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Effect of Mevalonic Acid on Lactate Fermentation Using Pediococcus sp. ISK-1

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The growth of a newly isolated strain of *Pediococcus* sp., designated ISK-1 was very slow and the concentration of cells in the medium remained low. Fermentation with an initial 30 g/l glucose required about 60 h. In order to stimulate fermentation, we attempted to optimize of the medium by flask culture and jar fermentation tests. Mevalonic acid and *mieki (soy* bean hydrolyzate) stimulated fermentation and increased the rate of formation of x-lactate. Kinetic analysis of the fermentation revealed that mevalonic acid increased markedly the specific glucose consumption rate and the specific lactate production rate. *Mieki* and mevalonic acid had a synergistic effect, but the effect of mevalonic acid was different from that of *mieki*.

INTRODUCTION

Pediococcus spp. are present as natural flora in various fermented foods and are used as commercial starter cultures in the production of fermented sausages (Amy and Hoover, 1988), sauerkraut and pickles (Blickstad and Goran, 1981). Many types of fermented food are produced from plant sources throughout the world and they can be divided into several groups. *Pediococci* are found as very common microorganisms in various foods, but, the studies of this genus have been limited. *Pediococcus* sp. ISK-1 was isolated in our laboratory from *nukadoko* which is the name given to the rice-bran packed fermentation bed used for preparation of home-made Japanese traditional pickled vegetables. Strain ISK-1 has very interesting characteristics; it produces a novel bacteriocin; it has strong arginine-hydrolyzing activity; it generates oxygen efficiently from hydrogen peroxide. However, its growth in nutrient medium is very poor. In complete medium with glucose (CMG), which consisted of yeast extract and polypeptone as nutrients, about 60 h were required for consumption of 30 g/l glucose at a cell density of approximate 4.5 g/l. For reasonable growth and the production of lactate, it is important to improve the growth medium for this microorganism. It seem that the genus *Pediococcus* has rather complex nutritional requirements, and that amino acids, vitamins and special growth promoters, such as mevalonic acid, are required for its rapid growth.

The effects of mevalonic acid on the growth of lactic acid microorganisms were first reported by Kitahara et *al.,* (1957). *Lactobacillus homohiochi,* which was isolated from sake, failed to growth in MRS broth (Sneath et al., 1986). However in Rogosa SL broth (Sneath et *al., 1986)* supplemented with DL-mevalonic acid (30 g/l) and ethanol (40 g/l) copious growth is obtained after a marked lag phase of 4-7 days. Thus, it seems that mevalonic acid is essential or highly stimulator-y and that ethanol is promotive for growth of *L. homohiochi.* However, no reports on the effects of mevalonic acid on the genus *Pediococcus* are available. We describe here the stimulatory effects of mevalonic acids as

well as of amino acids, on the growth of *Pediococcus* sp. ISK-1, a new strain isolated from *nukadoko*. We also describe our investigations of the fermentation kinetics of this strain in improved media during jar culture.

MATERIALS AND METHODS

Microorganism

The microorganism used in this study was *Pediococcus* sp. ISK-1 that had been stored in a deep freezer (-80°C). The microorganisms were grown at 37°C for 18 h in thioglycolate (TGC) medium (Difco Laboratories, Detroit, MI) that had been autoclaved at 121 °C for 15 min. for rejuvenate.

