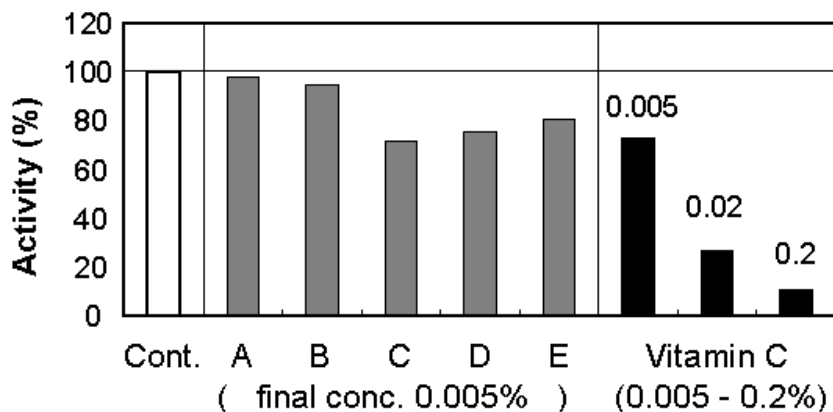


Fig. 11. Tyrosinase inhibition activities of compounds A-E.

### 2.8.2.3 The production of farnesol

Polyprenols are polyisoprenoid alcohols that contain anywhere from 5 to 25 or more multiprenyl chains with a hydroxyl group at the end (Fig. 12). They are present in all living cells, and referred to as dolichol (C75-C115), bactoprenol (C55), and ficaprenol (C50-C145) (Rezanka et al., 2001). Polyprenyl phosphates are essential intermediates that act as lipid carriers in several biochemical pathways, including N-linked protein glycosylation and cell wall biosynthesis. Certain polyprenols are suggested to be biomarker for aging (Parentini et al., 2005).

Farnesol (C15) is a major fragrance component found in the flowers of many plants, and it is a common intermediate in several essential components, such as sterols and quinones (see Fig. 9). Additionally, it has been utilized as a starting material in synthetic pharmaceuticals. Recently, derivatives of farnesol have become candidates in anti-cancer reagents, as they induce apoptosis in various tumor cell lines (Gibbs et al., 1999; Burke et al., 2002). Thus, recent research efforts are aimed at enhancing the production of polyprenols.

A mutant strain auxotrophic for ergosterol, which blocks farnesyl diphosphate synthase, was reported to produce a low concentration of farnesol (1 mg/L) (Chambon et al., 1990). Although the amount of farnesol produced by the squalene synthase-deficient mutant *Saccharomyces cerevisiae* ATCC 64031 was low (4-6 mg/L), combining glucose with soybean oil results in more than 28 mg/L of farnesol in the soluble fraction of the broth. Thus, this method allows for the over-production of hydrophobic and useful compounds, such as tocopherol and carotenoids. Furthermore, an alkaline pH (7-8) also enhances farnesol production, where it was reported that a concentration of 102.8 mg/L was produced in a jar fermentor (Muramatsu et al., 2008; 2009).

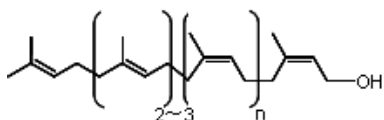


Fig. 12. Structure of a polyprenol.

2.8.2.4 The production of polyprenols from *B. subtilis* KSM6-10 (C-E)

Using a metabolic regulatory fermentation process rather than a genetic modification, we have previously determined a way of increasing the amounts of our isolated polyprenols (C-E). Briefly, *B. subtilis* KSM 6-10 was grown for 2 days at 30°C in a 500-ml flask containing 100 ml of K medium (Takigawa et al., 2010). The whole broth was then extracted with n-hexane, and the extract was analyzed via gas chromatography (GC) (Fig. 13).

