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Malate Stimulation on Growth Rate of *Leuconostoc oenos*

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*Leuconostoc oenos*의 생장률에 대한 사과산의 촉진 작용

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Abstract

The presence of L-malic acid in culture media contaniing glucose stimulated the growth rates of *Leuconostoc oenos* strains. The L-malic acid also stimulated the synthesis and activity of D-malate dehydrogenase (D-LDH) resulting in rapid production of D-lactate from glucose. The rapid utilization of glucose under the presence of L-malic acid may explain, in part, the stimulatory effect of the compound on the growth rate of leuconostocs.

Introduction

In 1976, Pilone and Kunkee (9) reported that the presence of L-malic acid in culture medla increased the growth rate of *Lauconostoc oneos* ML34 remarkably, specially at low pH. The stimulation was not the result of external pH change during the culture and L-malic acid was not an energy source for the organism. Since energy is not involved in this effect the mechanism of L-malic acid stimulation growth rate should be sought elsewhere. It has been observed that the addition of L-malic acid to a glucose-containing medium induced the synthesis of lactate dehydrogenase (LDH) using a heterofermentative strain of *Leuconostoc* (3). Doelle (2) reported that *Leuconostoc mesenteroides* increased its lactic acid production from glucose threefold when malic acid was added to the culture. Addition of malic acid increased 6.5-fold the specific activity of nicotinamide adenine dinucleotide (NAD)-linked L-LDH and increased 3,2-fold that of NAD-linked D-LDH according to the report. *L. oneos* is an heterofermentative lactic acid bacterium (G. J. Pilone, Ph. D. thesis, University of California, Davis, 1971) and it forms D-lactate exculsively (4). James (5) proposed a metabolic pathway adding an acetate yielding branch to the phosphoketolase pathway fcr heterolactic fermentation of glucose by leuconostocs. In this pathway six NAD-linked oxidation-reduction steps are intimately coupled to yield four endproducts, D-lactate, acetate, ethanol, and carbon dioxide from glucose. In

the present study the malate stimulation on growth rate of *L. oneos* was reconfirmed and the relationship between growth rate, NAD-linked LDH, and fermentation balance was carefully observed to explain the mode of stimulation.

Materials and Methods

Organisms and culture media

Two strains of *Leuconostoc oenos* were used. Strain ML34 was a gift from Dr. G. J. Pilone, The Christian Brothers, Mont La Salle Vineyards, Napa, California, and strain A25 was isolated from Korean wine (6) recently. The organisms were cultivated in a modified Rogosa medium (8) which contained per liter; 5g glucose, 20g tryptone, 5g peptone, 5g yeast extract, 1g liver infusion broth (dehydrated), and 0.05*ml* Tween 80. All contents were Difco products. The same medium lacking glucose was used as basal broth.

Growth rate measurements

The growth of organisms in a phototube of Bausch and Lomb spectrometer was measured periodically by reading the optical density at 600 nm. And the specific growth rates were calculated from the generation times which were determined from straight-line portions of plots of the logarithm of the optical densities versus time as described by Pilone and Kunkee (9), Temperature was maintained at 28 °C throughout the experiments.

Continuous culture

A New Brunswick BioFlo C 30 chemostat (New Brunswick Scientific. Co., New Brunswick, N. J.) having working volume of 360 *ml* was used for continuous cultures. Flow rates were varied to give dilution rates in the range between 0.022 and 0.046 hr^{-1}. The level of pH in the reactor was controlled automatically with 1 N KOH and 1 N HCl by use of an automatic pH controller (Model pH-40, New Brunswick Scientific Co.). Samplings for analyses were made when the growth reached a steady state which was confirmed by periodical measurments of optical densities after each shift of culture conditions.

Preparation of cell-free extracts

Cells grown batchwise in a 4-liter glass bottle or collected by receiving effluents of steady state continuous cultures were harvested by centrifugation at 13,000g for 10 min and washed three times with 0.05M Tris (hydroxymethyl) aminomethane-HCl buffer (pH 7.0). The washed cells were resuspended in the same buffer (ca 1.5 g wet weight cells per 10 *ml*) and then subjected to disintegration in a sonic oscillator (Sonic 300 Dismembrator, Artek Systems Co., Framingdale, N. Y.) at 80% relative output using an intermediate tip. During the total 10min oscillation, the glass vessel was cooled in an ice bath allowing 1.5-min intervals between the 0.5-min oscillations. The cell debris was removed by centrifuging at 15,000g for 20min and the resultant supernatant was used as cell-free extract.

