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Effect of Nitrogen Sources in Culture Medium on L-Lactate Fermentation Employing *Lactococcus Zactis* IO-1

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Effect of amino acids, yeast extract and polypeptone on cell growth and L-lactate production of *Lactococcus lactis* IO-1 was investigated. The growth rate of the microorganism, cultivated in a synthetic medium consisting of amino acid, vitamins and minerals, is lower than that of the media containing yeast extract. It was considered that yeast extract was essential for rapid growth of *L. lactis* IO-1. An increase in yeast extract concentration in the culture medium increased specific growth rate and final cell concentration of *L. lactis* IO-1, and as a result the fermentation time was shortened.

INTRODUCTION

Recently, L-lactic acid has been used as a raw material for manufacturing poly-L-lactic acid, which is a kind of biodegradable plastics. Lactic acid is manufactured by fermentation employing lactic acid bacteria or by chemical synthesis, but D(-) and L(+) lactic acid are manufactured by fermentation only. Further, it is known that many lactic bacteria produce antimicrobial peptides (Meghrou et al., 1992), especially nisin produced by *Lactococcus lactis*, is already used as a food preservative in some countries in Europe. However, expensive culture media, which contain natural complex-organic nitrogen sources such as yeast extract, malt extract and/or polypeptone, are necessary for the cultivation of lactic bacteria because nutritional requirement of lactic bacteria is very complicate. *Lactococcus lactis* especially has numerous growth requirements (Marshall et al., 1984). The growth mechanism and the nutritional requirement of lactic acid bacteria have not been clarified yet. Recently, many workers have been studying the effects of nutrients (Amrane et al., 1993 ; Amrane, 1991; Desmazeaud, 1991; Ledesma et al., 1977 ; Vahvaselka et al., 1987) on the growth and product formation of lactic bacteria. Among the nutrients contained in the culture medium for lactate fermentation, the nitrogen source such as yeast extract seems to be very important for cell growth and acid production. Some workers have reported the promotion effect of yeast extract on the growth of lactic bacteria (Aeschlimann et al., 1990 ; Orberg et al., 1984). However the growth promotion effect of these complex nitrogen sources have not been completely clarified yet.

In this paper, we report on the effect of amino acids and yeast extract on the cell growth and L-lactic acid production of *Lactococcus Zactis* IO-1, which produces L-lactic acid from glucose and/or xylose with a high production rate (Ishizaki et al., 1992a), and nisin-like antimicrobial peptide (Ishizaki et al., 1992b).

MATERIALS AND METHODS

Microorganism The strain used was *Lactococcus Zactis* IO-1 (JCM) which was isolated and characterized in our laboratory (Ishizaki et al., 1990).

Culture media In L-lactate fermentation employing *L. Zactis* IO-1, the culture medium composed of $5\text{-g}\cdot\text{dm}^{-3}$ yeast extract (Difco), $5\text{-g}\cdot\text{dm}^{-3}$ polypeptone (Nihon Seiyaku Co., Ltd., Japan), $5\text{-g}\cdot\text{dm}^{-3}$ NaCl, and glucose and/or xylose has been usually used (this medium is hereafter referred as CM medium). In this study, several kinds of nutrients were used to further investigate their growth promotion effect on *L. Zactis* IO-1. The composition of the culture media used will be described in each paragraph for experimental results. All the culture media used were prepared with distilled water and the initial pH was adjusted to 6.5 with 1N NaOH.

Cultivation The stock culture stored at 5°C were refreshed in TGC medium by static culture in a test tube for 18 h at 37°C . Three milliliter of the TGC-culture broth was added to 50 ml of CM medium containing $10\text{ g}\cdot\text{dm}^{-3}$ glucose in a 100 ml flask and the suspension was incubated for 4 h with reciprocal shaking (60 strokes/min). The culture broth was aseptically centrifuged and the harvested cells were used as the seed for mainculture. The mainculture were carried out by shaking with 200-ml flask or by pH-stat jar cultivation. The pH-stat jar cultivation was carried out using a 1-dm^3 glass jar fermenter under the following conditions :- agitation speed of 400 rpm and working volume of 500 ml without gas feeding. The pH was maintained at 6.0 by automatic feeding of 3N NaOH using a pH controller (PHC-2201, Biott, Co., Ltd., Japan). The temperature was kept at 37°C .

Analyses Cell concentration was determined by converting the optical absorbance at 562 nm of the culture broth to dry cell weight (gram per 1 dm^3) with a standard curve prepared previously. The concentrations of L-lactic acid and glucose were determined by a L-lactate analyzer (Model 23L, Yellow Spring Instrument Co., Ltd., USA) and a glucose analyzer (Model 23A, YSI, Co., Ltd.), respectively.