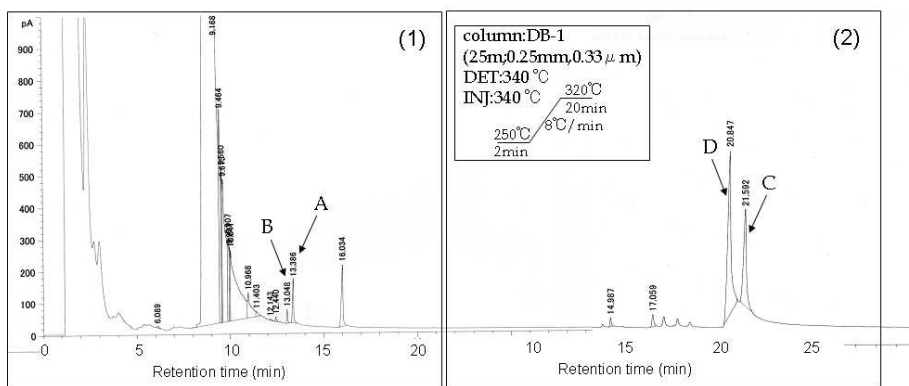


Fig. 13. Gas chromatographic analysis of isolate A and B (1), and C and D (2).

Of the additives tested, we found that monoterpenes, such as *p*-cymene and β-myrcene, enhanced the production of C35-terpenes (A and B). Three-hundred ppm of *p*-cymene demonstrated the most prominent effect, which was then further accelerated by the addition of a high concentration of glucose or yeast extract in the medium (Fig. 14).

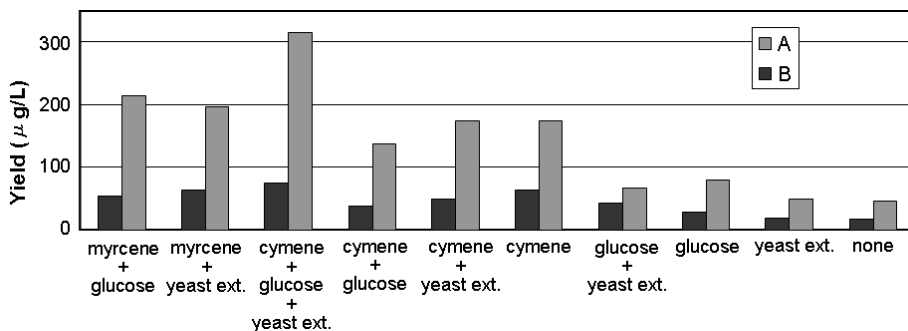


Fig. 14. The effects of additives on the production of compounds A and B.

In our preliminary studies, however, we were not able to enhance the production yields of polyprenols (C-E). Given that prenyltransferase present in *Bacillus subtilis*, which synthesizes undecaprenyl pyrophosphate (see Fig. 9), is stimulated markedly by the addition of monovalent cations, such as K<sup>+</sup> and NH<sub>4</sub><sup>+</sup> (Takahashi et al., 1982), changing the composition of the liquid medium may increase the amount of polyprenols (C-E) produced. In particular, the addition of KH<sub>2</sub>PO<sub>4</sub> into the K medium during the mid-log phase of the liquid culture of *B.subtilis* KSM 6-10 may enhance the production of undecaprenyl pyrophosphate synthase, including the amount of polyprenol E produced. This is currently being studied by our research group.

## 2.9 Sake lees

The traditional Japanese alcoholic beverage (Sake in Japanese) is produced from steamed rice by the simultaneous addition of two microorganisms, *Saccharomyces cerevisiae* and *Aspergillus oryzae* (koji in Japanese). Sake lees (sake kasu in Japanese) is a fine sediment left after the sake filtering process. While a small amount of sake lees is used as a pickling agent for making fermented vegetables (Tsukemono in Japanese), as well as a food material, most of it is considered industrial waste. Recently, the biological activities of sake lees have been investigated. For example, sake less has been shown to inhibit ACE (Saito et al., 1994) and tyrosinase (Jeon et al., 2006). We have recently been assessing whether sake lees has other potential biological activities.

