EnzymeWave

Volume 1

Sake and Enzymes

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AMANO

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New Start:

It is my great pleasure to announce that Amano Pharmaceutical Co., Ltd. has changed its corporate name to Amano Enzyme Inc. as of October 1, 2000. This name change represents our commitment to become the number one speciality enzyme producer and supplier in the world.

Over 100 years has passed since our company was established. Our dream has been to contribute to the betterment of society through the introduction of novel enzymes that provide a healthy life style for all people. During the last 50 years we have continuously worked to fulfill this dream. Today, enzymes play important roles in many fields. For example, enzymes increase the efficacy of nutraceutical products, increase the precision of diagnostic analysis, and provide safer and more delicious foods. We believe there will be many more products that will gain high added value because an enzyme is used at some stage of the production process.

Our vision is that enzymes will be used potentially in every product line and used to develop environmentally friendly production processes in the near future. In order to accomplish this vision, we opened a new Technical Center in February, 2000.

Finally, we hope that this newsletter will provide an open communication with you and let us share ideas and dreams for a bright future.

Motoyuki Amano
President

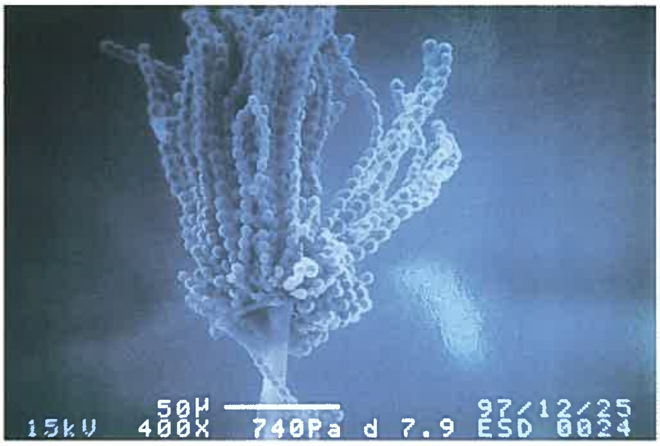
Gifu R&D Center opened in February, 2000

Japanese Fermented Foods and Koji

Biotechnology in the food industry, especially the production of fermented foods, has been practiced for more than 8,000 years. In the fermentation process, microorganisms transform food raw materials to value added foods by producing enzymes which catalyze various chemical reactions. Traditional fermented foods reflect local culture and history.

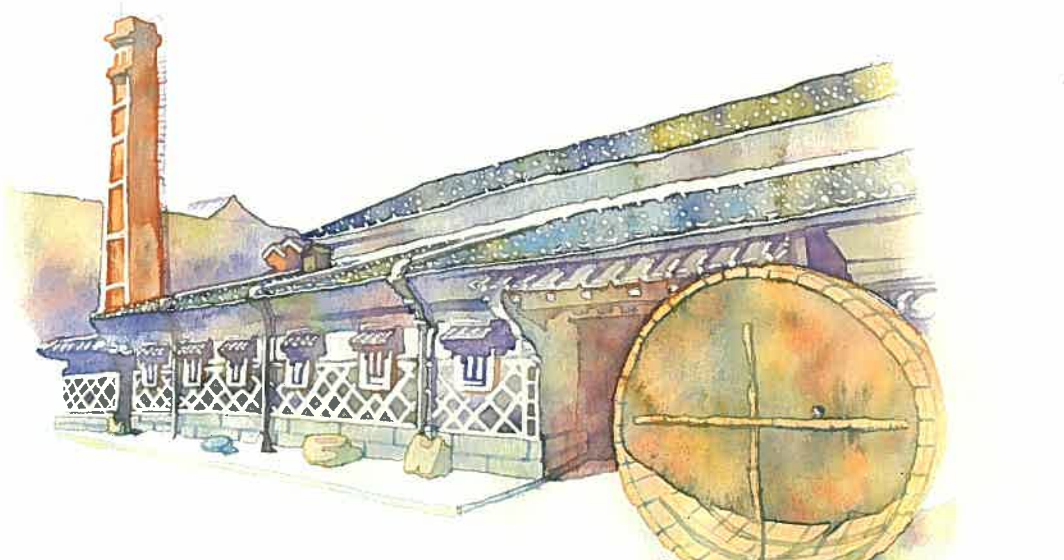
Examples of unique Japanese fermented foods are *sake* (rice wine), *miso* (fermented soybean paste), *shoyu* (soy sauce), *su* (rice vinegar) and *shochu* (a distilled liquor). The unique Japanese fermented food process relies on koji as the crucial ingredient. Koji is steamed grain onto which mold, such as *Aspergillus oryzae*, has been cultivated. The grain can be rice, wheat or soybean and the resulting koji is called rice-koji, wheat-koji or bean-koji. Koji is an abbreviation for *kamudachi*, which means, "bloom of mold". The main role of koji is to provide enzymes to degrade starch in the grain to fermentable sugar for fermentation by yeast and/or lactic bacteria. A comparison between the production methods of sake versus western alcoholic beverages demonstrates the role of koji. In beer brewing, malt (sprouted barley seed) provides enzymes to convert the starch present in barley into glucose needed for fermentation. In wine making, fermentable sugars are present naturally in ripened grapes and readily fermented when yeast is added. Since sake is brewed from white rice stripped of its husk, enzyme production by malting is not possible. Therefore koji is required to provide enzymes to hydrolyze starch to fermentable sugar (glucose).

