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Growth Stimulation of *Pediococcus* sp. ISK-1 by *Sakekasu* (*Sake Filter Cake*) as a Substitute for Mevalonic Acid

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Pediococcus sp. ISK-1, isolated in our laboratory, has been found to inhabit in well-aged *Nukadoko* which is believed to have lasted for 360 years. We compared the influence of ethanol between *Lactobacillus homohiochii* JCM 1199^T and strain ISK-1 and found that the growth of strain ISK-1 was seriously inhibited by ethanol. However, fermentation of ISK-1 was strongly stimulated by *Yamahai-moto Sake* and *Sakekasu* (*Sake Filter Cake*). *Yamahai-moto Sake* is obtained from traditional brewing, therefore, it was possible that *Sakekasu* would have been used for the first *Nukadoko* preparation at that time. Addition of 8.0% NaCl and 2.0% *Sakekasu* in MRS broth resulted in substantially improving the cell population and lactate formation. Based on those facts, it is presumed that the first *Nukadoko* may have been prepared by the supplementation of *Sakekasu*, *Soy souce*, and *Salt* with selective pressure resulting in enrichment of strain ISK-1 of the bacteriocin producer.

INTRODUCTION

Pediococcus sp. ISK-1 was isolated in our laboratory from well-aged *Nukadoko* which is a rice bran packed fermentation bed for home-made Japanese traditional vegetable pickles (Herawati and Ishizaki, 1997). It is believed that this special *Nukadoko*, the source of isolation, had first been prepared by Lord Ogasawara, the feudal Lord of Kokura castle (Kitakyushu city) 360 years ago and had lasted until now without spoilage by bacterial contamination. We found that strain ISK-1 produces a new bacteriocin (Kimura *et al.*, 1997), and from this fact we introduce the hypothesis that this compound may have contributed to the long-time preservation of healthy microflora in this *Nukadoko*. In addition to bacteriocin, strain ISK-1 has very interesting characteristics such as strong arginine hydrolyzing activity, and vigorous oxygen formation from hydrogen peroxide. However, the growth of strain ISK-1 in nutrition medium such as complete medium with glucose (CMG) which consists of yeast extract and polypeptone as nutrition sources was very poor. About 60 h was needed to consume 30.0 g/l glucose at a cell density of approximately 4.5 g/l. It is, therefore, a question of how Lord Ogasawara had made the first *Nukadoko* to stimulate the growth of strain ISK-1 among other microorganisms inhabiting the environment. The special medium ingredient prepared by Lord Ogasawara at that time may provide the selective pressure of the screening of strain ISK-1 resulting in long-time preservation with ideal microflora in his *Nukadoko*. However, we cannot confirm this hypothesis because he did not leave any record of his experimental work.

In a previous paper, we found that D-mevalonic acid (MVA) strongly stimulated the growth of strain ISK-1 (Herawati and Ishizaki, 1997). Mevalonic acid the first found by

Tamura from *Koji* as hiochic acid (Tamura, 1956; Tamura, 1978; Tamura, 1994) and at almost the same time Skeggs *et al.* (1956) and Wright *et al.* (1956) reported isolation of mevalonic acid as a new acetate replacing factor. Tamura and Folkers (1957) then confirmed that hiochic acid and mevalonic acid were the same compound. The effect of MVA on the growth of a lactic acid microorganism was first reported by Kitahara *et al.* (1957a and 1957b) on *Lactobacillus homohiochii* isolated from *Sake* (Kitahara *et al.*, 1957a; 1957b). However, it is absolutely certain that no information on MVA was available in the Lord's time. We therefore presumed that the Lord used *Sakekasu* (*Sake* Filter Cake=SFC) as a substitute material to supply MVA for his first *Nukadoko* preparation. In his time, the *Sake* (Japanese rice wine) brewing process was a conventional one in which lactic acid fermentation was produced concomitantly by natural microflora (*Yamahai-moto* starter). In modern *Sake* brewing, pure lactic acid is added before vigorous alcohol fermentation for rapid brewing and proper process control to avoid contamination.

This paper deals with the stimulative effect of *Sakekasu* for the culture of *Pediococcus* sp. ISK-1, as a substitute for MVA. Also, the growth of strain ISK-1 was stimulated by *Sake* brewed by *Yamahai-moto*. Based on these observations, we can presume that the old *Nukadoko* was prepared by a special recipe for a medium using *Sakekasu* from the traditional *Sake* brewing process (*Yamahai-moto*).

