Improvement of Diacetyl Production by *Lactococcus lactis* ssp. *lactis* CNRZ 483 Through Oxygen Control

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ABSTRACT

This study illustrated the effect of controlled oxygenation on diacetyl production by citrate-utilizing *Lactococcus lactis* ssp. *lactis* CNRZ 483. Effects of soluble oxygen concentrations at 21 and 50% of the medium saturation and pressurization of the reactor under 1 and 2 atm (atmospheres) of oxygen were tested. Oxygen had a slightly negative effect on the growth of *L. lactis* ssp. *lactis* CNRZ 483. In contrast, oxygen had a substantial effect on diacetyl production. Under nitrogen, the maximal concentration of diacetyl in the medium was 0.015 m*M*; diacetyl maxima were 0.13 and 0.26 m*M* when soluble oxygen concentrations were 21 and 50%, respectively. Diacetyl production reached a maximum of 0.39 and 0.45 m*M* when oxygen pressures of the culture were 1 and 2 atm, respectively. The effect of oxygen on lactate dehydrogenase activity and on chemical decarboxylation of α-acetolactate explained the higher diacetyl production.

(**Key words**: *Lactococcus lactis* ssp. *lactis*, diacetyl, oxygen)

Abbreviation key: **ALS** = α-acetolactate synthase, **Cit⁺** = citrate-utilizing, **LDH** = lactate dehydrogenase.

INTRODUCTION

Diacetyl is responsible for the characteristic aroma of some fresh cheeses and is produced mainly from citrate by citrate-utilizing (**Cit⁺**) *Lactococcus lactis* ssp. *lactis* (25). Citrate permease transports citrate into the cell, where citrate is cleaved into acetate and oxaloacetate by citrate lyase (10). Oxaloacetate is converted into CO_2 and pyruvate and then decarboxylated to form CO_2 and acetaldehyde-thiamine pyrophosphate. The α-acetolactate synthase (**ALS**) condenses the latter with a second molecule of pyruvate, producing α-acetolactate. Diacetyl originates from the chemical oxidative decarboxylation of α-acetolactate (11, 26, 30), and acetoin originates from the decarboxylation of α-acetolactate by α-acetolactate decarboxylase, from reduction of diacetyl by acetoin dehydrogenase, or from chemical nonoxidative decarboxylation.

Because the amount of diacetyl produced by mesophilic starter cultures in milk is relatively low (0.017 to 0.023 m*M*) (31), factors such as pH, temperature (20), citrate concentration (9, 17, 21), the presence of metallic ions (Cu^{2+} and Fe^{2+}) or hemin (12), and particularly oxygen (2), have been evaluated for effects on diacetyl production. Certain strains of Cit⁺ *L. lactis* ssp. *lactis* produced diacetyl only under aerated conditions (4). Similarly, *Lactobacillus casei* 2206 produced 10 times more diacetyl in the presence of oxygen than in its absence (14). Diacetyl production by immobilized cells showed that high diameter gel beads inhibited oxygen transfer, resulting in decreased production of diacetyl (27). Recent studies (2) have shown that Cit⁺ *L. lactis* ssp. *lactis* CNRZ 483, grown under initial oxygen concentrations of 21, 50, and 100% of medium saturation, produced 2, 6, and 18 times more diacetyl, respectively, than under nitrogen. Those experiments were carried out in static cultures, which were hermetically sealed after oxygen incorporation; therefore, oxygen concentration of the medium decreased during growth. The present study tested the effect of controlled oxygenation of the medium by regulation of the concentrations of soluble oxygen or by pressurization of the reactor with oxygen.

MATERIALS AND METHODS

Culture Preparation

The Cit⁺ *L. lactis* ssp. *lactis* CNRZ 483 (from the collection of Institut National de la Recherche Agronomique, Jouy-en-Josas, France) was grown for

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8 h at 30°C in skim milk (100 g/L) (Elle & Vire; Union Laitière Normande, Condé-sur-Vire, France) that had been sterilized for 15 min at 110°C. Stock cultures were obtained by inoculating 200 μl of the culture, grown in skim milk, into 2 ml of sterilized litmus milk followed by storage at −20°C.

Fermentation Conditions

The growth medium contained whey (60 g/L; Besnier, Bourgbarré, France), trisodium citrate·$2H_2O$ (2 g/L; Prolabo, Paris, France), bactopeptone (5 g/L; Difco Laboratories, Detroit, MI), and yeast extract (3 g/L; OSI, Maurepas, France). The medium was sterilized at 110°C for 20 min and then inoculated at 30 ml/L with a culture grown in the same medium. The 7-L reactor (Inceltech, Toulouse, France) was maintained at 30°C and 400 rpm. The oxygen concentration of the medium was measured with an oxygen probe (Ingold, Urdof, Switzerland) that had been calibrated previously with identical media saturated with nitrogen and oxygen, respectively. The 0% oxygen concentration was maintained with nitrogen, and the 100% was maintained with pure oxygen at the pressure used during fermentation. Controlled oxygen intake of the medium was ensured either by pressurization or automatic control of the oxygen concentration. In all cases, oxygen was introduced in the headspace. For pressurization, the headspace above the culture medium was maintained at 1 atm (atmosphere) with nitrogen and oxygen and at 2 atm with oxygen only. Under 1 and 2 atm, the degree of saturation by oxygen corresponded to 100% of the medium saturation at the working pressure and decreased to 60 and 90%, respectively, during fermentation. In the second case, oxygen concentration of the medium was regulated automatically (Inceltech) at 21 and 50% of the medium saturation. Temperature, pH, and oxygen concentrations were measured hourly through a data acquisition system. All fermentations were duplicated.