RESULTS AND DISCUSSION

Comparison of growth of *L. lactis* IO-1 on a synthetic medium and on media containing yeast extract The flask culture of *L. Zactis* IO-1 was carried out using a synthetic medium and then the media containing yeast extract. Cell growth was compared to investigate the nutritional requirement. The media used were as follows ; (i) CM medium containing $50\text{-g}\cdot\text{dm}^{-3}$ glucose (ii) $5\text{-g}\cdot\text{dm}^{-3}$ yeast extract, $5\text{-g}\cdot\text{dm}^{-3}$ NaCl and $50\text{-g}\cdot\text{dm}^{-3}$ glucose (iii) $5\text{-g}\cdot\text{dm}^{-3}$ polypeptone, $5\text{-g}\cdot\text{dm}^{-3}$ NaCl and $50\text{-g}\cdot\text{dm}^{-3}$ glucose (iv) $2\text{-g}\cdot\text{dm}^{-3}$ casamino acid, $5\text{-g}\cdot\text{dm}^{-3}$ NaCl and $50\text{-g}\cdot\text{dm}^{-3}$ glucose (v) synthetic medium. The composition of the synthetic medium is shown in Table 1. Table 2 shows the fermentation results. The highest cell concentration and specific growth rate were obtained when CM medium was used. Cell concentration and specific growth rate obtained in the medium (ii) were almost the same as those in CM medium. The cell growth in the media containing no yeast extract, namely (iii), (vi) and (v), were inferior than those in the media containing yeast extract. It is thought

Table 1. Composition of synthetic culture medium used for flask culture of *L. Zactis* IO-1.

Components	Concentrations (g·dm ⁻³)
D-Glucose	50
NaCl	5
NH ₄ Cl	3
K ₂ HPO ₄	0.5
KH ₂ PO ₄	0.5
MgSO ₄ ·7H ₂ O	0.2
MnSO ₄ ·4H ₂ O	0.01
FeSO ₄ ·7H ₂ O	0.01
L-Alanine	0.2
L-Arginine·HCl	0.2
L-Aspartic acid	0.2
L-Cystine·HCl	0.1
L-Glutamic acid (Na)	0.5
Glycine	0.1
L-Histidine·HCl	0.1
L-Isoleucine	0.1
L-Lysine·HCl	0.2
L-Methionine	0.1
L-Phenylalanine	0.1
L-Proline	0.1
L-Serine	0.05
L-Threonine	0.1
L-Tryptophan	0.05
L-Tyrosine	0.1
L-Valine	0.1
Adenine·HCl	0.01
Guanine·HCl	0.01
Uracil	0.01
Xanthine	0.01
Thiamine·HCl	0.001
Riboflavin	0.001
Niacin	0.001
Ca-pantothenate	0.001
Pyridoxine·HCl	0.001
Pyridoxal·HCl	0.001
p-Amino benzoic acid	0.0002
Folic acid	0.00001
Biotin	0.00001

that the amount of amino acids contained in these culture media is sufficient but its composition might not be suitable for the cell growth. Hence, the culture experiment was carried out with a synthetic medium in which the composition of amino acids and vitamins were prepared to match that of yeast extract. In this modified synthetic medium, L-leucine was introduced as an essential amino acid of *L. Zactis* IO-1 which

Table 2. Composition of growth of *L. lactis* IO-1 cultivated on a synthetic medium and media containing yeast extract.

Media ^a	μ (h ⁻¹)	Cell concentration after 4h (g·dm ⁻³)
(i)	0.93	0.776
(ii)	0.92	0.763
(iii)	0.35	0.302
(iv)	0.31	0.299
(v)	0.32	0.311

a : (i) CM medium; (ii) Yeast extract; 5 g·dm⁻³, NaCl 5 g·dm⁻³; (iii) Polypepton ; 5 g·dm⁻³, NaCl; 5 g·dm⁻³; (iv) Casamino acid; 5 g·dm⁻³, NaCl 5 g·dm⁻³; (v) Synthetic medium.

All the culture media contained 50·g·dm⁻³ glucose.

was determined by amino-acid requirement test (the other essential amino acids were L-glutamic acid and L-valine; the data is not show). The composition of the modified synthetic medium is shown in Table 3. Table 4 shows the fermentation result. Specific growth rate and cell concentration after 4 hours of cultivation were 0.32 h⁻¹ and 0.335 g·dm⁻³ respectively, which were lower than those obtained in the media containing yeast extract. On the other hand, addition of 1·g·dm⁻³ yeast extract to the synthetic medium increased specific growth rate of *L. Zactis* IO-1 to the level obtained in CM medium. Such a growth promotion effect was not obtained by the addition of polypeptone. These results suggested that yeast extract may contain some kind of essential growth factor for *L. lactis* IO-1 except amino acids and vitamins.