MATERIALS AND METHODS

Microorganism

The microorganism used in this study was a bacteriocin producer (Kimura *et al.*, 1997), *Pediococcus* sp. ISK-1, isolated from well-aged *Nukadoko* preserved by Mrs. Teruko Sato in Kokura-ku, Kitakyushu city (it is believed that the originator of this *Nukadoko* is Lord Ogasawara) and stored in a deep-freeze refrigerator (-80°C). The microorganisms were grown at 37°C for 18 h in thioglycollate (TGC) medium (Difco Laboratories, Detroit, Mich.,) autoclaved at 121°C for 15 min. for rejuvenation. *Lactobacillus homohiochii* JCM 1199^T was purchased from the Japan Collection of Microorganisms (JCM), (Riken Wako, Saitama), and was grown at 30°C for 20 h in *Sake* medium.

Medium

The complete medium with glucose (CMG) used for flask culture was the same as that previously described (Herawati and Ishizaki, 1997). The basal medium for jar fermentation was MRS (deMan, Rogosa, and Sharpe) broth using 3.0% initial glucose. Synthetic medium was prepared according to the method described previously (Herawati and Ishizaki, 1997). The medium for *Lactobacillus homohiochii* JCM 1199^T consisted of *Sake* (*Fukutokucho Yamahai-moto*, Fukutokucho Co., Ltd., Kurume, Fukuoka) 700 ml, yeast extract (Difco Laboratories, Detroit, Mich.,) 5 g, liver extract concentrate (SIGMA Chemical Co., St. Louis, MO) 0.2 g and deionized water 100 ml. The pH of the medium was adjusted to 5.4, and the medium was autoclaved at 105°C for 10 min.

Sake used in this experiment is *Fukutokucho Ginjoshu* and *Fukutokucho Yamahai-moto* (brewed by *Yamahai* starter described above), a gift from Fukutokucho

Co., Ltd. (Kurume, Fukuoka), Gold prize (in a new brewed *Sake* contest for fiscal 1996) *Ginjoshu*, a gift from the Office of Brewing Technology, Fukuoka Regional Taxation Bureau, (Fukuoka) and *Kizakura* (Kizakura Brewing Co., Kyoto), purchased from a local *Sake* shop in Fukuoka. *Sakekasu* (cake discharged from the filter press in *Sake* filtration) of *Fukutokucho Yamahai-moto* is a gift from Fukutokucho Co., Ltd. (Kurume, Fukuoka). The dry matter content of *Sakekasu* was 37.7%. *Mieki* is a gift from Ajinomoto Co., Ltd. (Tokyo). (\pm) Mevalonic lactone (97%) was purchased from Aldrich Chem. Co., (Milw. WI).

Culture method

The culture method for the flask and jar cultures was the same as that described in the previous paper (Herawati and Ishizaki, 1997).

Bioassay

The medium for the bioassay of MVA by *Lactobacillus homohiochii* JCM 1199^T has been established by Tamura *et al.* (1968). However we simplified the medium composition based on the medium for *Lactobacillus homohiochii* given by JCM catalogue (1992) and obtained satisfactory result and good reproductivity. The bioassay medium for *Lactobacillus homohiochii* JCM 1199^T consisted of 8 parts of solution A and 2 parts of solution B. Solution A, the basal medium, consisted of casein peptone, tryptic digest (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD) 10 g, yeast extract (Difco Laboratories, Detroit, Mich.,) 5 g, meat extract (Kyokuto Seiyaku Co., Tokyo) 2 g, glucose 20 g, KH_2PO_4 0.5 g, K_2HPO_4 0.5 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 10 mg, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g, $\text{MnSO}_4 \cdot x\text{H}_2\text{O}$ 7.5 mg, Tween 80 1 ml, ethanol 40 ml, and sodium acetate 20 g, in 800 ml of deionized water. Solution B, standard and samples, was prepared as follows. In the case of MVA, 30 mg of (\pm) mevalonic lactone was dissolved in 200 ml deionized water. 1, 2, 3, 4, and 5 ml of this solution were then mixed with 9, 8, 7, 6, and 5 ml of deionized water, respectively. In the case of *Sake*, 10 ml of *Yamahai-moto Sake* was used and filled to 100 ml with deionized water. Then 1, 2, 3, 4, and 5 ml of this *Sake* solution were mixed with 9, 8, 7, 6, and 5 ml of deionized water, respectively. In the case of *Sakekasu*, 10 g of *Sakekasu* was weighed and dissolved into 80 ml of deionized water. The suspended solid material in the *Sakekasu* solution was then removed by filtration using Advantec 1011 filter paper 125 mm ϕ (Toyo Roshi Kaisha, Ltd., Tokyo), and the filtrate was filled to 100 ml. Then 1, 2, 3, 4, and 5 ml of *Sakekasu* solution were mixed with 9, 8, 7, 6, and 5 ml of deionized water respectively. Eight parts of solution A and 2 parts of solution B were mixed and the pH of the mixture was adjusted to 5.2. The mixture was subjected to autoclaving at 105°C for 10 min. Fifty μl of *Lactobacillus homohiochii* JCM 1199^T precultured by *Sake* medium was inoculated and cultured at 30°C for 65 h. Growth of the microorganism in individual test tubes was measured by the optical absorbance at 562 nm with a spectrophotometer (UVIDEC 320 JAS Co., Tokyo).