Medium

A 100-m*l* aliquot of CMG consisting of 5.0 g yeast extract, 5.0 g polypeptone, 5.0 g NaCl and 10.0 g glucose in 1 liter of deionized waters was used for preculture. A 400-ml aliquot of CMG prepared with only 3.0 g/t yeast extract and with 30.0 g/*l* glucose was used for jar cultures. The pH of the medium was adjusted to 7.0 with 1 N KOH and 1 N HCl and the medium was sterilized at 121 °C for 15 min. An amino acid mixture was prepared such that the amino acid composition of the medium would be equivalent to medium that contained 5.0% *mieki*. Thus, 130 mg of L-alanine, 250 mg of L-arginine, 500 mg of L-aspartic acid, 12 mg of L-cysteine, 850 mg of L-glutamic acid, 220 mg of glycine, 110 mg of L-histidine, 180 mg of L-isoleucine, 200 mg of L-leucine, 300 mg of L-lysine, 25 mg of L-methionine, 200 mg of L-phenylalanine, 200 mg of L-serine, 170 mg of L-threonine, 60 mg of L-tyrosine, and 235 mg of L-valine were dissolved in 50 ml of deionized water. The amino acid solution was then added to 50 ml of 2 × CMG and the solution was brought to 100 ml. Synthetic medium was prepared by the method described by Uchimura and Okada (1992). The medium consisted of 20 g of glucose, 10 g of potassium acetate, 3 g of NH_4Cl, 500 mg of K_2HPO_4, 500 mg of KH_2PO_4, 200 mg of $MgSO_4 \cdot 7H_2O$, 10 mg of $MnSO_4$ $4H_2O$, 10 mg of $FeSO_4 7H_2O$, 10 mg of NaCl, 1 mg of thiamine HCl, 1 mg of riboflavin, 1 mg of niacin, 1 mg of Ca-pantothenate, 1 mg of pyridoxine HCl, 1 mg of pyridoxal HCl, 200 μg of p-amino benzoic acid, 10 µg of folic acid, 10 µg of biotin, 200 mg of L-alanine, 200 mg of L-arginine HCl, 200 mg of L-aspartic acid, 100 mg of L-cystein HCl, 500 mg of L-glutamic acid (sodium salt), 100 mg of glycine, 100 mg of L-histidine HCl, 100 mg of L-isoleucine, 100 mg of L-leucine, 200 mg of L-lysine HCl, 100 mg of L-methionine, 100 mg of L-phenylalanine, 100 mg of L-proline, 50 mg of L-serine, 100 mg of L-threonine, 50 mg of L-tryptophane, 100 mg of L-tyrosine, 100 mg of L-valine, 10 mg of adenine H_2SO_4, 10 mg of guanine HCl, 10 mg of uracil, and 10 mg of xanthine per liter of deionized water. The pH was adjusted to 6.8 with 1 N NaOH and 1 N HCl and the medium was autoclaved at 110°C for 10 min. All amino acids (medical grade) and *mieki* (soybean protein hydrolysate) were a gift from Ajinomoto Co. Ltd. (Tokyo, Japan).

(±) Mevalonic lactone (97%) was purchased from Aldrich Chemical. Co., (Milw, WI).

Flask culture for optimization of the medium

Flask culture was used for the screening of effective components of the medium. Preculture was carried out in 100 ml of CMG in an Erlenmeyer flask. After inoculation with 10 ml of subculture, the flask was incubated in a water bath at 37°C for 6 h with agitation of 100 rpm. The pH of the culture broth was maintained at an almost constant value by addition of 2.0% (w/v) $CaCO_3$ after the initial stage of the culture. Flasks were shaken at 100 oscilations per min. with a stroke of 25 mm in a water bath whose temperature was maintained at 37°C. The "one at a time" method was used for screening (Chen et al., 1981; Chynier *et al.*, 1983; Bowman and Geiger, 1984; Zertuche and Zall, 1985; Houng et al., 1989; Silveira *et al.*, 1991). In this method, one component at different concentrations is tested without any change in the other constituents of the basal medium. The effects of the target component on various kinetic parameters of fermentation were then evaluated. The optimum medium was prepared with the optimum levels of the selected ingredients.

Jar culture experiment

Twenty ml of the preculture were inoculated into a 1-Z jar fermentor with a working volume of 400 m*l*. The culture was incubated at 37°C with agitation of 400 rpm without gas flow. The pH was controlled at 6.0 by feeding 2 N NaOH.

Analysis

The cell density of the culture was determined from the optical absorbance at 562 nm, measured with a spectrophotometer (Uvidec 320; JAS Co., Tokyo, Japan). The absorbance was then converted to DCW (dry cell weight) by reference to a pre-determined standard curve. Glucose and L-lactate were quantitated by enzymatic analyzers (models 23A and 23L respectively; Yellow Spring International Co. Ltd., Ohio, USA). DL-lactate was quantitated by HPLC (model 638-30; Hitachi, Tokyo, Japan with BIO-RAD an HPX-87H column) and an mobile phase of 0.005 N H_2SO_4. Optical absorbance was monitored with a UV-VIS detector (model SPD-10 A; Shimadzu Co., Kyoto, Japan). The DL-lactate concentration in the culture broth in which DL-lactate formed a sediment as calcium compound was determined from the following formula :

$$Y = 1.812\ X$$

where Y is the true concentration of L-lactate (g/l) in a culture medium that contains 2.0% $CaCO_3$ at concentrations of L-lactate from 0 to 5.74 g/Z and X is the concentration of L-lactate (g/l) measured by an enzyme sensor at concentrations of L-lactate from 0 to 10.4 g/*l*.