Enzyme assays

Since the cell-free extracts thus obtained did not show any appreciable activity of oxidizing NADH in control cuvettes lacking substrate, the activity of NAD linked LDH in the cell-feee extracts was assayed spectrophotometically at 340 nm by using a Beckman Spectrophotometer Acta C111 DB (Beckman Instruments Inc., Fullerton, Calif.). The 3.0 *ml* standard assay system contained; 2.5 mM sodium pyruvate, 0.167mM NADH, 0.1*ml* cell-free extract, and Tris (hydroxylmethyl) aminomethane-HCl buffer (0.25M, pH 7.0). The reaction was initiated by the addition of the cell-free extract at 25 °C. A unit of enzyme activity was defined as a rate of NADH oxidation of 1 micromole per min under the above assay conditions. Specific activity of the enzyme was expressed as micromoles of NADH oxidized per min per mg of protein. Protein was determined by the method of Lowry et al (7) with bovine serum albumin (Sigma Chemeical Co., Saint Louis, Missouri) as a standard.

Analytical methods

Glucose was determined spectrophotometrically with a glucose oxidase peroidase kit (Sigma Chemical Co.) according to Sigma Technical Bulletin No. 510 (1978). Lactate was measured following the method of Barker and Summerson (1). Ethanol and acetate were determined using a gas chromatograph (Shimadzu GC-4CM with dual hydrogen flame ionization). The column used was a coiled

stainless steel column (1.8 m×4 mm) packed w Chromosorb 101 (80/100 mesh, Sigma Chemical Co.) and was run isothermally at 120 °C for ethanol and at 115°C for acetate with the inlet at 150 °C and the detector at 220 °C. Flow rates for hydrogen and air were 30 ml/min and 300ml/min, respectively. Samples were acidified (6N H_2SO_4, 20 ml) just prior to injection and then 5 ul portions were applied to the column. Uninoculated culture media were included as blanks and isobutyric acid was used as an internal standard.

Results

Malate stimulation on growth rate

Two strains of *L. oenos*, ML34 and A25, were grown batchwise with and without L-malic acid at several initial pH levels (4.0 to 6.0) and specific growth rates at 28 °C are shown in Fig. 1. The optimal pH for ML34 cells growing in the modified Rogosa medium without the addition of L-malic acid was around 5.0 with a specific growth rate of μ= 0.028h^{-1} (g=25h). In the presence of mM L-malic acid, the optimal pH was 4.5 with a specific growth was more than doubled, μ=0.063h^{-1} (g=11 h). This picture is consistent, except a slight deviation in optimal pH values, with the observations made by pilone and Kunkee (9) who used the same strain. The similar trend was observed in another strain of this species, A25, which was isolated from Korean wine (6), suggesting a common nature of the organism.

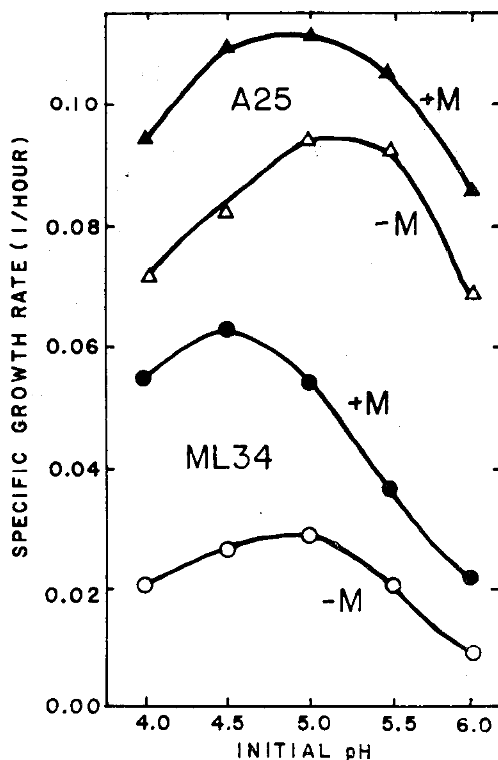

Fig. 1. Effects of L-malic acid and pH on specific growth rates of *L. oenos* strains ML34 and A25 in modified Rogosa medium. Symbols: ○, ●, ML 34; △, ▲, A25; -M, without L-malic acid; +M, with L-malic acid, 15mM.

