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Characteristics and Role of Feruloyl Esterase from *Aspergillus Awamori* in Japanese Spirits, 'Awamori' Production

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1. Introduction

Feruloyl esterases (EC 3.1.1.73), known as ferulic acid esterases, which are mainly from *Aspergillus* sp. (Faulds & Williamson, 1994), can specifically cleave the (1→5) ester bond between ferulic acid and arabinose. The esterases show high specificity of hydrolysis for synthetic methyl esters of phenyl alkanoic acids (Kroon and others, 1997). The reaction rate increases markedly when the substrates are small soluble feruloylated oligosaccharides derived from plant cell walls (Faulds and other, 1995; Ralet and others, 1994).

These enzymes have high potential for application in food production and other industries. Ferulic acid links hemicellulose and lignin. In addition, cross-linking of ferulic acids in cell wall components influences wall properties such as extensibility, plasticity, and digestibility, as well as limiting the access of polysaccharides to their substrates (Borneman et al., 1990).

Actually, feruloyl esterase is used for *Awamori* spirit production. *Awamori* spirits are Japanese spirits with a distinctive vanilla-like aroma. Feruloyl esterase is necessary to produce that vanilla aroma. Actually, lignocellulosic biomass is one means of resolving energy problems effectively. It is an important enzyme that produces bio-fuel from lignocellulosic biomass.

As explained in this paper, *Awamori* spirit production is described as an application of feruloyl esterase. The vanillin generating pathway extends from ferulic acid as precursor, with isolation of *Aspergillus* producing feruloyl esterase, which is characteristic of the enzyme. Moreover, the application of feruloyl esterase for beer production and bio-fuel production is explained.

2. *Awamori* spirits

2.1 *Awamori* spirit characteristics

Awamori spirits have three important features. First, mash of *Awamori* spirit is fermented using *koji*, *Aspergillus* sp. are grown on steamed rice, which is the material and saccharifying agent used in *Awamori* spirit production. That fermentation is done in a pot still. Mash used in *Awamori* spirit processing is different from beer brewing, in which fermenting is done with saccharified mash by malt. Their fermentative form is called 'parallel fermentation' which progresses simultaneously with saccharification and fermentation. The resultant

fermentative yeast can produce high concentrations of ethanol, approximately 16–18%, from mash of *Awamori* without osmotic injury.

Secondly, highly concentrated citric acid is produced in this process from *koji* made by black *Aspergillus* sp., classified as *Aspergillus awamori*. Because of this acid, the mash maintains low pH. It is usually made in the warm climate of Okinawa, with average temperatures of 25°C in all seasons. Koizumi (1996) describes that spoiling bacteria are able to grow in mash under pH 4.0 conditions. Moreover, although amylase from *Aspergillus oryzae* is inactivated at less than pH 3.5, that from *Aspergillus awamori* reacts stably at pH 3.0. Furthermore, the mash ferments soundly under those warm conditions.

Finally, aging is an important feature of *Awamori* spirits, which have a vanilla aroma that strengthens during aging. The *Awamori* spirit is aged in earthen pots for three years or more. Particularly, the spirit aged for more than three years, called ‘*Kusu*’, is highly prized. The vanilla aroma in Scotch whisky, bourbon, or brandy is produced from lignin in barrel wood during aging. *Kusu* is not aged in barrels, but it does have a vanilla aroma resembling those of aged Scotch whisky, bourbon, and brandy.

Differences between *Awamori* spirit and other beverages are shown in the table. History and production methods of *Awamori* spirits are described below.

	<i>Awamori</i>	Sake	Whisky	Brandy
Type	Distilled beverage	Brewed beverage	Distilled beverage	Distilled beverage
Place	Okinawa	Mainly Japan	Worldwide	Worldwide
Production Temperature	All seasons average annual temperature (25°C)	Mainly winter 0–4°C	Room temperature (10–15°C)	Room temperature (15–20°C)
Material	<i>Indica</i> rice	<i>Japonica</i> rice	Barley, corn	Grapes
Mash	Parallel Fermentation Containing citric acid produced by	Parallel fermentation, Containing lactic acid produced by Lactic acid	Single Fermentation Not Containing Acid	Single Fermentation containing Malic acid from Material
Saccharifying agent	<i>Koji</i>	<i>Koji</i>	Malt	-
Microorganisms	<i>Aspergillus awamori</i> <i>Awamori</i> yeast	<i>Aspergillus oryzae</i> Sake yeast Lactic acid	Whisky yeast	Wine yeast

Fermentative Temperature	High temperature (27–30°C)	Low temperature (10–15°C)	Middle temperature (15–25°C)	Middle temperature (15–26°C)
Alcohol concentration	25–30%	15–16%	40–50%	40–50%
Aging period	Approximately 3 years or more	Very short term	More than 3 years	More than 3 years
Aging vessel	Mainly earthen pot	Mainly stainless tank	Barrel	Barrel
Taste and aroma	Vanilla like	Estery, fruit-like	Vanilla like	Vanilla like

Table 1. *Awamori* spirit and other alcoholic beverages

2.2 History of *Awamori*

Awamori spirits are traditionally produced in Okinawa, which has 47 production sites. *Awamori* spirits are produced from long-grain rice and rice imported from Thailand. Partly because it uses long grain rice imported from Thailand for production, it is believed that *Awamori* spirit production methods were brought from Thailand (Koizumi, 1996). According to one account (Koizumi, 1996) of Okinawa’s history, ‘Ryukyu’ was an independent country ruled by king Sho in 1420, which traded with the countries of Southeast Asia. At the time, the port of Naha bustled as a junction port between Japan and the South China Sea Islands, Indonesia, Cambodia, Vietnam, the Philippines, and Thailand. *Awamori* spirits were brought from there and also traded. In 1534, ‘Chen Kan's Records’, reported to his home country, China, noted that *Awamori* spirits have a clear aroma and were delicious; he noted also that *Awamori* spirits had been brought from Thailand. Moreover, it was written that long-grain rice harvested in Thailand was used in *Awamori* spirit production, and the distilled spirits were aged in earthen pots. Their ancient technology of *Awamori* spirit production is followed by the present technology. Distillation technology was brought also via Thailand from China, as it was with *Awamori* spirits. Furthermore, they transported the technology eventually to the main islands of Japan. The cradle of distillation technology is actually ancient Rome. In that era, distillation methods were used to produce essential oils in the following manner: plant resin was boiled in a pan on which a wool sheet had been placed. After boiling, the upper wool sheet was pressed to obtain the essential oil. That is a primitive distillation method. The distillation method brought to Okinawa was superior to the Roman method, but the efficiency of distillation was low, according to Edo period accounts: 360 mL of distillate was obtained from 18 l of fermented alcohol beverages. Eventually, 72 ml of spirits were distilled from the first distillate (Koizumi, 1996). We can infer the alcohol concentration experimentally: fermentative alcoholic beverages (*Sake*) have approx. 10% alcohol concentration, the first distillate has approximately 20% alcohol concentration, and final spirits have approximately 30% alcohol concentration. The distillate yield by the original method was lower than that of the present method because the condenser was not a water-cooled system.