RESULTS

Growth of Pediococcus sp. ISK-1 in CMG

In a standard experiment, strain ISK-1 was cultured in a jar fermentor in CMG (30 g/*l* glucose) to serve as the control culture. Figure 1 shows the time course of fermentation. Culture conditions for the jar culture were a temperature of 37°C, pH of 6.0, and gentle agitation without gas flow, as described in Materials and Methods. Samples were

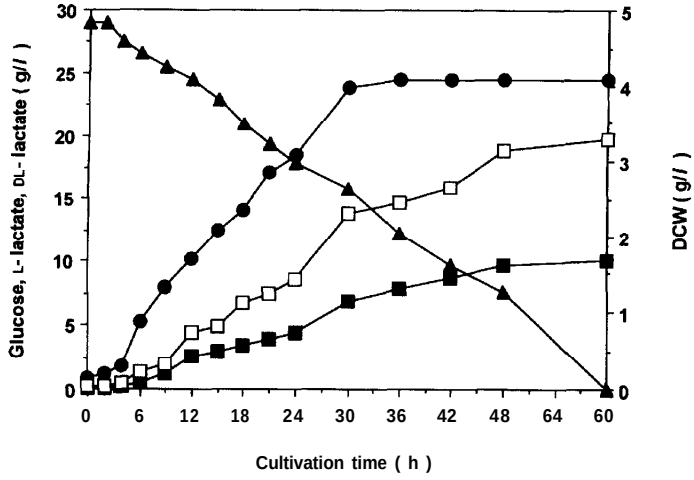

Fig. 1. Time course for jar culture of *Pediococcus* sp. ISK-1 grown in CMG. DCW (●), glucose consumption (A), L-lactate production (■), DL-lactate production (□).

withdrawn at approximately two or three hour intervals during the lag phase, at six hour intervals during the logarithmic phase and twelve hour intervals during the stationary phase and analized immediately. The concentration of cells reached its a maximum value of 4.08 *g/l* at stationary phase, when nearly 50% of the initial glucose had been consumed at 36 h and the concentration of L-lactate was 7.80 g/*l* (14.75 g/*l* of DL-lactate). Fermentation was completed a within 60 h when 10.1 *g/l* L-lactate and 19.7 g/E DL-lactate had accumulated. Although CMG contains excellent sources of nutrients, such as yeast extract and polypeptone, and with glucose is present as a carbon source, the growth of strain ISK-1 was very slow and the rate of accumulation of lactic acid was unsatisfactory. When sucrose was used in place of glucose as a carbon source, the fermentation rate increased slightly (data not shown). Therefore, in order to study the characteristics of this strain, it was necessary to improve the medium and culture conditions to obtain higher cell densities and concentrations during relatively short times.

Effect of Tween 80, *mieki* and amino acid mixture

Tween 80 has often been used in the medium for growth of anaerobic microorganisms, such as MRS medium and Bifidobacterium medium (Oiki *et al.*, 1996). We tested the effects of Tween 80 on the growth and fermentation of strain ISK-1 in flask culture. Then we studied the effects of amino acids using *mieki*. *Mieki* which is soybean protein hydrolysate provided by Ajinomoto Co, Ltd, Tokyo. The total nitrogen content of mieki was equivalent to 3.5 *g/l*.

The basal medium used in the experiments in flask culture was CMG containing 2.0% $CaCO_3$. As shown in Figure 2, Tween 80 had the opposite effect on both growth and

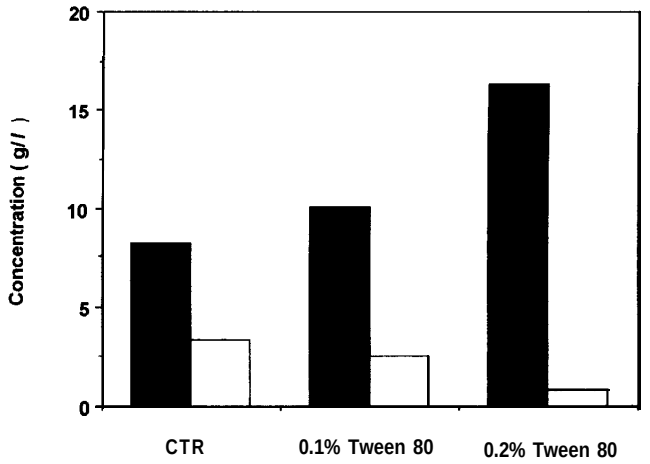

Fig. 2. Effect of Tween 80 on fermentation by *Pediococcus* sp. ISK-1 in flask culture at 24 h. Glucose consumption (■), L-lactate production (□).