The medium for bioassay of MVA for *Pediococcus* sp. ISK-1 was the same as the medium for *Lactobacillus homohiochii* JCM 1199^T except for the omission of ethanol, Tween 80 and sodium acetate in solution A. In Tamura's medium, acetate was used to increase buffer capacity (Tamura *et al.*, 1968), and at the same time, we presume that it was taking into account that MVA was found as a acetate-replacing factor (Wright *et al.*,

1956). Strain ISK-1, however, produced acetic acid as by product and it inhibited the growth of strain ISK-1 (data not shown). Thus, we omitted acetate from the bioassay medium for strain ISK-1. Fifty μ l of strain ISK-1 precultured by TGC medium was inoculated and cultured at 37°C for 65 h. Growth of the microorganism in individual test tubes was measured. The increase in absorption for both microorganisms, *L. homohiochii* JCM 1199^T and strain ISK-1, was linear relative to the standard MVA concentration and the MVA activity in *Sake* and *Sakekasu*.

Analysis

The cell density of the broth and the concentrations of L-lactate, DL-lactate, and glucose were measured by the same method described previously (Herawati and Ishizaki, 1997).

RESULTS

Influence of ethanol on fermentation of *Pediococcus* sp. ISK-1

The influence of ethanol on strain ISK-1 was investigated. The basal medium used in this experiment was the synthetic medium (Herawati and Ishizaki, 1997). Ethanol (99.5%) was added to the autoclaved basal medium aseptically through a syringe with DISMIC-25 (Toyo Roshi Co., Ltd., Tokyo). The result is shown in Fig. 1. As shown in this figure, ethanol seriously inhibited the fermentation of strain ISK-1 and the fermentation was not entirely observed with 10.0% (v/v) ethanol. Because the fermentation of *Lactobacillus homohiochii* is stimulated by ethanol (Tamura, 1978; Tamura, 1994), the fermentation characteristics of strain ISK-1 are not the same as those of *L. homohiochii* JCM 1199^T.

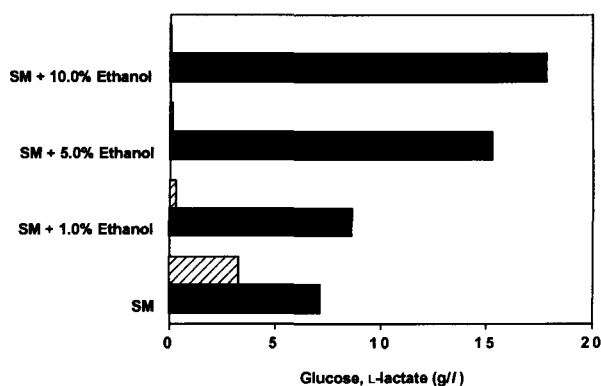


Fig. 1. Effect of Ethanol Supplementation in Synthetic Medium (SM) on Lactate Fermentation Using *Pediococcus* sp. ISK-1 in Flask Culture at 24 h.