2.3 Awamori spirits production method

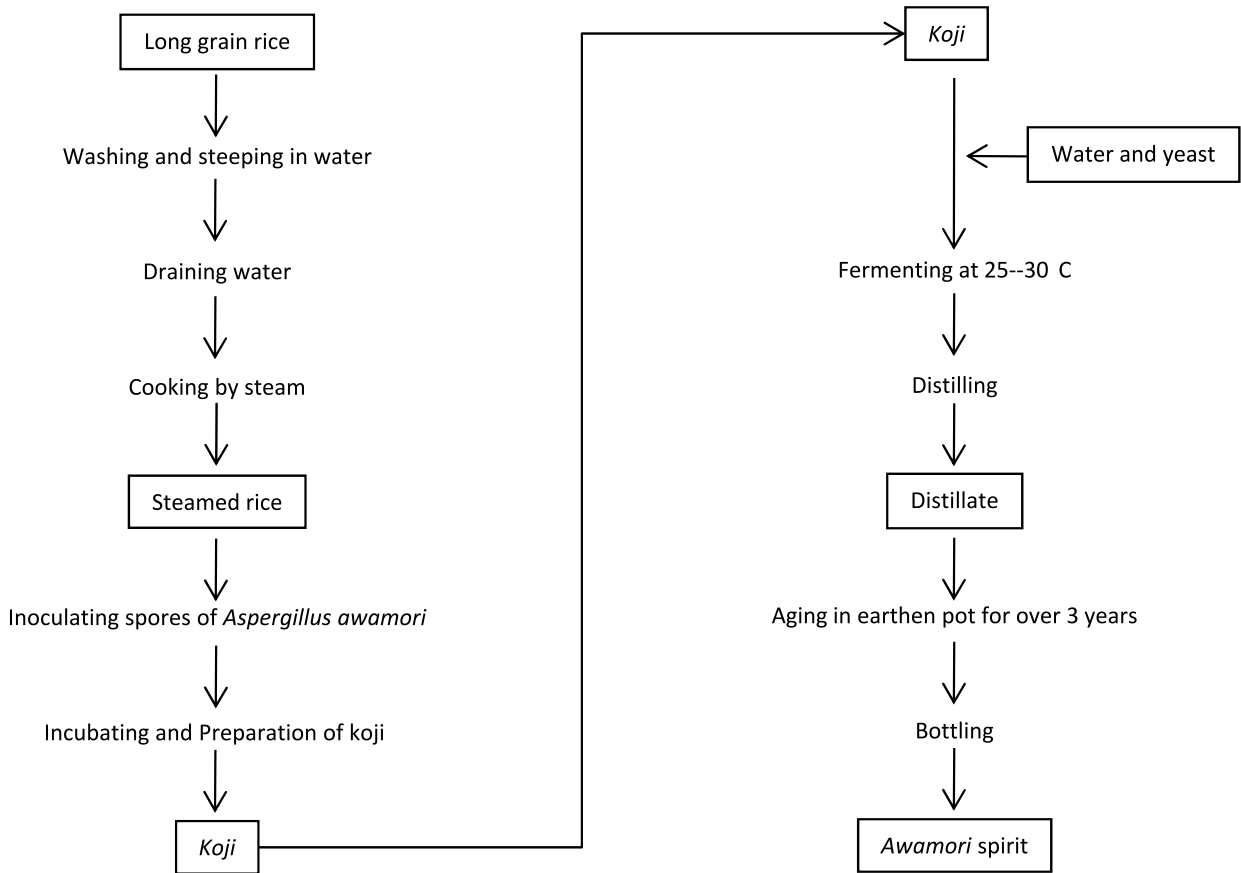


Fig. 1. Schemes of Awamori spirit production.

2.3.1 Rice

Awamori spirits are produced using long-grain rice imported from Thailand. *Oryza sativa* is a perennial plant. Kato (1930) reported rice taxonomy. He reported some differences in *Indica* rice strains (imported from Thailand) and Japonica rice strains (grown in Japan): rice grains of *Indica* strain are longer than those of Japonica rice strain. Its leaves are light green. Moreover, *Indica* rice grains are longer than Japonica rice grains. The two strains sterilize in mating with each other. Furthermore, they relate to each other as subspecies, *Oryza sativa* subsp. *japonica* and *Oryza sativa* subsp. *indica*. Some merits exist for the use of imported Thailand rice as the material for production.

1. The rice material is cheaper than Japanese rice. 2. Because the indica rice is not sticky, it is easy to work with during *koji* preparation. 3. Mash temperatures of mash using *Indica* rice are easy to control because this rice is hard and saccharifies slowly. 4. The alcohol yield from *Indica* rice is higher that from *Japonica* strains. 5. Indica rice has been used to produce Awamori spirit since it was brought from Chiame, Thailand (Nishiya, 1991).

The rice strains differ not only in grain size and shape but also in starch characteristics. Rice contains starches of two types: amylose and amylopectin. The structure is shown in the figure.