fermentation. The residual glucose and L-lactate concentrations in the CMG used as control medium (CTR) were 8.16 and 3.33 g/*l* respectively. Supplementation of 0.1% and 0.2% Tween 80 inhibited the consumption of glucose and the production of L-lactate. The residual glucose and the L-lactate production in CMG with 0.1% Tween 80 and 0.2% Tween 80 were 10.06 and 2.49, and 16.33 and 0.83 g/Z respectively. Next we postulated that strain ISK-1 might have very complex requirements for ammo acid. The amino acid composition of mieki, according to the supplier, is 5.4 g of L-alanine, 10.0 g of L-arginine, 20.5 g of L-aspartic acid, 0.5 g of L-cysteine, 34.5 g of L-glutamic acid, 8.8 g of glycine, 4.4 g of L-histidine, 7.4 g of L-isoleucine, 8.0 g of L-leucine, 12.2 g of L-lysine, 0.5 g of L-methionine, 8.0 g of L-phenylalanine, 8.0 g of L-serine, 7.0 g of L-threonine, 2.5 g of L-tyrosine, 9.5 g of L-valine per liter. We examined the effects of concentrations of *mieki* from 1.0% to 5.0%. Figure 3 shows that mieki stimulated fermentation and product formation. As compared with the control culture, *mieki* at 3.0% stimulated glucose consumption and lactate formation. At 5.0% mieki, the initial glucose (30 g/*l*) was completely consumed within 24 h and almost 50% of the glucose was converted to L-lactate. Further studies were carried out to investigate whether the effect of mieki was due to amino acids or to growth promoters other than amino acids. A flask culture with medium plus an amino acid mixture with concentrations of ammo acids that were almost the same as those in 5.0% mieki was carried out. Flask culture tests using synthetic medium consisted of minerals, vitamins and amino acids were also carried out. Results are shown in Figure 4. CMG with *mieki* was markedly better than CMG with ammo acid mixture. Amino acid concentrations in the synthetic medium were almost the same as those in medium supplemented with *mieki* or with the ammo acid mixture. However, the fermentation

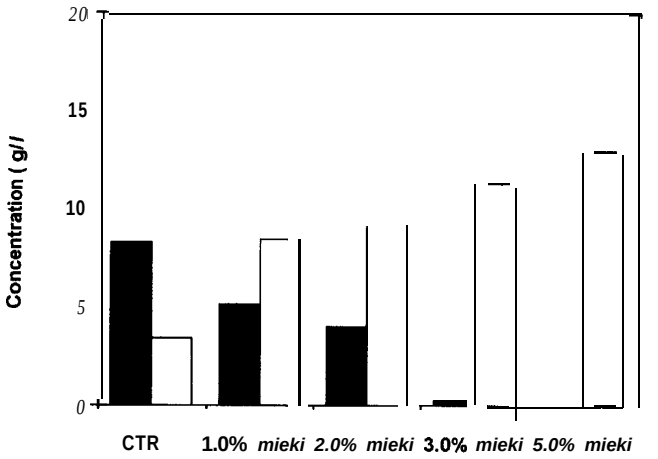

Fig. 3. Effect of *mieki* on fermentation by *Pediococcus* sp. ISK-1 in flask culture at 24 h.
Symbols are the same as in Fig. 2.

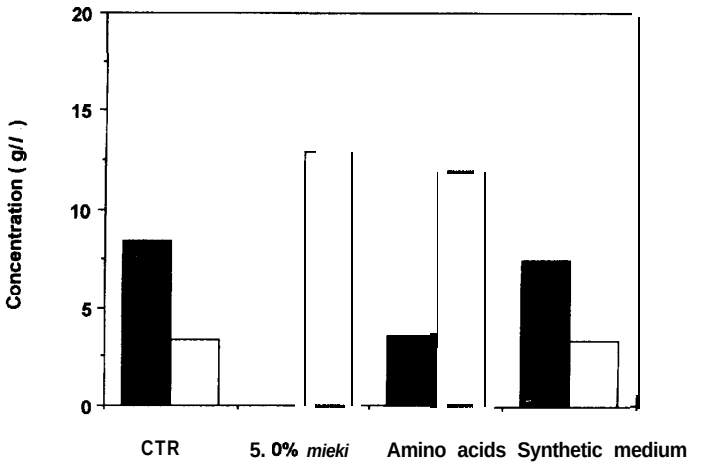

Fig. 4. Effect of *mieki*, amino acids and synthetic medium on fermentation by *Pediococcus* sp. ISK-1 in flask culture at 24 h.
Symbols are the same as in Fig. 2.