Effect of yeast extract on cell growth and lactate production in batch culture of *L. Zactis* IO-1 In the above flask culture experiments, satisfactory cell growth of *L. Zactis* IO-1 was obtained when yeast extract was used. The effect of yeast extract on the cell growth and L-lactate production of *L. Zactis* IO-1 was then investigated by pH-stat jar cultivation. Yeast extract was added to the medium composed of 5·g·dm⁻³ polypeptone, 5·g·dm⁻³ NaCl and 50 g·dm⁻³ glucose at various concentration. Figure 1 shows the fermentation time courses. When 5·g·dm⁻³ yeast extract was used (Fig. 1a), maximum specific growth rate observed was $\mu=0.93$ h⁻¹ and growth ceased at the cell concentration of 2.38 g·dm⁻³. Glucose in the medium was exhausted after 18 hours of cultivation, while L-lactic acid concentration reached 37.16 g·dm⁻³. When the concentration of yeast extract was 3.0 g·dm⁻³ (Fig. 1b), maximum specific growth rate was $\mu=1.01$ h⁻¹ and growth ceased at a cell concentration of 1.53 g·dm⁻³. After 20 hours of cultivation, 7.90 g·dm⁻³ of glucose still remained in the culture liquid. The concentration of L-lactic acid after this cultivation time was 24.42 g·dm⁻³. When the concentration of yeast extract was 1.0 g·dm⁻³ (Fig. 1c), maximum specific growth rate was $\mu=0.91$ h⁻¹ and the final cell concentration was 0.82 g·dm⁻³. After 19 hours of cultivation, 23.4 g·dm⁻³ of glucose still remained and L-lactic acid produced was only 16.38 g·dm⁻³. In these cultivations, maximum specific growth rates observed were almost the same, however the final cell concentration decreased as yeast extract concentration in the medium decreased. This is especially the case, when using 1·g·dm⁻³ yeast extract medium ; cell growth

Table 3. Composition of synthetic culture medium to match that of $5\text{-g}\cdot\text{dm}^{-3}$ yeast extract.

Components	Concentrations ($\text{g}\cdot\text{dm}^{-3}$)
D-Glucose	50
NaCl	5
NH_4Cl	3
K_2HPO_4	0.5
KH_2PO_4	0.5
$\text{MgSO}_4\cdot 7\text{H}_2\text{O}$	0.2
$\text{MnSO}_4\cdot 4\text{H}_2\text{O}$	0.01
$\text{FeSO}_4\cdot 7\text{H}_2\text{O}$	0.01
L-Alanine	0.145
L-Arginine·HCl	0.06
L-Aspartic acid	0.035
L-Glutamic acid (Na)	0.186
Glycine	0.035
L-Histidine·HCl	0.03
L-Isoleucine	0.031
L-Lysine	0.053
L-Lysine·HCl	0.17
L-Methionine	0.028
L-Phenylalanine	0.027
L-Serine	0.016
L-Threonine	0.016
L-Tryptophan	0.01
L-Tyrosine	0.01
L-Valine	0.05
Adenine·HCl	0.01
Guanine·HCl	0.01
Uracil	0.01
Xanthine	0.01
Thiamine·HCl	0.00075
Riboflavin	0.00039
Niacin	0.0028
Ca-pantothenate	0.001
Pyridoxine·HCl	0.00019
Cyanocobalamin	0.0000013
Folic acid	0.000008
Biotin	0.00001

Table 4. Cell growth of *L. Zactis* 10-1 in a modified synthetic culture medium.

Media ^a	μ (h^{-1})	Cell concentration after 4h ($\text{g}\cdot\text{dm}^{-3}$)
Modified synthetic medium	0.32	0.335
Modified synthetic medium added $1\text{ g}\cdot\text{dm}^{-3}$ yeast extract	0.89	0.711

a : The composition of modified medium is shown in Table 3.