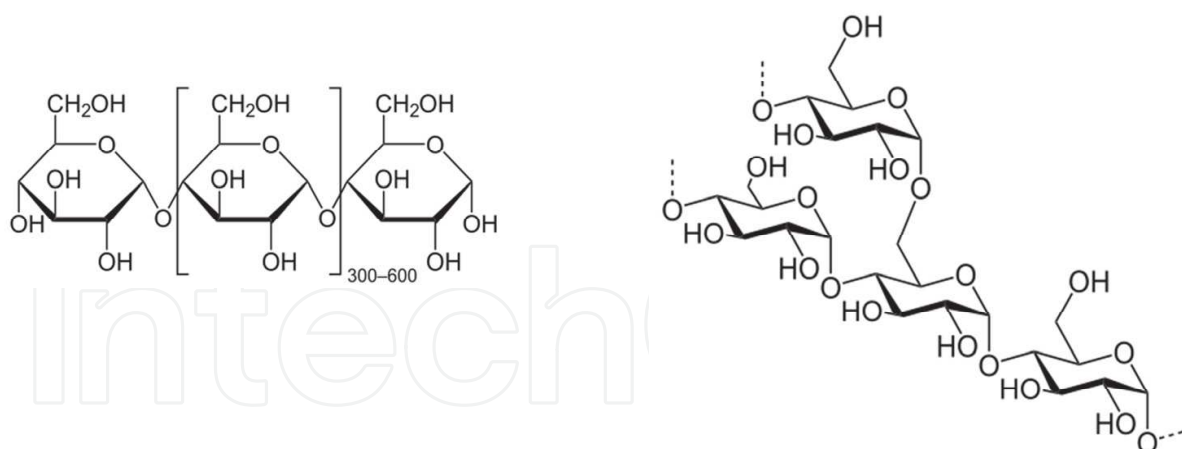


Fig. 2. Structures of amylose and amylopectin
Wikipedia (<http://www.wikipedia.org>)

Generally, *Japonica* rice strains contain 10–22% amylose, and amylose is not present in *Japonica* waxy strains. The starch is almost entirely composed of amylopectin (Juliano, 1985). In contrast, *Indica* rice contains about 18–32% amylose (Juliano, 1985). High concentrations of amylopectin make cooked rice sticky. Therefore, *Indica* rice is not sticky and is therefore suitable for preparation of *koji* for *Awamori* spirit production. Moreover, Horiuchi and Tani (1966) reported amylograms of nonwaxy starch prepared from some *Japonica* rice and *Indica* rice. The gelatinization temperature of *Indica* rice is 71.5°C (69.5–73.5°C), although the mean pasting temperature for *Japonica* varieties is 63.5°C (59–67%). No definite differences were found in the values of maximum viscosity and breakdown between *Japonica* and *Indica* varieties. Juliano et al. (1964a) obtained narrower pasting temperature ranges for *Japonica* rice flour (62–67°C) than for *Indica* (62–76.5°C). Gelatinization temperature of *Indica* rice was the highest among major cereals as waxy (*Japonica*) 62°C, 66°C for maize, and 62°C for wheat. (Dendy and Dobraszczyk, 2000).

2.3.2 Preparation of 'koji' growing *Aspergillus awamori* on steamed rice

A. awamori have a role of saccharification during fermentation. Furthermore, the strain has a black area (conidia), and it differs completely from *A. niger*. *A. awamori* was isolated by Inui from *koji* for *Awamori* spirit production and named *A. luchuensis*, Inui (Inui, 1991). The strain was later renamed *A. awamori*. The strain is an important strain for alcohol production in Japan. The mold strain prevents contamination during fermentation processing by produced citric acid and acid tolerance α -amylase supplied by the strain saccharified starch as substrate under acid conditions during fermentation processing. Raper and Fennell (1965) investigated Black *Aspergillus*. Murakami (1982) compared the size of conidia, lengths of conidiophores, and characteristics of physiology, and classified two groups as *Awamori* Group and *Niger* Group in Black *Aspergillus*. *A. awamori* can grow at 35°C. It assimilates nitrite, and it has a short conidiophore: less than 0.9–1.1 mm.

2.3.3 Preparation of *koji*

Koji is used for alcohol beverage production or production of Asian seasonings such as miso paste and soy sauce. However, *koji* for *Awamori* spirits is prepared with a unique method (Nishiya, 1991). After steaming the rice, the seed mash is inoculated to the steamed rice.

Furthermore, the inoculated rice is incubated at 40°C and reduced step-by-step. Generally *koji* for seasoning or *sake* production is prepared at 30–35°C or with temperatures raised step-by-step. At high temperatures, amylase is produced and other enzymes such as feruloyl esterase break the cell walls. At the same time, fungi grow in rice grains. After 30 hr, the temperature is decreased gradually. At low temperatures of less than 35°C, *A. awamori* produces very much citric acid. The characteristic vanilla aroma of *Awamori* spirits is produced from ferulic acid as a precursor. Fukuchi (1999) reported that *A. awamori* grown at 37–40°C has high feruloyl esterase activity.

2.3.4 Fermentation of *Awamori* mash

Awamori spirit yeast, ‘*Awamori* yeast’, that had been isolated from *Awamori* brewery was used during fermentation process and it might be peopling in brewer’s house. The yeast is tolerant of low pH and high alcohol contents, and it can grow and ferment at low pH and high citric acid concentrations, producing very high alcohol concentrations (Nishiya, 1991). Nishiya (1972) and Suzuki (1972) reported that yeast growth was inhibited in conditions of greater than 11% alcohol and 35°C temperatures. However, the yeast ferments up to approximately 17% alcohol.

Awamori mash is prepared with *koji* and water only, and the water as 170% of *koji* weight is added. Fermented *Awamori* mash has 3.8–4.8% citric acid. The range of mash temperatures is 23–28°C. The yeast grows rapidly and fermentation finally ceases at temperatures higher than 30°C. At temperatures less than 20°C, the yeast grows slowly, and the mash is contaminated by bacteria. Generally, after 3 days, the mash has more than 10% of alcohol concentration. After 4 days, it has approx. 14%. After 7 days, it has more than 17%.