Glucose consumption (■), L-lactate production (▨)

Stimulative effect of *Yamahai-moto Sake* on fermentation of *Pediococcus* sp. ISK-1

The effect of *Yamahai-moto Sake* which may contain various amino acids from rice protein and MVA on the growth of strain ISK-1 was investigated. Fig. 2 shows the results of the experiment using a synthetic medium as the basal medium. As shown in this figure, *Yamahai-moto Sake* stimulated the fermentation of strain ISK-1 in spite of the ethanol contained in the *Sake*. To observe the effect of amino acid on the fermentation of strain ISK-1, 5.0% *Mieki* was added. *Mieki* strongly stimulated the fermentation, however, the effect of *Yamahai-moto Sake* (10.0%) was slightly greater than that of *Mieki* (5.0%). Taking into account the inhibitory effect of the ethanol existing in *Sake*, the stimulative effect of *Yamahai-moto Sake* may be due to the MVA contained in *Sake*. The same result was obtained in an experiment using CMG as the basal medium (Fig. 3). As shown in Fig. 3, the fermentation using CMG + 5.0% *Mieki* + 10.0 ppm MVA was better than the fermentation using the medium containing 10.0% *Yamahai-moto Sake* in place of 10.0 ppm MVA. This may be due to the ethanol contained in *Sake*.

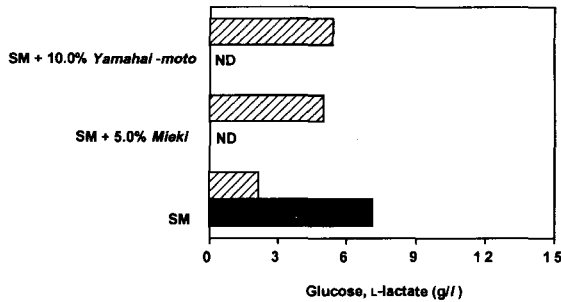


Fig. 2. Effect of *Mieki* and *Yamahai-moto Sake* Supplementation in SM on Lactate Fermentation Using *Pediococcus* sp. ISK-1 in Flask Culture at 24 h.

Symbols are the same as in Fig. 1.
ND : not detected.

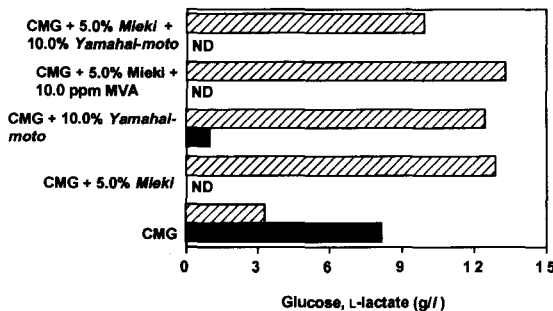


Fig. 3. Effect of *Mieki*, MVA and *Yamahai-moto Sake* Supplementation in CMG Medium on Lactate Fermentation Using *Pediococcus* sp. ISK-1 in Flask Culture at 24 h.

Symbols are the same as in Fig. 1.
ND : not detected.

Evaluation of the activity equivalent to mevalonic acid contained in *Sake* and *Sakekasu* by bioassay employing *Lactobacillus homohiochii* JCM 1199^T and Strain ISK-1

The growth of *L. homohiochii* JCM 1199^T and strain ISK-1 at MVA concentrations of 3, 6, 9, 12, and 15 ppm was measured as a standard. The results are shown in Fig. 7. From the result, relationship between the OD (optical density) of *L. homohiochii* JCM 1199^T and the MVA concentration (Xm ppm) was expressed as :

$$(OD)_h = 0.260 + 2.667 \times 10^{-2} X_m \quad (r^2 = 1.000) \quad (\text{Fig. 7a})$$

while that of strain ISK-1 was

$$(OD)_p = 0.520 + 6.667 \times 10^{-3} X_m \quad (r^2 = 1.000) \quad (\text{Fig. 7b})$$

where $(OD)_h$ is the OD of *L. homohiochii* JCM 1199^T, $(OD)_p$ is the OD of *Pediococcus* sp. ISK-1 and r^2 is a regression coefficient. Thus, the MVA unknown concentration can be determined from the OD value by the relationship for *L. homohiochii* JCM 1199^T

$$X_m = \{(OD)_h - 0.260\} / 0.027$$

and for strain ISK-1

$$X_m = \{(OD)_p - 0.520\} / 0.007.$$

Fig. 8. shows the results of the bioassay for *Sake*. The relationship between the OD