Because koji also develops desirable flavors and texture in the final products, the choice of koji starter (conidia) and koji production is the most critical step in fermented food production. In Japan each provider of koji starter possesses hundreds of proprietary strains of *Aspergillus*. The annual production of koji starter in Japan is estimated to be about 40 tons.



Blooming *Aspergillus oryzae*

Koji is a treasure house of valuable enzymes. In fact, the first commercial microbial enzyme preparation in Japan was a koji-extract, "Takadiastase", which is still a popular digestive enzyme in Japan. Also, the traditional koji production process is the origin of the current solid fermentation process for enzyme production in Japan. The same strain of microorganism can produce different enzymes or different relative amounts of enzymes if produced by solid fermentation or submerged fermentation. Investigation of gene expression levels in solid and submerged fermentation is currently being carried out in Japan. In future issues of "EnzymeWave", we will report to you on the progress of these studies. In this issue, we would like to provide some information about the Japanese national beverage, sake, and the role of enzyme preparations in sake production.



Sake

Sake is a traditional alcoholic drink in Japan. It has played a central role in Japanese life and culture for centuries. Wedding celebrations and New Year's festivals are not complete without sake on hand to bestow blessings. You will find the most beautiful artifacts for serving sake in every household in Japan. In Japan, sake is the second most consumed alcoholic beverage after beer and its annual production is about one billion liters. Today, about 2,200 breweries of all sizes are engaged in sake production in Japan.