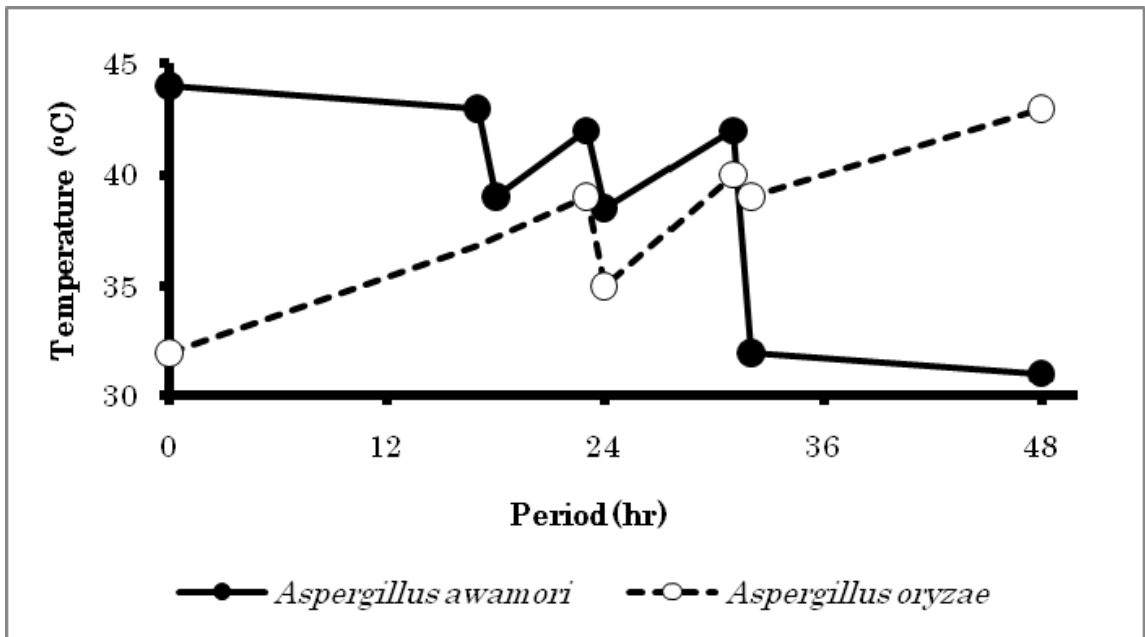


Fig. 3. Temperatures during two *Koji* preparations.

2.3.5 Mash distillation

After fermentation, the mash is distilled quickly because the mash aroma sours after yeast is digested by high alcohol and high temperatures. Two distillation systems exist as

atmospheric distillation systems and reduced pressure distillation systems. *Awamori* spirits distilled with atmospheric distillation systems have three features (Nishiya, 1991).

1. Spirits have many components and a rich taste.
2. The spirit quality is good after aging.
3. Spirits have a distinctive aroma like scorching as furfural, which is produced by heat during distillation.

The distillation system with reduced pressure has no scorching aroma or higher alcohol, and it has a softer taste. It requires no long period of aging. During distillation, aldehydes and esters are distilled in the initial distillate. Then ethanol is distilled. The continuation of distillation decreases ethanol. The scorching aroma, that of furfural, is increased by heat during distillation. A direct heat distillation system is used traditionally: mash is heated in a kettle directly by fire. It has a scorched aroma. The compounds in distillate are shown during distillation of spirits.

2.3.6 Aging

Awamori spirits are aged in earthen pots using the *Shitsugi* method. The spirits show accelerated aging in earthen pots. Fatty acids or volatile acids as acetic acid in spirit are neutralized by calcium or magnesium released from earthen pots during aging. The acid or fatty smell in spirit is removed by aging. Recently, consumers favor a dry taste *Awamori* spirit aged in stainless steel tank after filtrate by ion exchange resin or activated charcoal.

The *Shitsugi* method is conducted by aging in earthen pots as follows. The distilled spirits are aged in earthen pots for 3–5 years. The spirits in the oldest earthen pots are consumed, and the consumed volume is supplied from the second oldest pot, and second oldest pot is supplied from third oldest pot is supplied from fourth oldest pot. Finally, the distilled spirit is poured in the newest pot. It resembles the solera system of sherry wine production. It is a specific aging method that has been used for *Kusu Awamori* spirits.

3. Mechanisms of making vanillin via ferulic acid in 'Awamori' during aging within an earthen pot

3.1 Vanilla aroma of 'Awamori spirit'

Awamori spirits have a vanilla aroma. Koseki (1998) reported mechanisms of vanilla aroma production in *Awamori* spirits. They found that not only vanillin but also phenol compounds as 4-vinyl guaiacol and ferulic acid from sufficient aged *Awamori* spirit. It does not age in barrels containing lignin. Therefore, they consider that the phenol compound is extracted from the material as rice.

3.2 Vanilla from ferulic acid in rice cell walls

Ferulic acid is contained in cell walls of rice. Then the ferulic acid is converted to vanilla during production. Koseki (1998) reported that ferulic acid dissolved in citric acid buffer was distilled, and that 4-vinyl guaiacol was detected in the distillate. Furthermore, the vanillin was detected from aged distillate containing 4-vinyl-guaiacol. Koseki mentions that the phenol compound was converted by heat. The ferulic acid is released from rice, and converts 4-vinyl guaiacol by heat when distilled. Furthermore, 4-vinyl guaiacol converts vanillin during aging. 4-Vinyl guaiacol is a well known compound as an off-flavor of beer and orange juice. Also, it is known as a characteristic flavor in weizen beer, or wheat beer. It is a distinct flavor.

Recently however, ferulic acid is converted to 4-vinyl guaiacol by microorganisms as *Saccharomyces cerevisiae* (Huang and others, 1993), *Pseudomonas fluorescens* (Huang and others, 1993), *Rhodotorula rubra* (Huang and others, 1994), *Candida famata* (Suezawa, 1995), *Bacillus pumilus* (Degraasi and others, 1995) and *Pseudomonas fluorescens* (Zhixian and others, 1994) via ferulic acid decarboxylase.

Furthermore, lactic acid bacteria converting ferulic acid to guaiacol were isolated from *Awamori* mash, and their ferulic acid decarboxylase was purified and compared (Watanabe, 2009). The lactic acid bacteria identified *Lactobacillus paracasei*, and their enzyme was the protein inferred as 47.9 kDa. The *p*-coumaric acid decarboxylase in *Lactobacillus plantarum* was 93 kDa; ferulic acid decarboxylase disagreed with those for *p*-coumaric acid decarboxylase (Cavin, 1997).