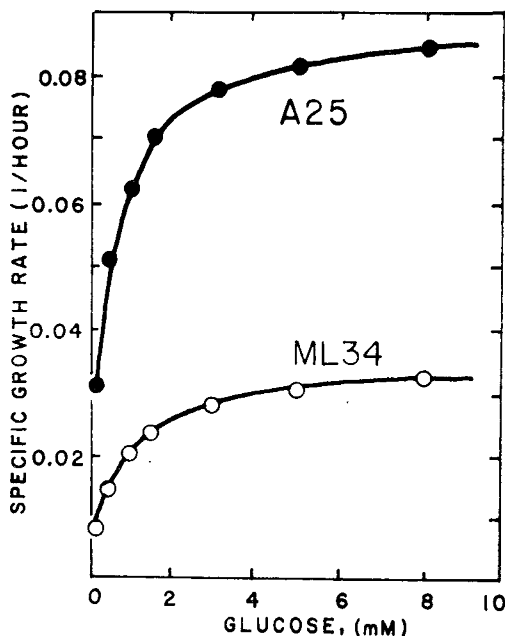

Fig. 2. Effect of concentration of glucose added to modified Rogosa medium on specific growth rates of *L. oenos* strains ML34 and A25. Symbols: ○, ML 34; ●, A25.

The specific growth rate of strain A 25 at its optimal pH with L-malic acid, μ=0.112h^{-1}, was about 1.8 times higher than the highest specific growth rate observed with ML34. The faster growth of strain A25 made a continuous culture easier later. Figure 2 shows the effect of glucose concen-

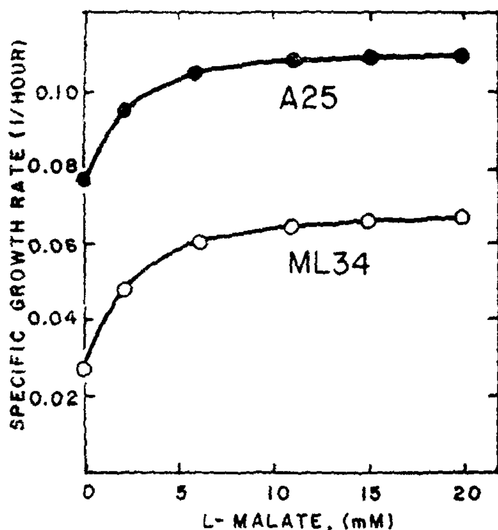

Fig. 3. Effect of concentration of L-malic acid added to modified Rogosa medium having 12mM glucose on specific growth rates of *L, oenos* strains ML 34 and A25. Symbols: ○, ML34; ●, A 25.

tration on the specific grow rates of the two strains. Through this experiment we decided to use 2 millimole of glucose as growth limiting amount and 12 millimole as excess amount of glucose to be added to the basal broth for the later experiments. In the presence of excess amount of glucose the excess concentration of L-malic acid, 15 millimole, to be added for the full stimulation of specific growth rates of the two strains was determined (Fig. 3).

Malate stimulation of LDH

It has been reported that malate has stimulatory effect on the synthesis and activity of NAD-linked LDHs in *Leuconostoc mesenteroides* (2). This was also true in both strains of *L. oenos* tested as shown in Table 1. When 15 millimole of L-malic acid was added to the modified Rogosa media with excess glucose, the synthesis of D-LDH in ML34 increased 5.5 fold and 2.3 fold in A25. L-malic acid stimulated the activity of D-LDH also; the presence of L-malic acid in the reaction mixture almost doubled the enzyme reaction in both strains.