rate and product formation in the synthetic medium were very much lower than those in CMG with mieki and CMG with the amino acid mixture. These observations suggest that natural nutrients, such as yeast extract and polypeptone, contain excellent growth promoters that cannot be provided by the synthetic medium. Moreover, *mieki* might contain special growth promoters for strain ISK-1 in addition to amino acids. It is known that *mieki* contains large amounts of levulinic acid. However, this acid did not affect the growth of strain ISK-1 (data not shown). To identify the growth promoter of strain ISK-1, we tested the effects of mevalonic acid.

Effects of mevalonic acid

In order to obtain a better understanding of the growth and lactic acid production by ISK-1 in complete medium, we carried out flask cultures test with added mevalonic acid. Using the same basal medium as in the flask cultures described in the previous section, we investigated the effects of mevalonic acid at concentrations from 1 ppb to 100 ppm on the growth and lactate production by ISK-1. The results are shown in Figure 5. As shown in this Figure 5, the effect of mevalonic acid was remarkable observed. At 100 ppm mevalonic acid, substrate consumption and lactate formation were stimulated and results of fermentation after a 24 h culture were almost the same as those obtained with CMG plus 5.0% mieki.

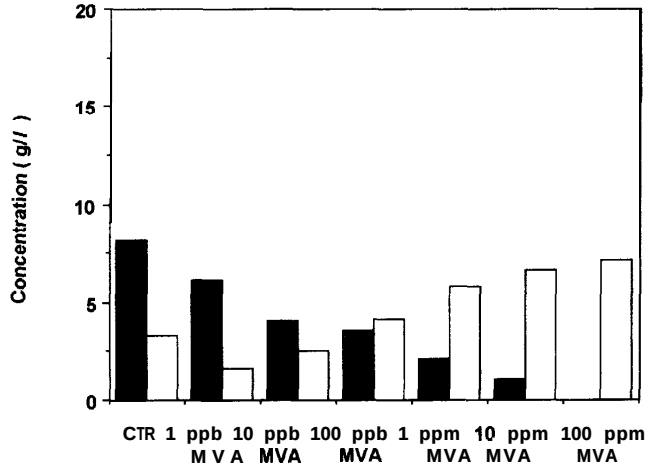

Fig. 5. Effect of mevalonic acid on fermentation by *Pediococcus* sp. ISK-1 in flask culture at **24** h.
Symbols are the same as in Fig. 2.

Optimization of jar culture

To confirm the results obtained in the flask culture tests, we performed a jar fermentation experiment at a regulated pH. CMG with 5.0% mieki, CMG with 10 ppm mevalonic acid and CMG with 5.0% ***mieki*** and 10 ppm mevalonic acid were tested. The results are shown in Figure 6, 7 and 8. Figure 6 shows that in CMG with 5.0% ***mieki,*** the

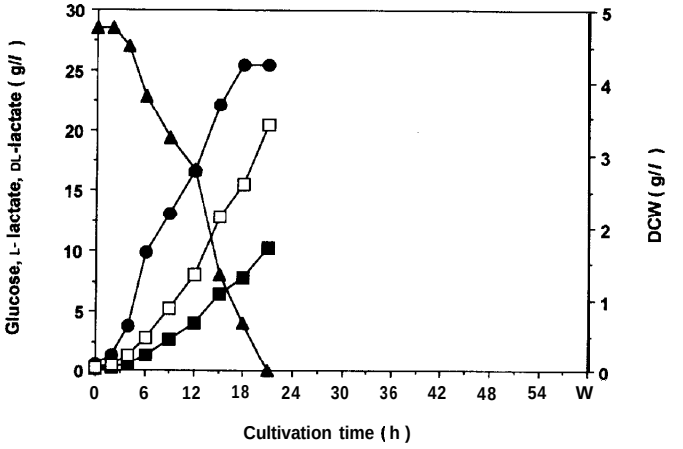

Fig. 6. Time course for jar culture of Pediococcus sp. ISK-1 grown in CMG with 5.0% *mieki*.
Symbols are the same as in Fig. 1.