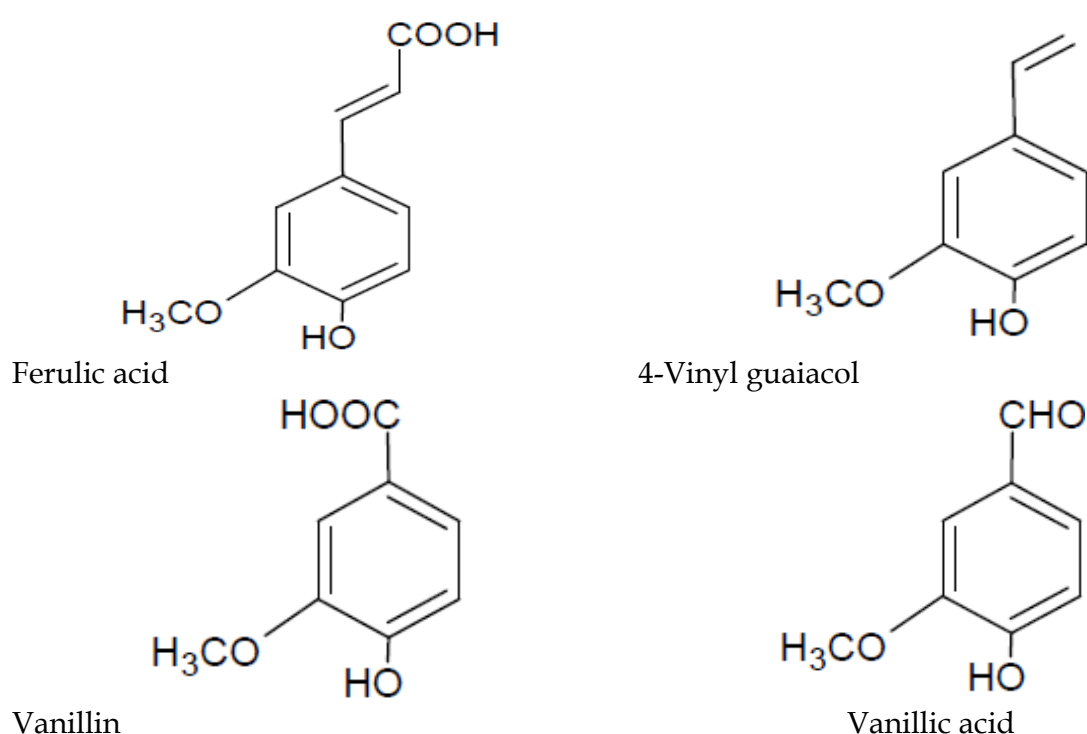


Fig. 4. Structure of phenol compound.

The optimum temperature was 60°C, and the optimum pH was approximately pH 3.0. The *p*-coumaric acid decarboxylase from *Lactobacillus plantarum* disagreed with that reported by Cavin (1997).

The optimum temperature was 30°C; the optimum pH was 5.5–6.0. The *Awamori* mash has an acid condition that is maintained by citric acid produced using *A. awamori*. Consequently, the mash is not contaminated by other microorganisms. The enzyme was regarded as having been grown optimally at pH 3.0: high acidity and low pH conditions. The vanillin is produced not only by chemical conversion but also through bio-conversion in *Awamori* spirit or the mash during *Awamori* spirit production.

3.3 Feruloyl esterase is an important enzyme for high-quality *A. awamori*

Ferulic acid is supplied from combining xylan on rice cell walls. The ferulic acid is released by feruloyl esterase. In this *awamori* production, *A. awamori* on *koji* provides the enzyme.

According to their reports, microorganisms are important to produce vanillin in *Awamori* spirits. The released ferulic acid is converted to 4-vinyl-guaiacol by lactic acid bacteria. Then vanillin is produced from guaiacol. The amount of ferulic acid is obtained in relation to feruloyl esterase activity: high concentration ferulic acid gives *Awamori* spirits a high vanilla aroma. Namely, the amount of vanillin in *Awamori* spirits is determined by the feruloyl esterase activity.