### 2.9.1 Peroxisome-proliferator activated receptors (PPAR)

Nuclear peroxisome proliferator-activated receptors (PPAR) have been shown to play critical roles in the regulation of energy homeostasis, including lipid and carbohydrate metabolism, inflammatory responses, and cell proliferation, differentiation, and survival. Since PPAR agonists have the potential to prevent or ameliorate diseases, such as hyperlipidemia, diabetes, atherosclerosis, and obesity, we have investigated whether the certain food metabolites can act as natural agonists for PPAR.

#### 2.9.1.1 Peroxisome proliferator-activated receptor luciferase assay

The pBIND-GAL4-PPAR $\alpha$ LBD and pBIND-GAL4-PPAR $\delta$ LBD chimeric expression plasmids were prepared, as previously described (Murase et al., 2006). The pG5luc reporter plasmid with the GAL4 binding site was obtained from Promega (Madison, WI). The African green monkey fibroblast cell line CV-1 was obtained from Riken Cell Bank (Tsukuba, Japan). Following a day of cultivation in DMEM, CV-1 cells were transfected using Superfect transfection reagent (QIAGEN, Valencia, CA). The cells were incubated in a transfection mixture containing 6.25  $\mu$ l of SuperFect, 0.375  $\mu$ g of pBIND-GAL4-PPAR-LBD expression plasmid, and 0.375  $\mu$ g of pG5luc reporter plasmid for 3 h at 37°C. They were then incubated for 4 h in fresh DMEM (+5% charcoal-treated FBS). After treatment with or without each sample for 20 h, cells were lysed, and then firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). Wy-14643 (SIGMA) was used as a positive control for PPAR $\alpha$  and GW-501516 (Wako) for PPAR $\delta$ .

#### 2.9.1.2 The effects of sake lees extracts on PPAR activation

Although both Wy-14643, a PPAR $\alpha$  agonist, and GW-501516, a PPAR $\delta$  agonist, significantly enhanced PPAR-dependent luciferase activities, sake lees extracts (0.02%) also demonstrated

a marked effect (Fig. 15). It appears that all of the test samples contained direct ligands for PPAR $\alpha$  and PPAR $\delta$ . Of the sake lees extracts tested, only the water extract demonstrated no activity, suggesting that there are certain hydrophobic components that are related to PPAR activation. Currently, we are in the process of isolating the hydrophobic ligands from sake lees, and hope to report on these active components in the near future.

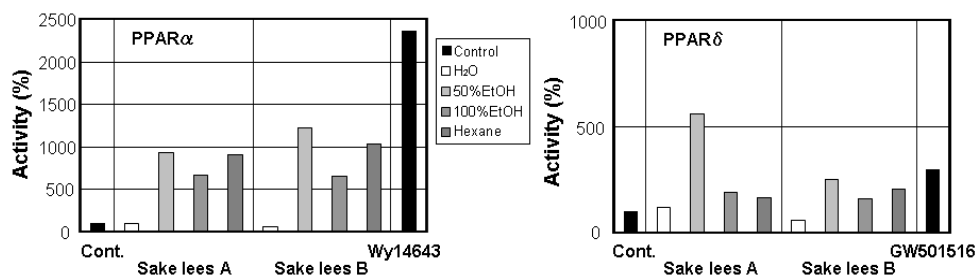


Fig. 15. PPAR activation with sake lees extracts.

### 2.9.2 Antioxidative effects of sake lees

The antioxidative effects of sake lees extracts were evaluated (Fig. 16). Briefly, the intracellular formation of reactive oxygen species was detected with a fluorescence probe, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (5,6-CM-H<sub>2</sub>DCFDA; Invitrogen). Leukocytes were isolated from male Sprague-Dawley rats (i.e. 10-16 weeks old) and then cultured in the presence of each test samples for 1 h at room temperature. The samples were then loaded with 10  $\mu$ M 5,6-CM-H<sub>2</sub>DCFDA for 20 min, and evaluated via flow cytometry. Of all of the sake lees extracts, the hexane extract demonstrated a significantly decrease in ROS generation, suggesting the presence of antioxidative activity. It is recognized that the antioxidative properties of sake lees result from their hydrophilic compounds, such as ferulic acid and vanillin, which originate from sake. Given that the hydrophobic components with antioxidative properties have not yet been isolated from a sake lees, we are currently attempting to isolate these active components.