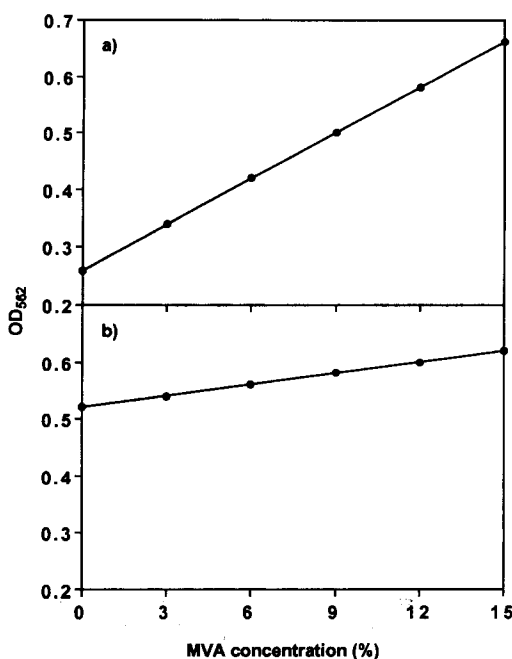


Fig. 7. Relationship between MVA Concentration and Growth of *Lactobacillus homohiochii* JCM 1199^T (a) and *Pediococcus* sp. ISK-1 (b).

to prevent contamination during *Sake* brewing. We presume that this forms a high MVA concentration in *Sake* so that *Yamahai-moto Sake* stimulates the fermentation of strain ISK-1.

However, it is suspicious that the different result observed among three brands may be due to different technology of individual brewer. Thus another experiment has been carried out that *Ginjoshu* of *Fukutokucho* and *Yamahai-moto* of *Fukutokucho* were compared at the same culture condition. Results are shown in Fig. 5. According to the results, it is clear that *Yamahai-moto Sake* gave excellent result. *Yamahai-moto Sake* may contain strong growth promotor than the one of *Ginjoshu* which is from ordinary brewing process.

Effect of *Sakekasu* (*Sake* Filter Cake)

From the results stated above, it is assumed *Yamahai-moto Sake* contains a high concentration of MVA. This suggests that *Sakekasu* must be a good source of MVA. *Sakekasu* from *Yamahai-moto* brewing, a gift from *Fukutokucho Co., Ltd.*, was used to confirm this hypothesis. The results are shown in Fig. 6. From this figure, *Sakekasu* is observed to stimulate the fermentation of strain ISK-1 with an increase in *Sakekasu* concentration for both CMG and bioassay medium in which ethanol, Tween 80 and sodium acetate were omitted.

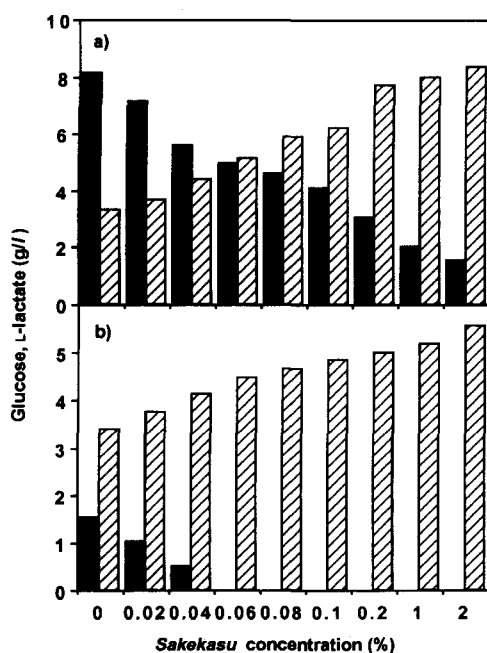


Fig. 6. Effect of *Sakekasu* Supplementation in CMG (a) and SM (b) on Lactate Fermentation Using *Pediococcus* sp. ISK-1 in Flask Culture at 24 h.

Symbols are the same as in Fig. 1.

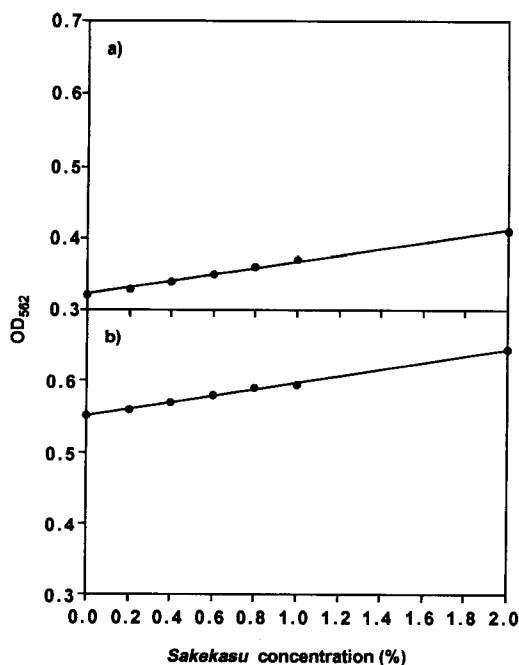


Fig. 9. Relationship between *Sakekasu* Concentration and Growth of *Lactobacillus homohiochii* JCM 1199^T (a) and *Pediococcus* sp. ISK-1 (b).

stronger than that on *L. homohiochii* JCM 1199^T.