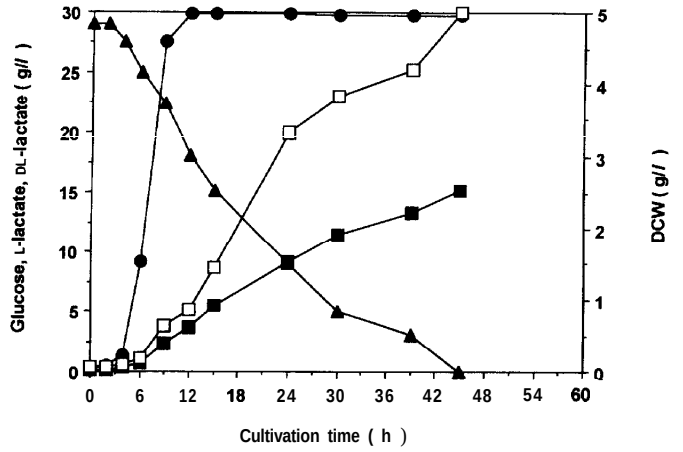

Fig. 7. Time course for jar culture of *Pediococcus* sp. ISK-1 grown in CMG with 10 ppm mevalonic acid.
Symbols are the same as in Fig. 1.

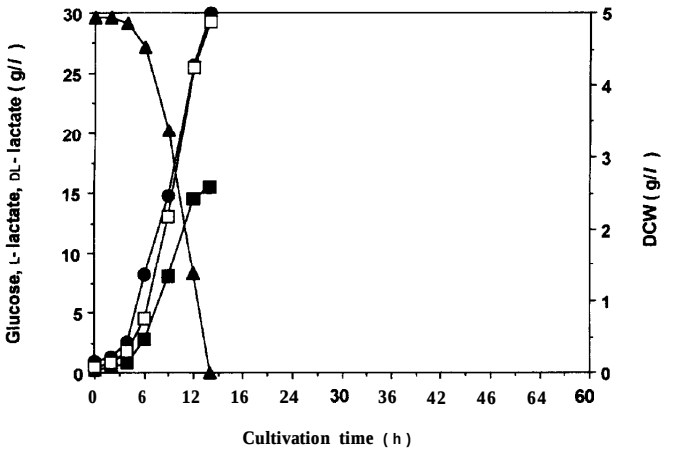

Fig. 8. Time course for jar culture of *Pediococcus* sp. ISK-1 grown in CMG with **5.0%** *mieki* and 10ppm mevalonic acid.
Symbols are the same as in Fig. 1.

fermentation time improved from 60 h to 21 h, with a maximum DCW of 4.24, L-lactate at 10.3 g/*l* and DL-lactate at 20.50 g/*l*. To confirm the effect of mevalonic acid, CMG with 10 ppm mevalonic acid was then tested. Figure 7 shows that mevalonic acid promoted growth and lactic acid production to yield a maximum DCW of 4.99, L-lactic acid at 15.15 g/*l* and DL-lactic acid at 29.94 g/*l*, respectively, although consumption of glucose was prolonged until 45 h after the start of culture. The results indicated that mieki stimulated glucose consumption while mevalonic acid had an effect on the growth and lactic acid production. Thus, we examined with both mieki and mevalonic acid. Data for the culture with CMG plus 5.0% *mieki* and 10 ppm mevalonic acid (Fig.8) showed that the lactate concentration was almost the same as that observed with CMG and mevalonic acid only. However, the culture with CMG and ***mieki*** plus mevalonic ascid reduced the fermentation time markedly.

DISCUSSION

The effects on cells of changes in the medium are quite difficult to quantify because of various cellular functions affect the transport of ions, atoms, and molecules that enter and leave the cell. We examined directly the effects generation time, and fermentation kinetics of strain ISK-1 in jar culture of CMG plus 5.0% *mieki*, CMG plus 10 ppm mevalonic acid and CMG plus 5.0% *mieki* and 10 ppm mevalonic acid.

Figures 9, 10 and 11 illustrate the changes in fermentation kinetics of *Pediococcus* sp. ISK-1. Using the three media we observed various growth phases, as seen from the growth curves. The concentration of cells at which a transition takes place from one growth to another depends on both the characteristics of the cells and the composition of