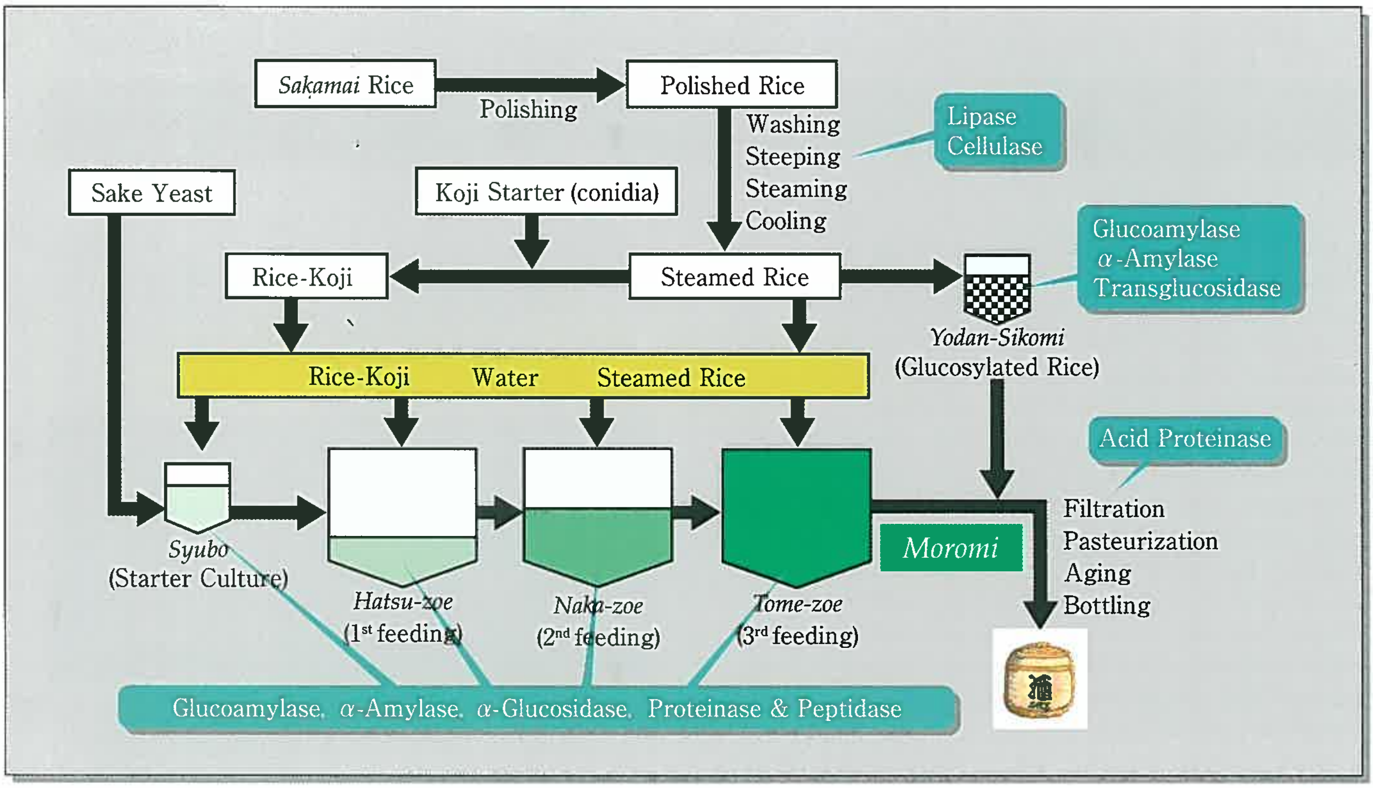
Most sake contains about 15% alcohol. It is made of rice, water, sake-yeast and rice-koji. The most suitable water for sake is found in what has become the famous sake producing regions of Japan. The water is high in potassium, magnesium and phosphoric acid, and low in iron and manganese, which cause discoloration and deteriorates the flavor of sake. *Sakamai*, a special variety of rice for sake is quite different from regular table rice. The grain of *Sakamai* rice is much larger and softer. In *Sakamai* rice, the center of the grain appears as a milky white core due to diffused light reflection from amyloplasts, where starch is synthesized. The starch core is surrounded by fats, proteins, amino acids and minerals which are carefully removed at the polishing stage. The rice is polished up to 65%, leaving as little as 35% of the rice, which is almost pure starch. A high degree of polishing gives sake a smooth mouth feel and a fruity flavor with low acidity. Since polishing has such a significant effect on the quality of the sake, one of the classification terms for sake is dependent on the degree of rice polishing.

The most popular way to drink sake is to drink it warm, though drinking it cold is becoming increasingly popular. In fact, the mark of quality sake, especially a *dai-ginjo* or premium sake with more than 50% polished rice, is its quality of flavor and enjoyability at lower temperatures.

Sake brewing starts in late fall after the rice fields turn to gold and the harvesting is finished. The first sake in the brewing period is called "*hatsu-shibori*" and brewers treat it to neighbors and customers. To announce the successful production of *hatsu-shibori*, the large cedar ball made of cedar leaves, called "*saka-bayashi*", that hangs in front of the brewery is changed from a year old brown ball to a freshly made green cedar ball. Sake barrels and *Masu* (a square wooden box which is the favored sake cup for ceremonies) are made of cedar, so the aroma of cedar is associated with sake. If you look for sake brewers in Japan, look for the cedar ball!

Cedar ball, "saka-bayashi"

It is said that Japanese get drunk much easier than westerners. This is because about half of all Japanese lack or have low levels of an enzyme called aldehyde dehydrogenase (ALDH2), which converts toxic acetaldehyde at low concentrations to acetic acid. (Alcohol is converted to acetaldehyde by alcohol dehydrogenase (ADH) in the liver.)