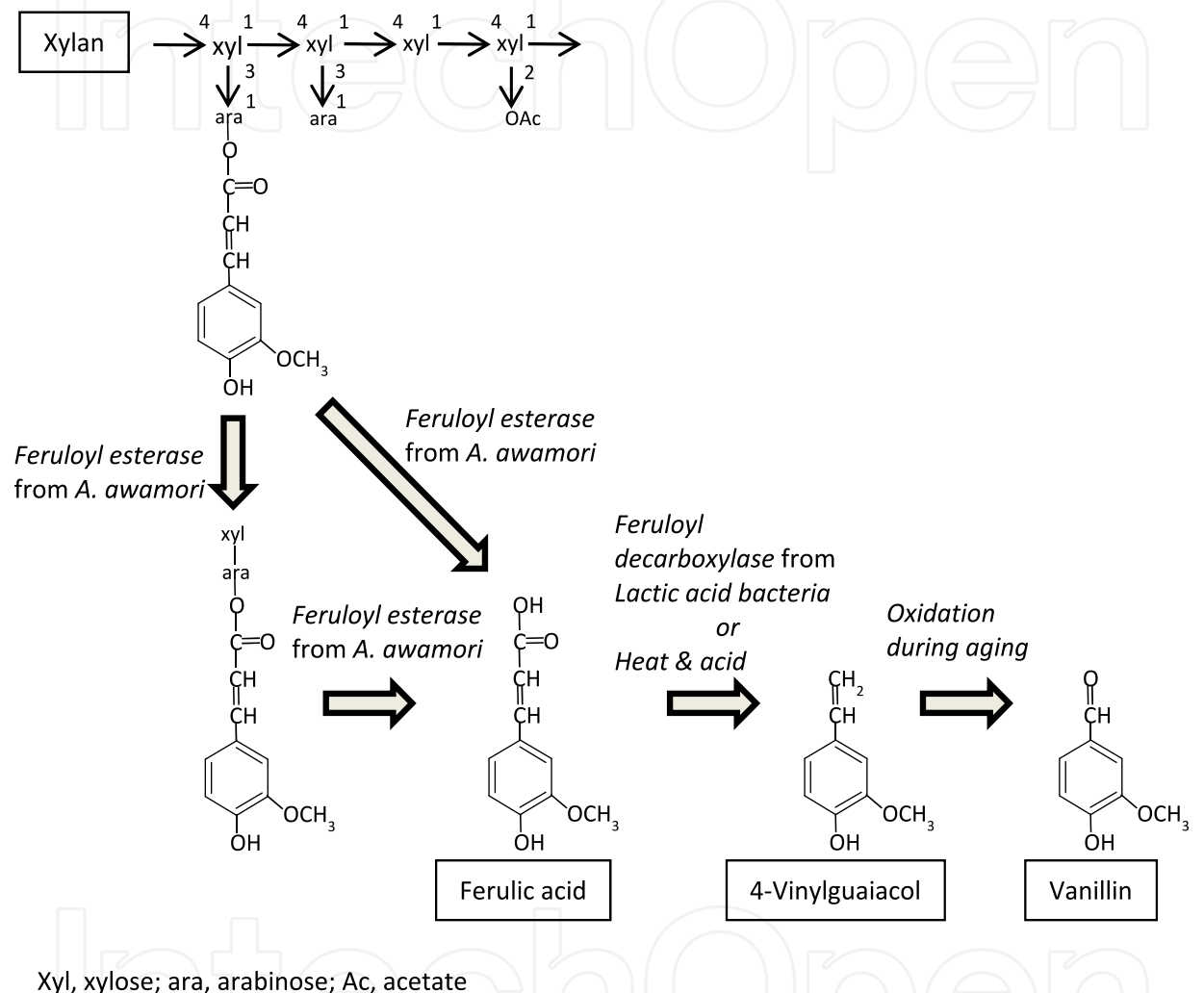


Fig. 5. Mechanisms of production of vanillin from ferulic acid during *Awamori* spirit

4. Selection of *Aspergillus awamori* strain producing the highest feruloyl esterase and characteristics of their enzymes

4.1 Screening of *Aspergillus awamori* producing the highest feruloyl esterase

We next describe the importance of feruloyl esterase during *Awamori* spirit production. We isolated and screened *A. awamori* to give high feruloyl esterase activity for *Awamori* spirits production. Mold strains were isolated and selected from some locations near *Awamori* spirits breweries in Okinawa. Consequently, black *Aspergillus* of many kinds was isolated. Then the strains were cultivated on xylan plate medium (1.5% xylan, 0.5% yeast extract, 0.5% polypeptone

and 1% agar) for the first screening. The strain with a clear zone on the plate medium was screened and the strain was assayed for feruloyl esterase activity. Results show that the largest clear zone was made by G-2. Its feruloyl esterase was 394 U/ml, which was 3–4 times higher than the others. As presented in Table 2, feruloyl esterase contents of G-2 were higher than those of either *Aspergillus awamori* NRIC1200 (118 U/ml) or *Aspergillus usami* IAM2210 (50 U/ml) as a standard strain. The mold strains that produced feruloyl esterase formed a black colony. Many reports have described feruloyl esterase from the black *Aspergillus* group. Results of this study concur with those previously reported results. Conidiophores were observed using a microscope. The conidia were observed using scanning electric microscopy (SEM). Conidiophores of G-2 were similar to those of *Aspergillus* sp.: their conidia were black with a smooth surface. production. In addition, nitrite was not assimilated by G-2. Generally, black conidia were *A. niger* or *A. awamori*; other *Aspergillus* were brown, yellow, or green (Pitt and Kich, 1988).

Strains	Feruloyl esterase (unit/ml)
<i>A. awamori</i> NRIC1200	118
<i>A. saitoi</i> IAM2210	50
G-2 strain	394
Other selected strains	130–293

Table 2. Activity of feruloyl esterase from the strains

Moreover, *A. awamori* were not assimilated and the surfaces of their conidia were smooth. In contrast, *A. niger* was assimilated; its conidia surfaces were rough (Murakami, 1979). For those reasons, G-2 was identified as *Aspergillus awamori*, used to produce Awamori spirits as Japanese traditional spirits produced in Okinawa. The G-2 strain does not produce ochratoxin or aflatoxin as a mycotoxin. This *A. awamori* is applicable of food production and Awamori production.

4.2 Feruloyl esterase characteristics

The enzyme was purified using ion-exchange, size-exclusion, and HPLC chromatography. After purification, the specific activity of the enzyme was 20-fold higher and the yield was 16% higher than that of the crude enzyme solution. The enzyme solution was then analyzed using sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS-PAGE). The molecular weight of feruloyl esterase in *A. awamori* was 35 kDa (Koseki, 1998b). The enzyme was 78 kDa, which was higher than that of *A. awamori*. The molecular weight of feruloyl esterase was measured using size-exclusion chromatography. The molecular weight was estimated as 80 kDa. The feruloyl esterase was inferred to be a monomer protein. The optimum pH of the feruloyl esterase was pH 5.0 and the optimum temperature was 40°C. The activity stabilized at pH 3.0–5.0. The feruloyl esterase of *A. awamori* is reportedly (Koseki and others, 1998b) unstable at pH 3.0, which was 30–50% of non-treated enzyme activity. However, the enzyme was stable in acid. The enzyme was stable at 50°C. Feruloyl

esterase of *A. niger* showed tolerance at 80°C (Sundberg and others, 1990). The enzyme is therefore acid-tolerant, but not heat-tolerant. Use of the enzyme is expected to be advantageous for food production under acid conditions, in which *A. awamori* produces citric acid very well.

Mercury ion (Hg²⁺) inhibited feruloyl esterase activity completely; Fe²⁺ inhibited 80% of the activity. Both PMSF and DFP completely inhibited feruloyl esterase activity. In general, feruloyl esterase, produced by black *Aspergillus* group, was classified into the serin-esterase group enzyme, which was inhibited by Hg²⁺ and Fe²⁺ (Koseki and others, 1998b). They reported that the active center of feruloyl esterase from *A. awamori* had Ser-Asp-His. It was therefore considered that the feruloyl esterase had similar catalysis.