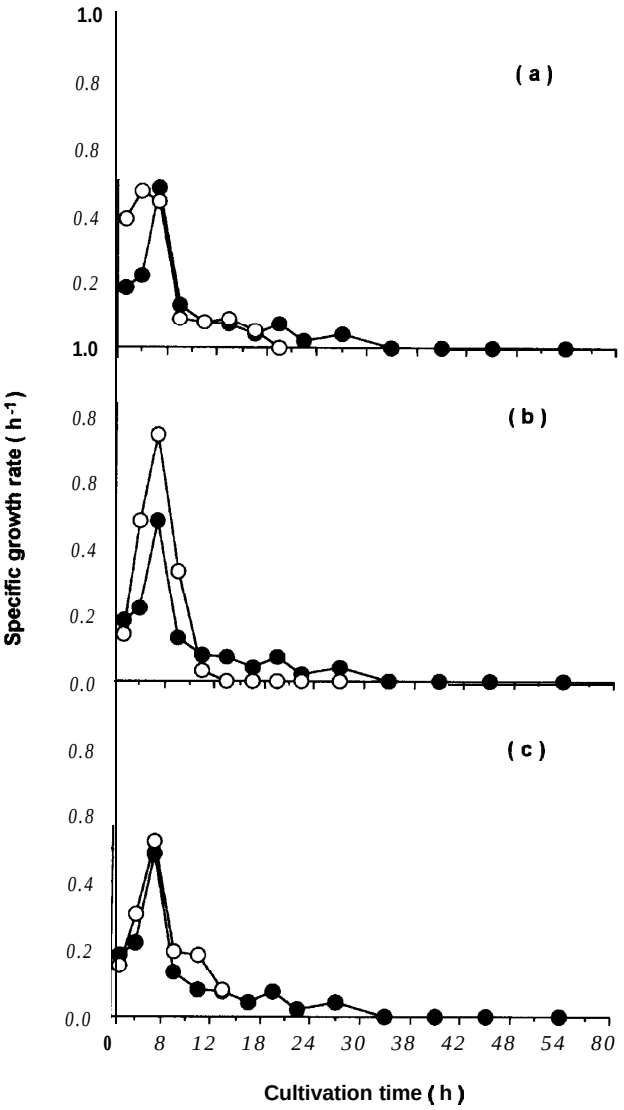

Fig. 9. Changes in the specific growth rate of *Pediococcus* sp. ISK-1 in CMG with 5.0% *mieki* (a), 10 ppm mevalonic acid (b), and 5.0% mieki and 10 ppm mevalonic acid (c).
Closed symbols indicate the data for the control culture (CMG). Open symbols indicate the date 'for CMG with 5.0% *mieki* (a), 10 ppm mevalonic acid (b), and 5.0% *mieki* and 10 ppm mevalonic (c).

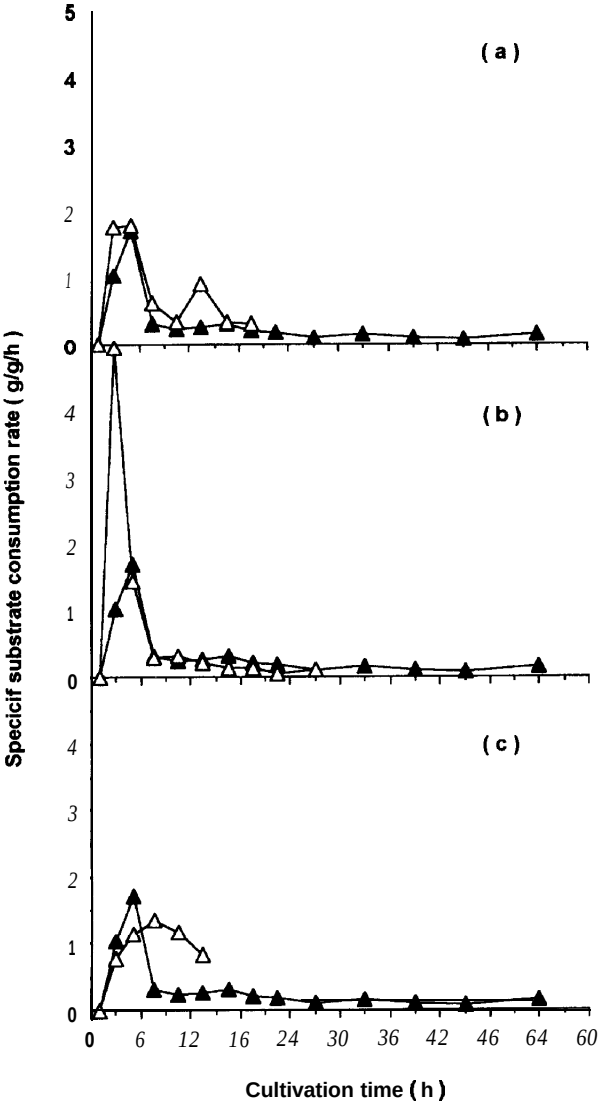

Fig. 10. Changes in the specific glucose consumption rate of *Pediococcus* sp. ISK-1 in CMG with 5.0% *mieki (a)*, 10 ppm mevalonic acid (b), and 5.0% *mieki* and 10 ppm mevalonic acid (c).
Symbols are the same as in Fig. 9.