Table 1. Effect of L-malic acid on the synthesis and activity of D-LDH of *L. oenos* strains ML34 and A25

Addition in culture medium	Addition of L-malic acid in reaction mixture	Sp act of D-LDH[a]	
		ML34	A25
Glucose[b]	−	0.154	0.456
	+[c]	0.283	0.852
Glucose, Malate[d]	−	0.842	1.034
	+	0.969	1.418

a Expressed as uM of NADH oxidized per milligram of protein per min.

b 12mM glucose added in basal broth.

c 15mM L-malic acid added in 3.0 *ml* reaction mixture.

d 15mM L-malic acid added in basal broth.

Table 2. Effect of cultural pH on the malate stimulation of D-LDH synthesis in *L. oenos* ML34 cells.

Initial pH of culture medium	Addition in culture medium	D-LDH	
		Sp. act.	Ratio[a]
3.8	Glucose[b]	0.049	1
	Glucose, Malate[c]	0.104	2.1
4.5	Glucose	0.154	1
	Glucose, Malate	0.842	5.5
5.5	Glucose	0.096	1
	Glucose, Malate	0.333	3.5

a Relative specific activities of D-LDH synthesized under absence and presence of L-malic acid under certain initial culture pH.

b 12mM glucose added in basal broth.

c 15mM L-malic acid added in basal broth.

The reaction stimulation, however, was less prominent on the D-LDH which was synthesized under the presence of L- malic acid.

It was also observed that the pH level of culture medium affected the degree of malate-stimulation on the synthesis of D-LDH by the strain ML 34 markedly (Tab. 2). At pH 4.5, which was optimal level for growth rate (Fig. 1), the presence of L-malic acid in the basal medium along with glucose, stimulated the synthesis of D-LDH and its specific activity increased 5.5 fold compared to that with

glucose alone. When the pH level raised or downed to the levels where the growth rates were lower, the stimulation effect of L-malic acid on D-LDH synthesis was also reduced. In other words, the malate stimulation effects on growth rate and on D-LDH synthesis appear to be correlated.

Relation between growth, D-LDH, and fermentation balance

According to the metabolic pathway proposed by James (5) heterolactic bacteria leuconostocs ferment glucose yielding three major endproducts, D-lactate, acetate, and ethanol with evolution of carbon dioxide as shown in Fig. 4. D-LDH participates in the last step of D-lactate formation. In order to relate this fermentation pattern to the growth of *L. oenos*, continuous cultures under various dilution rates were performed using the faster growing strain A25 and the results are shown in Table 3. As the dilution rate increased, the intracellular D-LDH levels also increased. At a steady state of an continuous culture dilution rate represents specific growth rate. Therefore, the data imply the fact that when D-LDH level in the cell grows faster. Furthermore, when the intracellular D-LDH level increased, two endporducts, L-lactate and ethanol, increased leaving the third endproduct acetate decreased. Interestingly enough, the balance between L-lactate and ethanol remained constant at the rate approximately 4 to 3 throughout the dilution rates tested. In other words the higher D-LDH level increased the efficiency of glucose utilization resulting in the faster growth of *L. oenos*.

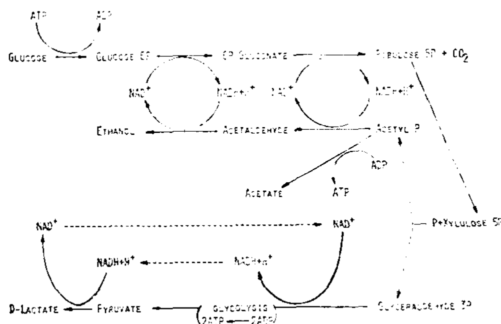

Fig. 4. Heterolactic fermentation of glucose by leuconostocs (5).

Discussion

The distinct stimulation of L-malic acid on the specific growth rate of *oenos* at low pH reported by pilone and Kunkee (9) using strain ML34 was reconfirmed (Fig. 1.). The phenomenum is quite general because we observed the similar effect with two other *L. oenos* strains (data not shown) besides ML34 and A25. L-malic acid stimulated the synthesis and activity of D-LDH in both strains of ML 34 and A25 (Table 1). Dolle (2) observed similar

Table 3. Relation between growth rate, intracellular D-LDH level, and fermentation balance during continuous culture of *L. oenos* A25 under various dilution rates[a].