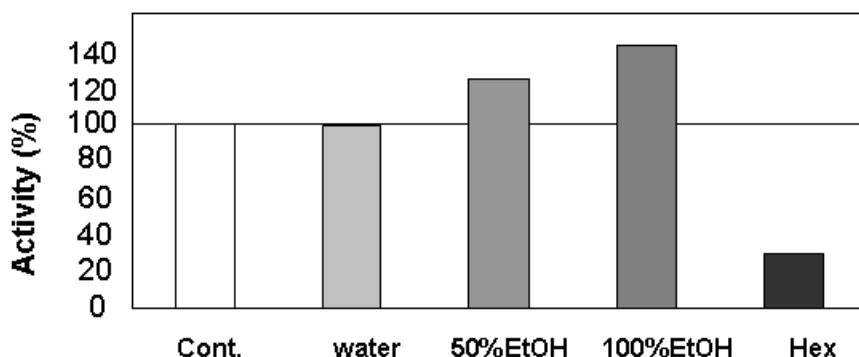


Fig. 16. Antioxidative effects of sake lees extracts.

### 2.9.3 Antihypertensive activity

The effects of hexane extract of sake lees on blood pressure was investigated in spontaneously hypertensive rats (SHR; 9 weeks old). Specifically, the tail blood pressure was measured using an indirect blood pressure meter (BP-98A, Softron) at 0, 1, 3, 6, and 24 h after a single intravenous injection of the hexane extract of sake lees (100 mg/kg) (Fig. 17). We also confirmed that the hexane extract did not have an effect on the blood pressures of normal Sprague-Dawley rats (data not shown). The active compound appears to be hydrophobic, and different from the other compounds (Saito et al., 1994).

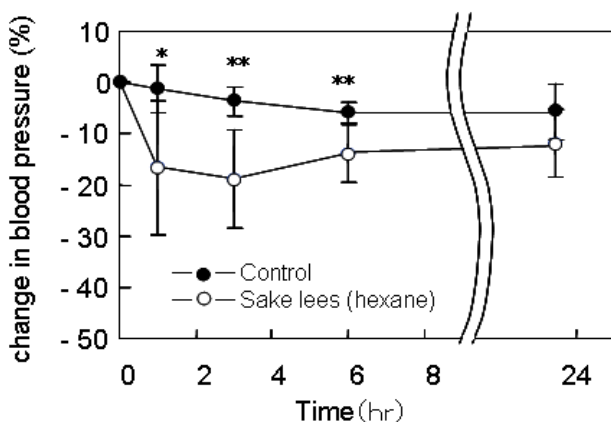


Fig. 17. The antihypertensive effects of the hexane extract containing sake lees.

### 2.10 Other biological activities

In many Asian countries, there are numerous traditional fermented foods other than tempeh, natto, and dou-chi. For example, kimchi (a fermented vegetable in Korea), fish sauce (Nam pla in Thailand, and Nuoc mam in Vietnam), and fermented tea (Oolong and Pu-erh) all warrant further investigation in regards to their potential biologically active metabolites.

## 3. Conclusion

Fermented foods contain various metabolites that are produced by their starter microorganisms, and are reported to have many desirable activities, for example antihypertensive (GABA and lactotriptides), antioxidative (isoflavones), and anticancer (polyprenols) properties. Since they are not digested within the intestine, they may present their biological activities after absorption. By regularly ingesting these foods, it may be possible to prevent and alleviate certain types of diseases.

#### 4. Acknowledgment

We hope that Asian fermented foods will become more popular and contribute to improving the health of individuals worldwide. Finally, we deeply appreciate the editorial board for providing us with the opportunity to discuss the use of fermented foods.

#### 5. References

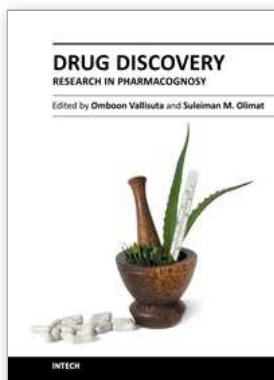
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