The feruloyl esterase activity was the highest among four substrates with 1-naphthyl acetate. The activity of the feruloyl esterase had 40% of the activity related to 1-naphthyl acetate, against 1-naphthyl propionate (composed of three carbons). In addition, 1-naphthyl butyrate (comprising four carbons) showed 5% activity related to 1-naphthyl acetate. The activity of feruloyl esterase was decreased against substrates containing more than three carbons; 2-naphthyl acetate did not react completely. The reported feruloyl esterase showed activity against 1-naphthyl propionate, as did 1-naphthyl acetate (Ishihara and others, 1999). The feruloyl esterase was inferred to be substrate-specific.

Adsorption of the feruloyl esterase to cell walls was not observed. The enzyme was not absorbed by xylan or starch that were present in the supernatant. However, it was absorbed by cellulose in the supernatant. It was also found in the precipitant with cellulose.

Strain	Mycelium color	Conidium color	Conidiophore (mm)	Conidial head diameter (μm)	Conidium diameter (μm)	Conidium surface	Assimilation of NaNO ₂
<i>A. awamori</i> NRIC1200	White	Black	0.61	220	3.8	Smooth	-
<i>A. niger</i> NRIC1221	White	Black	0.86	170	3.4	Spinky	+
<i>A. saitoi</i> IAM2210	White	Black	0.91	190	4.1	Rough	-
G-2	White	Black	0.68	200	3.4	Smooth	-

+, positive; -, negative

Table 3. Morphological and physiological characteristics of black *Aspergillus* spp.

Feruloyl esterase from *A. niger* and *A. awamori* reportedly adsorbed specifically to cellulose (Ferreira and others, 1993), which agrees with the results shown for this study. The intensity of absorbing the enzyme with cellulose was conducted to wash cellulose by boiling for 10 min in SDS solution (1–11%). The enzyme had a cellulose binding domain, as reported also by Koseki and others (2006). The protein was unbound by 10% and 11% SDS solution. It is considered that they were bound strongly.

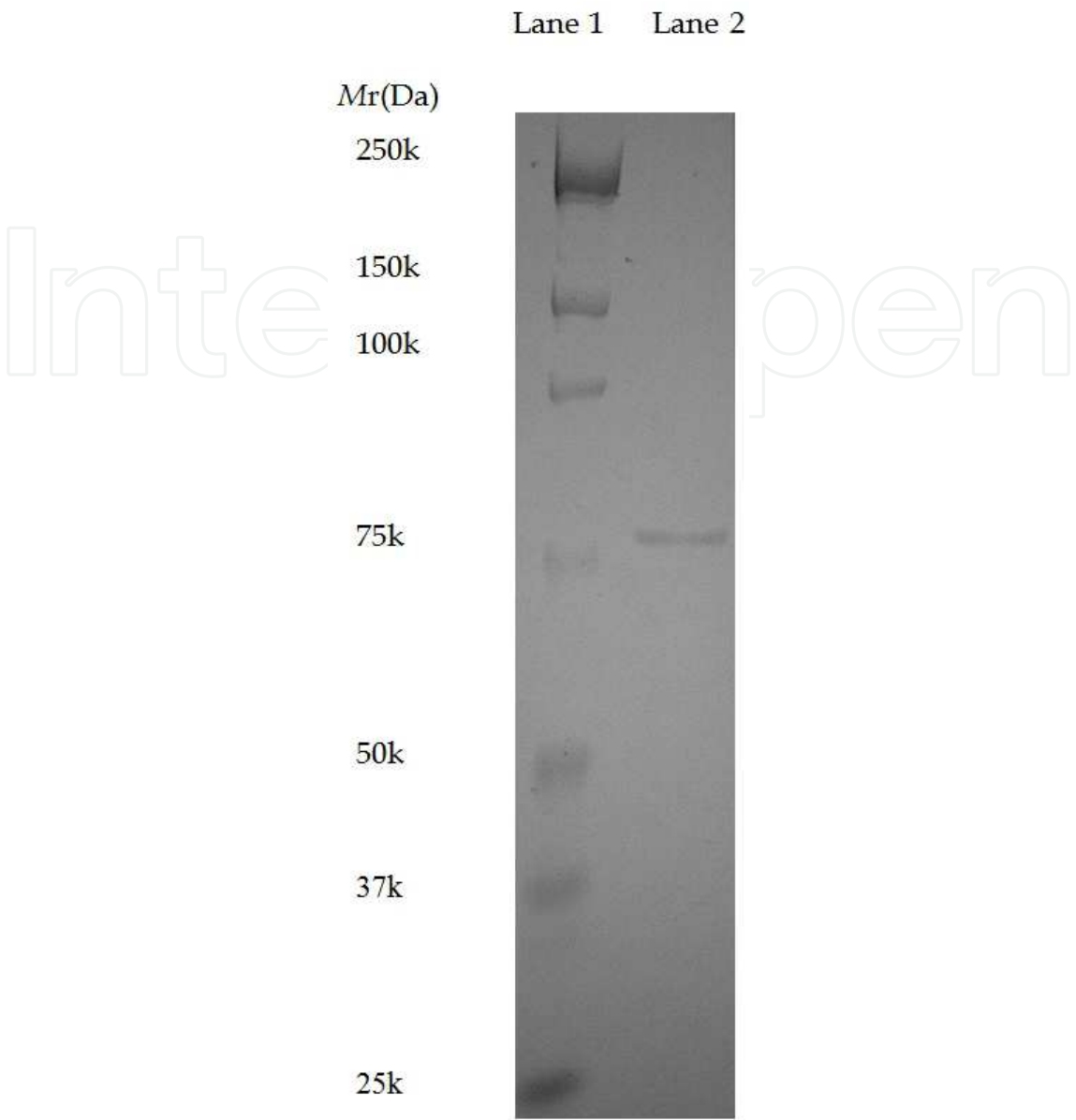


Fig. 6. SDS-PAGE of feruloyl esterase.