Effect of *Sakekasu* on MRS medium

Strain ISK-1 can grow very well on an MRS medium (Kimura *et al.*, 1997). The effect of *Sakekasu* on MRS medium was investigated. As shown in Fig. 10, a high NaCl concentration (8.0%) stimulated lactate formation. With a high NaCl concentration in MRS, *Sakekasu* increased the lactate formation in accordance with the increase in *Sakekasu* concentration.

Jar culture test

To confirm the results obtained in the flask culture tests, we performed a jar culture experiment at a regulated pH. The results are shown in Fig. 11. Addition of 8.0% NaCl and 2.0% *Sakekasu* to MRS medium containing 3.0% glucose (control medium) is shown to stimulate cell population tremendously and to reduce fermentation time to the control medium.

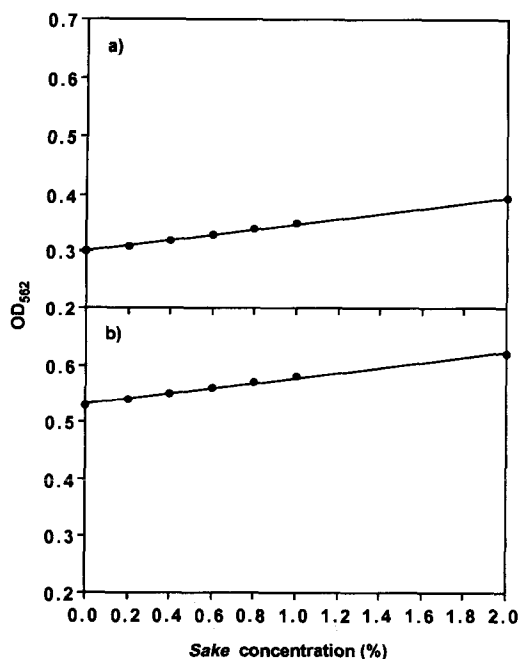


Fig. 8. Relationship between *Sake* Concentration and Growth of *Lactobacillus homohiochii* JCM 1199^T (a) and *Pediococcus* sp. ISK-1 (b).

of the microorganism and the *Sake* concentration (X_s %) was expressed as :

$$(OD)_h = 0.301 + 4.755 \times 10^{-2} X_s \quad (r^2 = 0.999)$$

for *L. homohiochii* JCM 1199^T (Fig. 8a) and

$$(OD)_p = 0.532 + 4.511 \times 10^{-2} X_s \quad (r^2 = 0.996)$$

for strain ISK-1 (Fig. 8b).

Therefore, the MVA concentration equivalent in *Sake*, $(MVA)_{seq}$, can be estimated by the relationship in which 1.0% *Sake* = 1.8 ppm $(MVA)_{seq}$ for *L. homohiochii* JCM 1199^T but 6.8 ppm $(MVA)_{seq}$ for strain ISK-1.

Fig. 9. shows the results of the bioassay for *Sakekasu*. The relationship between the OD of the microorganism and the *Sakekasu* concentration (X_k %) was expressed as :

$$(OD)_h = 0.322 + 4.522 \times 10^{-2} X_k \quad (r^2 = 0.996)$$

for *L. homohiochii* JCM 1199^T (Fig. 9a) and

$$(OD)_p = 0.551 + 4.701 \times 10^{-2} X_k \quad (r^2 = 0.998)$$

for strain ISK-1 (Fig. 9b).

Therefore, the MVA concentration equivalent in *Sakekasu*, $(MVA)_{keq}$, can be estimated by the relationship in which 1.0% *Sakekasu* = 1.7 ppm $(MVA)_{keq}$ for *L. homohiochii* JCM 1199^T but 7.1 ppm $(MVA)_{keq}$ for strain ISK-1.