Sake Brewing Process and Enzymes

The raw material of sake is steamed rice. Rice starch is broken down to glucose by koji and fermented to alcohol by sake-yeast (*Saccharomyces cerevisiae*) for 14 to 30 days. Here, saccharification and fermentation takes place in the same vat at the same time. This "multiple parallel fermentation" is a unique feature of traditional sake brewing which distinguishes it from other brewing processes and produces a high concentration of ethanol (up to 20%) in the *moromi* (sake-mash).

Koji contains various enzymes beneficial for sake production. Alpha-amylase liquefies rice starch and glucoamylase breaks down liquefied starch to glucose. Transglucosidase synthesizes isomalto-oligosaccharides, which contribute to the rich sake flavor. Acid proteinase and acid carboxypeptidase hydrolyze rice protein to amino acids which influence the sake flavor and aroma as well as provide nutrients for yeast. Proteinase also releases α-amylase from rice proteins, which tend to bind to α-amylase and inhibit starch liquefaction. Sake yeast also produces various enzymes that contribute to sake flavor and aroma development. Among these, alcohol acetyltransferase and alcohol acyltransferase are the most studied enzymes.

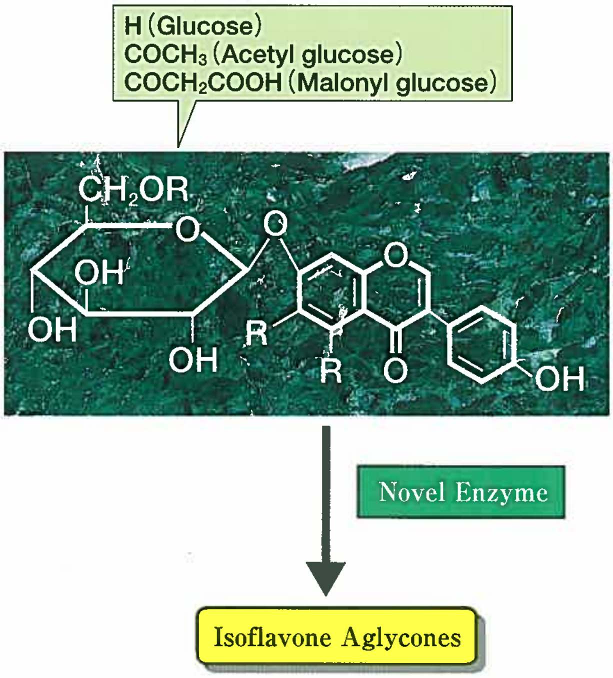
Today, to improve the quality of sake, enzyme preparations have come to play an important role just as they have in beer and wine brewing. Enzyme preparations containing multiple enzymes are used to supplement or replace koji. The main objective is to decrease the usage level of koji, the production of which is tedious and requires the most skill of any process in sake production. Less koji also gives a lighter flavor to the final product, which is preferred by some consumers. The enzyme preparations useful in sake production include α-amylase, glucoamylase, α-glucosidase (transglucosidase), proteinase & peptidase, lipase, cellulase and acid proteinase.