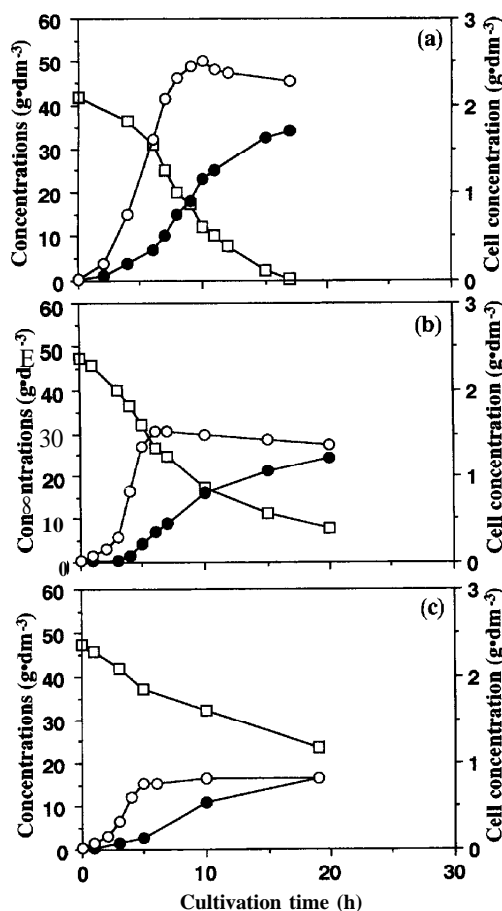


Fig. 1. Time courses of batch culture of *L. lactis* IO-1 at various yeast extract concentration in medium.

(a): Yeast extract concentration was 5 g·dm⁻³.

(b): Yeast extract concentration was 3 g·dm⁻³.

(c): Yeast extract concentration was 1 g·dm⁻³.

Symbols: (○), Cell concentration; (●) L-Lactic acid concentration; (□), Glucose concentration

suddenly stopped when the cell concentration reached 0.82 g·dm⁻³. When yeast extract concentration increased to 10 g·dm⁻³, specific growth rate was $\mu = 1.14 \text{ h}^{-1}$ and the final cell concentration increased to 2.83 g·dm⁻³. The fermentation was completed after 14 hours. When yeast-extract concentration was 10 g·dm⁻³, the fermentation result obtained was different when different types of yeast extract were used. Figure

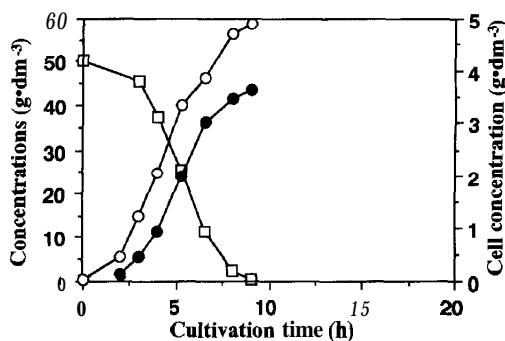


Fig. 2. Time courses of batch culture of *L. lactis* IO-1 using the medium containing 10-g·dm⁻³ yeast extract (Oriental Yeast Co., Ltd.).

Symbols: (○), Cell concentration; (●) L-Lactic acid concentration ; (□), Glucose concentration

2 shows the time courses of the cultivation using 10 g·dm⁻³ of yeast extract manufactured by Oriental Yeast Co., Ltd., (Japan). In this case, the specific growth rate and cell concentration increased to $\mu=1.54\text{ h}^{-1}$ and 4.91 g·dm⁻³, respectively. The fermentation was completed after 9 hours.

Jensen et al. (1993) reported that a synthetic culture medium consisting of glucose, acetate, vitamins, minerals and eight amino acids allowed for the growth of *Lactococcus lactis* subsp. *lactis* with a specific growth rate of $\mu=0.30\text{ h}^{-1}$, further with addition of 19 amino acids the growth rate increased to 0.70 h^{-1} and the exponential growth phase proceeded until high cell concentrations were obtained. However, our result showed that satisfactory cell growth of *L. Lactis* IO-1 could not be obtained even when using the synthetic culture medium supplemented with amino acids, therefore it is considered that the free amino acids do not very contribute to the growth promotion effect. On the other hand, it has been reported that the growth of lactobacilli is stimulated by various peptides that supply growth-limiting amino acids, which might not be transported into the cells in the form of free residues (Desmezeaud, 1983). Therefore, sources of peptides such as corn steep liquor, malt sprout extract, casein hydrolyzate, whey-protein hydrolyzates and yeast extract have to be supplemented in medium when milk and whey permeates are used for cultivation of lactic bacteria (Amrane et al., 1994). Then, an economical source containing the peptides that promote the growth of lactic bacteria, will be essential for reduction of the cost of lactate fermentation. We have already shown natural rubber serum (NRS), which is a waste from latex separation process, enables the satisfactory cell growth and ethanol production of *Zymomonas mobilis* without yeast extract (Tripetchkul et al., 1992). We are studying the effect of NRS on the growth and lactate production of *L. Zactis* IO-1 as the economical growth promoter resource.

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