Based on this fact, the MVA concentration in *Sake* can be said to be almost the same as that in *Sakekasu*. The MVA activity on strain ISK-1 is also said to be 4 times

DISCUSSION

Pediococcus sp. ISK-1 was isolated from well-aged *Nukadoko* which is believed to have been first prepared by Lord Ogasawara 360 years ago. Strain ISK-1 has very interesting characteristics in addition to producing a novel bacteriocin. There are a few other well-aged *Nukadokos* in Kokura and Fukuoka (Northern Kyushu territory) area and all those *Nukadokos* are believed to be originated by Lord Ogasawara. This is, however, a spread wide story among citizens without any scientific proof. However, it is a fact that these *Nukadokos* have been inoculated and transferred for many generations because we can prove it by the owner's word. We presume that the bacteriocin produced by the microorganism inhabiting this *Nukadoko* has the function of maintaining ideal microflora in the well-aged *Nukadoko*. Our next question was how the first *Nukadoko* was prepared to enrich the bacteriocin producer among the natural microflora. Lord Ogasawara did not leave any record of his experiment, so it is impossible to confirm his method from scientific records. However, as mentioned in this paper, we found that MVA or MVA equivalent activity stimulated tremendously the fermentation of strain ISK-1. In addition, the strong activity of this compound has been confirmed in *Sake* and *Sakekasu*, and very strong activity was observed in *Sakekasu* from a traditional *Sake* brewing process (*Yamahai-moto*). Mevalonic acid was first found as hiochic acid by Tamura (1956; 1978; 1994) from *Koji*, and it was presumed that hiochic acid in *Sake* came from *Koji*. However, Wright *et al.* (1956) isolated MVA from distillers dried soluble from whiskey distiller where no *koji* was used. Tamura surveyed the microorganism for fermentative production of MVA and found that lactic acid bacteria produced MVA (Wagner and Folkers, 1961; Tamura, 1994). We, therefore, presumed that lactic acid bacteria working in *Yamahai* process produced MVA thus *Yamahai-moto Sake* gave strong growth promoting effect on strain ISK-1. Because the *Yamahai-moto* process is the traditional method for *Sake* brewing, it is certain that, in the Lord's time, *Sake* and *Sakekasu* from the traditional *Yamahai-moto* brewing process were available. Considering that strain ISK-1 is sensitive to ethanol, it is presumed that Lord Ogasawara used *Sakekasu* but not *Sake* for his *Nukadoko* preparation. In addition to MVA, the fermentation of strain ISK-1 was stimulated by *Mieki* and NaCl. *Mieki* is a soybean protein hydrolyzate; therefore, this is very similar to the traditional fermented soy sauce. Based on those facts, we can imagine that the first *Nukadoko* was prepared using rice bran (*nuka*) mixed with *Sakekasu*, *Shoyu* (traditional fermented soy sauce), and salt. This composition may provide the selective pressure applied by the Lord to form the ideal microflora for good quality *Nukadoko*. We isolated another bacteriocin producer from other well-aged *Nukadokos* which are also believed to originate from Lord Ogasawara but have been preserved by other persons. The characteristics and similarity of this new strain compared to strain ISK-1 are now under investigation.

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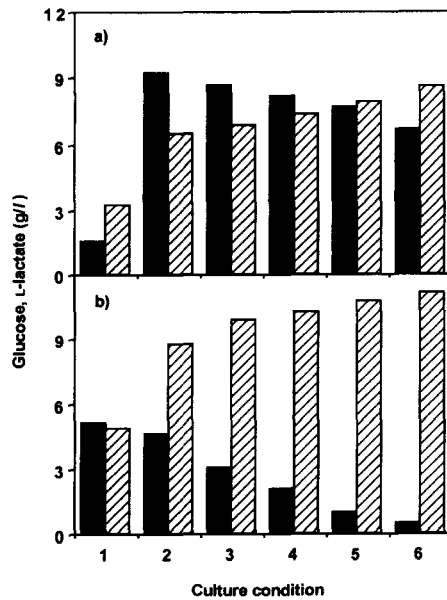


Fig. 10. Effect of *Sakekasu* and NaCl Supplementation in SM (a) and MRS Medium (b) on Lactate Fermentation Using *Pediococcus* sp. ISK-1 in Flask Culture at 24 h.