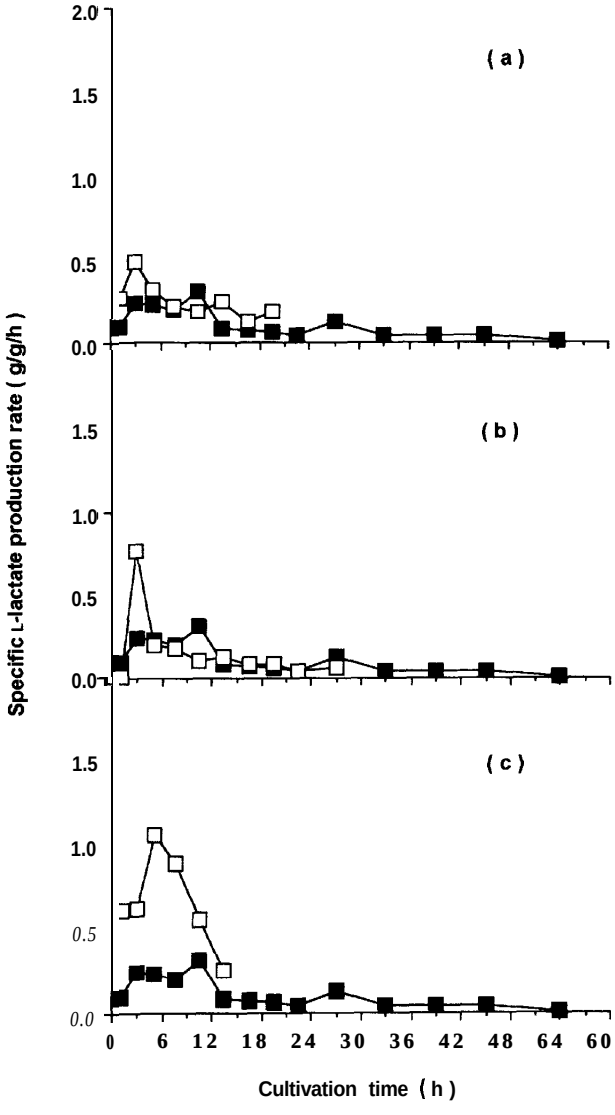

Fig. 11. Changes in the specific L-lactate production rate of *Pediococcus* sp. ISK-1 in CMG with 5.0% mieki (a), 10 ppm mevalonic acid (b), and 5.0% mieki and 10 ppm mevalonic acid (c).
Symbols are the same as in Fig. 9.

the culture medium. Consequently, the relative durations of the growth phases vary with specific rates of growth, substrate consumption and product formation. In CMG, the maximum specific rates of growth and glucose consumption were observed after a 5 h incubation. The specific substrate consumption rate was enhanced during the first 6 h of incubation by addition of 5.0% mieki and by addition of 10 ppm mevalonic acid (Fig.lOa and 10b). However, the growth rate of strain ISK-1 was almost the same upon addition to CMG of 5.0% *mieki* and 10 ppm mevalonic acid (Fig. 9a and 9b). Figure 11 shows that the specific product formation rate enhanced during a 12 h incubation by addition of 5.0% *mieki* and 10 ppm mevalonic acid. Addition of 5.0% *mieki* and 10 ppm mevalonic acid enhanced the specific substrate consumption rate and the product formation rate after 9 h of incubation (Fig. 10c and llc), while the specific growth rate was unchanged (Fig. 9c). These results indicate that in cultures of *Pediococcus* sp. ISK-1 in CMG that contained 30 g/*l* glucose initially, 5.0% *mieki* and 10 ppm mevalonic acid markedly affected substrate utilization and product formation but this effect was not associated with growth. It is possible that two different effects of mevalonic acid were involved: mevalonic acid may have done as an activate to peptidoglycan transpeptidase to the degradation of amino acids in the cell walls; and it might have done as stimulator in the biosynthesis of vitamins A, D, E, and K (Ishimoto *et al.*, 1971). *Mieki* might act as a nitrogen source and mevalonic acid might act as a growth factor and function as a vitamin so that together they might have a synergistic effect, acting together to promote the growth of this strain and lactic acid production. The medium developed in this study should help us to study the metabolism of mevalonic acid in *Pediococcus* sp. ISK-1.

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