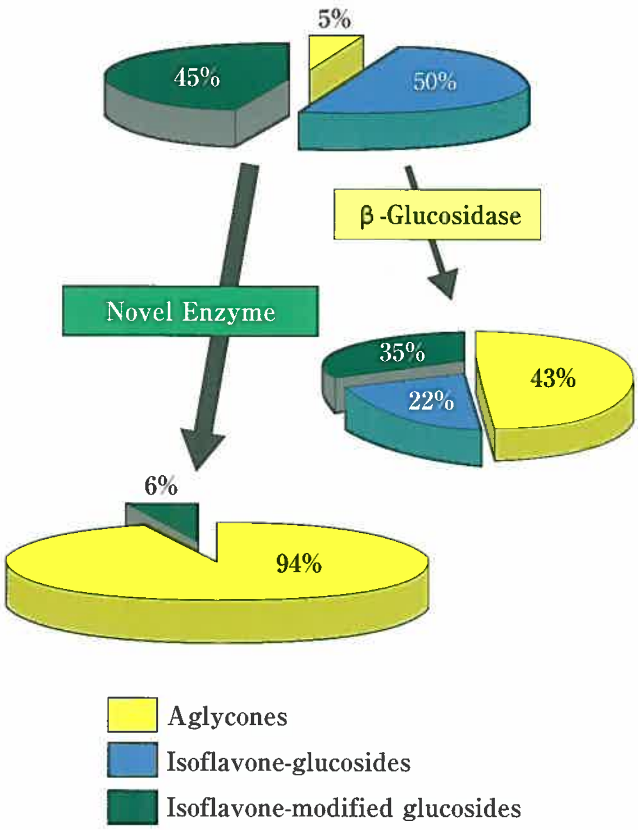
Advanced Sake Production

Automation is now an even more urgent theme in the sake brewing industry because of aging brew masters (*Toji*) and a shortage of their successors. Without spoiling the traditional taste of sake, some progress in the production process has been made, especially at the large breweries. One of the latest innovations in the production process is called "liquid rice brewing". Traditional brewing (multiple parallel fermentation) uses a ratio of 6 parts rice to 10 parts water by weight. This is a very high solid concentration and makes temperature control and mash stirring very difficult. In the "liquid rice brewing" process, heat stable α-amylase is mixed with rice in warm water and quickly "melts" the rice. The liquefied rice is pumped into a fermentation tank, and then yeast and koji are added to the mixture. Rice liquefaction makes it easier to automate sake production. The successful commercial production of heat stable α-amylase played an important role in developing this new process. In the future, new developments in the enzyme industry will make further contributions to sake brewing.

A Novel Enzyme for Isoflavone Aglycone Production

Soy food products are getting more attention from both ingredient suppliers and food manufactures in the fast growing functional foods/nutraceutical market. The soybean is unique in that it contains very high levels of isoflavones, up to about 4 mg/g dry weight. Mounting scientific evidence indicates that these unique phytochemicals can lower the incidence of heart disease and certain hormone-related cancers, reduce the risk of osteoporosis and ease menopausal symptoms. In soybean, isoflavones are present in the form of glycosides (with sugar) or aglycones (without sugar). The glycoside form is the major form of isoflavones in soy products. Recent studies show that isoflavones in the aglycone form are absorbed more efficiently into the blood stream. It is also reported that the isoflavone aglycones show greater antioxidant activity than their corresponding glycosides. Therefore, converting isoflavone glycosides in soy products to isoflavone aglycones may increase their bioavailability and improve their health benefits.





Amano has isolated a novel microbial enzyme capable of transforming isoflavone glycosides into isoflavone aglycones in soy products. This novel enzyme preparation utilizes all 9 isoflavone glycoside isomers found in soybeans. As a result, the novel enzyme preparation converts nearly all soy isoflavone glycosides to isoflavone aglycones (daidzein, genistein and glycitein) in a one step reaction. In contrast, current commercial β-glucosidase preparations convert isoflavone glucosides, but have little activity on modified glucosides (6"-O-acetyl glucoside and 6"-O-malonyl glucoside).

This novel enzyme and a new production method for isoflavone aglycones using the novel enzyme are the subject of a patent (WO0018931A) and were presented at "The Third International Soybean Processing and Utilization Conference" held in Japan (October, 2000). With this new production method, isoflavone glycosides in various soy products (defatted soy meal, soy concentrates, roasted soy flour and soymilk) can be efficiently converted to isoflavone aglycones, the readily bioavailable isoflavone form. We believe that this new process is superior to current conversion processes for soy isoflavones: acid hydrolysis, microbial fermentation and enzymatic hydrolysis by β-glucosidase combined with alkali pretreatment. *We have been working toward the commercialization of this novel enzyme preparation.*

Enzymes for Oligosaccharide Production

Functional oligosaccharides are the most frequently used ingredient for FOSHU claims (See back cover). Currently 9 different oligosaccharides are proved as functional oligosaccharides and are present in over 60 FOSHU food products. The functional oligosaccharides are difficult to digest with human digestive enzymes and are preferentially consumed by probiotic intestinal microflora. As a result the population of beneficial bacteria in the intestine (*Bifidobacteria* in the colon and *Lactobacilli* in upper gut) will increase. The known benefits of having abundant probiotics in the intestine are: suppress growth of harmful intestinal microflora, stimulate food digestion, boost the immune system, prevention of diarrhea, etc. Research to identify new health benefits of oligosaccharides is on going. Recent studies have shown that raffinose has anti-allergic and anti-atopic effects, and fructo-oligosaccharide increases calcium absorption.