Dilution rate (h^{-1})	Sp act of LDH	Fermentation balance				
		D-lactate		Acetate		Ethanol
0.022	0.212	0.97[b]		0.20		0.78
		(4)[c]	:	(1)	:	(3)
0.030	0.468	1.18		0.17		0.82
		(7)	:	(1)	:	(5)
0.033	0.986	1.17		0.10		0.88
		(12)	:	(1)	:	(9)
0.041	1.145	1.21		0.03		0.95
		(40)	:	(1)	:	(32)
0.046	1.216	1.23		0.03		0.98
		(41)	:	(1)	:	(33)

a Basal broth fortified with 15 mM L-malic acid and limited amount of glucose (2mM) was used.

b Mole of endproducts produced per mole of glucose used.

c Relative mole number of endproducts with acetate as unit mole under certain dilution rate.

effect that the addition of malic acid to the culture of *L. mesenteroides* stimulated L-lactate dehydrogenase not only in its synthesis but also in its activity. The data appeared in Table 2 and 3 suggest a close relationship between the specific growth rate and D-LDH both of which were stimulated by the presence of L-malic acid.

The operation of the heterofermentative metabolic pathway proposed by James (5) was substantiated in these strains tested by the endproducts formed from glucose (Table 3). The residual culture broth contained only D-lactate, acetate, and ethanol in significant amounts and no other soluble metabolites, such as formate, could be detected through the gaschromatographic analyses. The fourth metabolite, CO_2, of the pathway was not measured because it can be calculated theoretically; one mole CO_2 from one mole glucose. The diminishing production of acetate under higher dilution rates (Table 3), however, suggests *L. oenos* cells prefer to utilize glucose through the phosphoketolase pathway when they grow fast. According to the metabolic pathway proposed by James (5), as well as to the phosphoketolase pathway, the conversion of glucose to Xylulose-5-phosphate involves two oxidation steps, with the formation of 2 $NADH_2$, or the equivalent of 4H (Fig. 4). Metabolism of glyceraldehyde-3-phosphate to pyruvate yields a further 2H making 6H in all. Two of these are utilized for the reduction of pyruvate to lactate and, to balance the overall reaction, the remaining 4H are used to reduce acetyl phosphate to ethanol, via acetaldehyde. Altogether 6 steps, i. e, 3 oxidations and 3 reductions, are intimately coupled to yield D-lactate and ethanol from glucose leaving acetate as the side product. It may be assumed, therefore, that if any one of the 6 steps is accelerated by any means, the remaining 5 steps should also be accelerated simultaneously to balance the overall reaction resulting an efficient utilization of glucose and thus permiting a faster growth of the organism. From the data, (Table 1) we found that L-malic acid stimulated the synthesis and activity of D-LDH. The higher intracellular D-LDH together with its higher activity stimulated the reduction of pyruvate to produce more D-lactate (Table 3), suggesting that one of the 6 oxidation-reduction steps is accelerated. The simultaneous accelerations of the remaining 5 steps were proved by the simultaneous increase in ethanol production (Table 3). That is, when the reduction step at pyruvate was accelerated by the malate-stimulated D-LDH, the other two reduction steps at acetyl phosphate and acetaldehyde were also to be accelerated to produce more ethanol. This can only be done by supplying more hydrogen (H) through accelerating the three oxidation steps involved (Fig. 4). In summary, the presence of L-malic acid in the culture medium stimulated the growth rate of *L. oenos* cells. L-malic acid also stimulated the synthesis and activity of D-LDH in the organism. The high intracellular level and activity of D-LDH accelerated reactions of the six NAD-linked oxidation-reduction steps involved in the glucose metabolizing pathway increasing the efficiency of glucose utilization by the organism. Thus, the efficient utilization of glucose originally stimulated by the presence of L-malic acid increased, at least in part, the growth rate of *L. oenos*.

Acknowledgements

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요 약

포도당을 포함한 생장 배지속에 L-사과산을 첨가하면 *Leuconostoc oenos* 균들의 생장률을 크게 촉진 시켰다. L-사과산은 동시에 C-젖산탈수소 효소 (D-LDH)의 합성과 활성도를 촉진시켜 포도당에서 D-젖산의 생산을 빠르게 하였다. L-사과산 존재 하에서 이렇게 빨라진 포도당의 이용이 leuconostoc 균들의 생장률을 촉진시키는 것 같다.

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