Culture condition :

(1) Control (CTR) for SM (a) and MRS (b), (2) CTR+8.0% NaCl, (3) CTR+8.0% NaCl+0.1% *Sakekasu*, (4) CTR+8.0% NaCl+0.2% *Sakekasu*, (5) CTR+8.0% NaCl+1.0% *Sakekasu*, (6) CTR+8.0% NaCl+2.0% *Sakekasu*

Symbols are the same as in Fig.1.

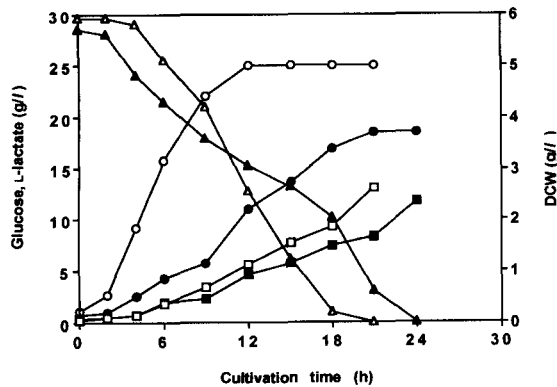


Fig. 11. Comparison of the Growth (DCW), Glucose Consumption and L-Lactate Production of *Pediococcus* sp. ISK-1 in Jar Culture.

DCW (●—○—), glucose consumption (—▲—△—), L-lactate production (—■—□—). Closed symbol is MRS medium, open symbol is MRS + 8.0% NaCl+2.0% *Sakekasu*.

DISCUSSION

Pediococcus sp. ISK-1 was isolated from well-aged *Nukadoko* which is believed to have been first prepared by Lord Ogasawara 360 years ago. Strain ISK-1 has very interesting characteristics in addition to producing a novel bacteriocin. There are a few other well-aged *Nukadokos* in Kokura and Fukuoka (Northern Kyushu territory) area and all those *Nukadokos* are believed to be originated by Lord Ogasawara. This is, however, a spread wide story among citizens without any scientific proof. However, it is a fact that these *Nukadokos* have been inoculated and transferred for many generations because we can prove it by the owner's word. We presume that the bacteriocin produced by the microorganism inhabiting this *Nukadoko* has the function of maintaining ideal microflora in the well-aged *Nukadoko*. Our next question was how the first *Nukadoko* was prepared to enrich the bacteriocin producer among the natural microflora. Lord Ogasawara did not leave any record of his experiment, so it is impossible to confirm his method from scientific records. However, as mentioned in this paper, we found that MVA or MVA equivalent activity stimulated tremendously the fermentation of strain ISK-1. In addition, the strong activity of this compound has been confirmed in *Sake* and *Sakekasu*, and very strong activity was observed in *Sakekasu* from a traditional *Sake* brewing process (*Yamahai-moto*). Mevalonic acid was first found as hiochic acid by Tamura (1956; 1978; 1994) from *Koji*, and it was presumed that hiochic acid in *Sake* came from *Koji*. However, Wright *et al.* (1956) isolated MVA from distillers dried soluble from whiskey distiller where no *koji* was used. Tamura surveyed the microorganism for fermentative production of MVA and found that lactic acid bacteria produced MVA (Wagner and Folkers, 1961; Tamura, 1994). We, therefore, presumed that lactic acid bacteria working in *Yamahai* process produced MVA thus *Yamahai-moto Sake* gave strong growth promoting effect on strain ISK-1. Because the *Yamahai-moto* process is the traditional method for *Sake* brewing, it is certain that, in the Lord's time, *Sake* and *Sakekasu* from the traditional *Yamahai-moto* brewing process were available. Considering that strain ISK-1 is sensitive to ethanol, it is presumed that Lord Ogasawara used *Sakekasu* but not *Sake* for his *Nukadoko* preparation. In addition to MVA, the fermentation of strain ISK-1 was stimulated by *Mieki* and NaCl. *Mieki* is a soybean protein hydrolyzate; therefore, this is very similar to the traditional fermented soy sauce. Based on those facts, we can imagine that the first *Nukadoko* was prepared using rice bran (*nuka*) mixed with *Sakekasu*, *Shoyu* (traditional fermented soy sauce), and salt. This composition may provide the selective pressure applied by the Lord to form the ideal microflora for good quality *Nukadoko*. We isolated another bacteriocin producer from other well-aged *Nukadokos* which are also believed to originate from Lord Ogasawara but have been preserved by other persons. The characteristics and similarity of this new strain compared to strain ISK-1 are now under investigation.

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