It is well known that bananas and milk contain some functional oligosaccharides, but 15 bananas or 10-L milk per day may have to be consumed to obtain the benefits. Since it is too expensive to isolate functional oligosaccharides from natural sources, most of the functional oligosaccharides are now produced by enzyme reactions. Table 1 lists the commercial enzymes and raw materials used for functional oligosaccharide production. The research and development of new enzymes for oligosaccharide production is on going at Amano Enzyme Inc. as new benefits of oligosaccharide are discovered.

Amano has developed novel enzyme mixtures (US Patent 6,042,823), which synthesize functional oligosaccharides efficiently from food components (starch, sucrose and lactose) in the stomach. The enzyme mixtures are composed of amylase (Biodiastase 2000), α-glucosidase (Transglucosidase) and/or fructosyltransferase (Levansucrase). It is known that functional oligosaccharides are difficult to digest and are therefore preferentially consumed by probiotic intestinal microflora. Table 2 shows that the *Bifidobacteria* population in colon of piglets fed the enzyme mixture for 14 days with meals increased significantly. Table 3 shows that the enzyme mixture may prevent weight gain due to transforming digestible saccharides to indigestible oligosaccharides. The animal study also indicates that the enzyme mixture prevents an increase in blood glucose levels after meals. *We have been working toward the commercialization of levansucrase preparation.*

Table 1: Enzymes used for oligosaccharide production.

Oligosaccharide	Raw Material	Used Enzyme
Fructo-	Sucrose	β-Fructosyltransferase
Galacto-	Lactose	β-Galactosidase
Xylo-	Corncob	β-Xylanase
Lacto-fructo-	Sucrose Lactose	β-Fructofuranosidase
Isomalto-	Starch	β-Amylase Pullulanase Transglucosidase

Table 2: Piglet feeding study: Effect of oligosaccharide producing enzymes on *Bifidobacteria* population in the colon

Enzymes Fed	*Bifidobacteria* (log CFU)*			
	C	AC	DC	R
Levansucrase Transglucosidase Biodiastase 2000	7.9	8.4	8.8	8.1
Levansucrase	7.3	7.5	7.8	7.8
None (Control)	<6.0	6.9	7.5	7.6

C: cecum, AC: ascending colon,
DC: descending colon, R: rectum

*: Values represent an average *Bifidobacteria* population in different parts of the colon of two piglets fed enzymes with a basal diet plus dextrin, sucrose and lactose for 14 days.

Table 3: Piglet feeding study: Effect of oligosaccharide producing enzymes on weight gain.

Enzymes Fed	Daily Weight Gain (g/day)*
Levansucrase Transglucosidase Biodiastase 2000	59.3±16.6
Levansucrase	59.4±30.9
None (Control)	76.5±15.5

*: Values represent an average weight. gain of two piglets fed enzymes with a basal diet plus dextrin, sucrose and lactose for 14 days.

FOSHU
Japanese Health Food Approval System

厚生省許可
特定保健用食品

There is a worldwide trend for regulatory agencies to permit functional food products to make specific health claims. In Japan, the concept of functional foods began in the late 1980s and the approval system, FOSHU (Foods for Specified Health Use) was established in 1991. FOSHU are food products designated by the Ministry of Health, Labor and Welfare as effective for preservation of health by addition of certain functional ingredients or removal of undesirable ones. The added functional ingredients must have proved medical and nutritional benefits. Examples of added functional ingredients are dietary fiber, functional oligosaccharides, lactic acid bacteria, functional peptides, polyphenols and anthocyanins.

To obtain the FOSHU designation, the health benefits and safety of the food must be proved by clinical trials in Japan. The FOSHU claim and safety must be proved through the clinical trial on Japanese. FOSHU branded food products with functional ingredients are individually approved to carry various health claims, such as:

- Improve intestinal track conditions.
- Reduce blood cholesterol levels.
- Reduce hypertension.
- Promote mineral (calcium, iron) absorption.
- Reduce blood glucose levels.
- Reduce serum triglyceride levels.
- Prevent tooth decay.

Etc.

The approved products can be labeled with the FOSHU symbol as shown here. As of January 2001, over 220 branded food products have been approved as FOSHU. The current market size for FOSHU is estimated to be about $ 2 billion and still growing.

AMANO

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