We investigated Michaelis constants of the feruloyl esterase. A Lineweaver-Burk plot is shown to calculate the K_m of the feruloyl esterase: its K_m was 0.0019% (0.01 mM). Koseki reported that K_m was 0.26–0.66 mM (Koseki and others, 2006). The feruloyl esterase has higher affinity to 1-naphthyl acetate than that described by Koseki. The feruloyl esterase from *A. awamori* G-2 was more stable under the acid condition than the other black *Aspergillus* groups. In general, the esterase was as unstable under the acid condition as *Awamori* mash or shochu mash during fermentation. However, results suggest that the feruloyl esterase survived under the acidic conditions. The ferulic acid was related to the aroma of *Awamori* spirits or shochu spirits. The ferulic acid released from the cell wall was converted to vanilla via 4-vinyl guaiacol by yeast or lactic acid bacteria during aging (Gabe and Koseki, 2000; Watanabe and others, 2007). In addition, results showed that the enzyme-stabilized activity under acid and heat conditions of pH 3.0 and 50°C is applicable to food production.

Substrates	Relative activity (%)
1-naphthyl acetate	100
1-naphthyl propionate	40
1-naphthyl butyrate	5
2-naphthyl acetate	0

Table 4. Substrate specificity of feruloyl esterase

5. Application of feruloyl esterase to beer brewing

In beer brewing processes, the mash is used differently from the mash of *Awamori* spirits. Beer mash is saccharified and then fermented. In the mashing process, barley malt produces not only maltose as base material of ethanol for fermentation. The malt also supplies β -glucan in mash. β -glucan (called (1-3) (1-4)- β -D-glucans) are components of endosperm cell walls in barley, occupying 75% of the cell wall (MacGregor and Fincher, 1993). The amount of β -glucans is negatively correlated with yields of the amount of wort in the mashing process (Bourne and others,1982; Kato and others, 1995) . The amount of β -glucan in grain shows levels of decomposition of cell wall in barley endosperm. The malt contained a great amount of β -glucan, showing insufficiency in decomposing β -glucan in malt during germination. The malt insufficiently decomposes β -glucans and does not have sufficient starch or protein in endosperm. Therefore the yields fall. Furthermore, the β -glucans in malt used to make mash have high viscosity, which reduces the mash filtration speed (MacGregor and Fincher, 1993). Speed is a limiting factor of beer brewing. The slow filtration of beer presents problems for breweries. Moreover, glucan is a factor of invisible haze or pseudohaze (Jacson and Bamforth, 1983) (1-3), (1-4)– β -D-glucanase is usually added to elevate the activity (Bamforth, 1985). Kanauchi and Bambort reported that β -glucan was decomposed not only by (1–3), (1–4)- β -D-glucanase but also by xylanase, arabinofuranosidase, and feruloyl esterase. At least, feruloyl esterase decomposed xylan or feruloylxylan layer covering β -glucan. The enzyme helps to access β -glucanase to b-glucan.

6. Application of feruloyl esterase for biomass processing

Global crude oil production is 25 billion barrels, but human societies are expected to reduce the use of fossil fuels after bio-fuel development (Benoit et al., 2006). Particularly in the US, these gasoline fuels contain up to 10% ethanol by volume. In the future, automobiles can use a blend of 85% ethanol and 15% gasoline by volume (Fazary and Ju, 2008). Under this situation, lignocellulosic and other plant biomass processing methods have been developed recently. The material is not only a renewable resource but it is also the most abundant source of organic components in high amounts on the earth: the materials are cheap, with a huge potential availability.

Plant lignocellulosic biomass comprises cellulose, hemicellulose, and lignin. Its major component is cellulose (35–50%) with hemicellulose (20–35%) and lignin (25%) following, as shown in Table 5 (Noor and others, 2008). Proteins, oils, and ash make up the remaining fraction of lignocellulosic biomass (Wyman, 1994b). Cellulose is a high-molecular-weight linear polymer of b-1,4-linked D-glucose units which can appear as a highly crystalline material (Fun and others, 1982). Hemicelluloses are branched polysaccharides consisting of the pentoses D-xylose and L-arabinose, and the hexoses D-mannose, D-glucose, D-galactose and uronic acids (Saka, 1991). Lignin is an aromatic polymer synthesized from phenylpropanoid precursors (Adler, 1977).

It is noted throughout the world that ethanol is producible from biomass material. For their bioconversion, pretreatment is an important procedure for practical cellulose conversion processes (Bungay, 1992; Dale and Moreira, 1982; Weil and others, 1994; Wyman, 1994a). Ferulic acid is found in the cell walls of woods, grasses, and corn hulls (Rosazza and others, 1995). It is ester-linked to polysaccharide compounds, where it plays important roles in plant cell walls including protein protection against pathogen invasion and control of extensibility of cell walls and growth (Fry, 1982).

	Composition (% dry weight basis)		
	Cellulose	Hemicellulose	Lignin
Corn fiber	15	35	8
Corn cob	45	35	15
Rice straw	35	25	12
Wheat straw	30	50	20
Sugarcane bagasse	40	24	25

Table 5. Composition of some agricultural lignocellulosic biomass (Noor and other, 2008)

In bio-ethanol production, complete enzymatic hydrolysis of hemicelluloses as arabinoxylan requires both depolymerizing and sidegroup cleaving enzyme activities such as FAEs.

Any hemicellulose-containing lignocellulose generates a mixture of sugars upon pretreatment alone or in combination with enzymatic hydrolysis. Fermentable sugars from cellulose and hemicellulose will fundamentally be glucose and xylose, which can be released from lignocellulosics through single-stage or two-stage hydrolysis. In Europe, portable alcohol manufacturing plants are based on wheat endosperm processing, with the hemicellulosic by-product remaining after fermentation consisting of approximately 66% (W/W) arabinoxylan (Benoit et al., 2006). A synergistic action between cellulases, FAEs, and xylanases might prove to be more effective when applied at a critical concentration in the

saccharification of steam-exploded wheat straw (Borneman and others, 1993; Kennedy and others, 1999; Tabka and others, 2006).

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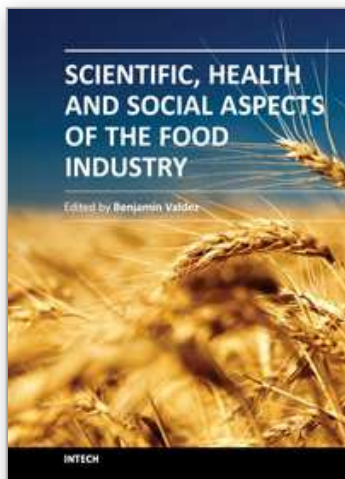
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