Bacterial Lysis and Enzyme Assays

When the culture reached pH 5.2, the cells were harvested by centrifugation at 14,000 × *g* for 15 min at 4°C, washed in 50 m*M* sodium phosphate buffer, pH 7.0, and suspended (1:20, vol/vol) in an identical buffer. Cells were lysed at 30°C for 30 min with 330 U/ml of lysozyme (Sigma Chemical Co., Isère, France) and 15 U/ml of mutanolysin (Sigma Chemical Co.). After ultrasonic treatment (50 W, three times for 30 s in a Sonifier 250, Branson, Carouge-Genève, Switzerland) and centrifugation (14,000 × *g* for 30 min), the supernatant was recovered and used for all enzyme assays. Butanediol, acetoin, and lactate dehydrogenase (**LDH**) activities were measured by monitoring the decrease in the absorbance at a wavelength of 340 nm (5). For butanediol and acetoin dehydrogenase assays, the reaction mixture contained 300 μl of cell-free extract, 0.13 m*M* of NADH, and 13 m*M* of diacetyl (or acetoin) in 100 m*M* sodium phosphate buffer (pH 7.0). These enzyme activities were corrected for NADH oxidase activity. Activity of NADH oxidase was measured with 0.13 m*M* NADH (1). One enzyme unit was equivalent to 1 μmol of NADH oxidized/min. The LDH assay was performed in 50 m*M* Tris-maleate buffer (pH 7.0) containing 100 μl of cell-free extract, 10 m*M* sodium pyruvate, 1 m*M* fructose-1,6-diphosphate, and 0.15 m*M* NADH (29).

Activity of ALS was determined by measuring the conversion of pyruvate to acetoin (5). The reaction mixture contained 100 μl of cell-free extract, 80 m*M* of sodium pyruvate, and 0.21 m*M* of thiamine pyrophosphate in 100 m*M* sodium phosphate buffer (pH 6.5). After 15 min of incubation at 45°C, 200 μl of HCl (0.5 *M*) were added to stop the reaction and to convert the α-acetolactate into acetoin. The acetoin formed during 30 min was measured by HPLC. One enzyme unit of activity represented 1 μmol of acetoin formed/min. Protein concentrations were determined by the method of Bradford (3) with BSA as the standard protein.

Bacterial Counts

Samples for total lactococcal counts were taken hourly, treated with a Turrax disperser (IKA, Labortechnik, Stafen, Germany) for 30 s, and dispensed on M17 agar plates (28) using a spiral system (Interscience, St-Nom la Bretèche, France). The Cit^+ colonies were enumerated on Kempler and McKay agar (15).

Analyses

Cell-free supernatant fluids, obtained by centrifugation at 14,000 × *g* for 10 min, were used for the assay of substrates and products. Diacetyl and acetoin were determined with the colorimetric method described by Walsh and Cogan (32). The concentrations of lactic acid, acetic acid, 2,3-butanediol, and citrate were determined by HPLC as previously described by Bassit et al. (2).

Maximum Rates of Lactic Acid Production, Acidification, and Citrate Consumption

For each fermentation, data were fitted to the following Weibull equation (16): $X = X_0 \pm a\,[1 - \exp(-bt^c)]$, where a, b, and c are fitted parameters calcu-

lated using nonlinear regression software (8), t is time (hours), X is a variable to study, and X_0 is its corresponding value at t = 0. The first derivative of this equation represented the rate of X as a function of time. Its maximum was reached when the second derivative was equal to zero. The first and second derivatives were calculated by using a numerical differentiation of two consecutive values.

RESULTS

Effect of Oxygen on Acidification and on Bacterial Growth

Figure 1 shows the evolution of pH and lactic acid production by Cit$^+$ *L. lactis* ssp. *lactis* CNRZ 483 grown at 30°C under different conditions of oxygenation. Mean pH was lowered to 4.5 after 9 h except for growth under 2 atm of oxygen, which lowered the pH to 5.0. Under nitrogen, the maximal acidification rate was 0.77 pH unit/h (Table 1). The rate of pH change was substantially lower, 0.55 to 0.27 pH unit/h, in the presence of oxygen. Furthermore, when 2 atm of pure oxygen were applied to the reactor, the maximum concentration of lactic acid produced was 24.1 m*M* but under 1 atm of nitrogen was 35 m*M*. The maximum rate of lactic acid production also decreased markedly in the presence of oxygen, varying from 13.1 m*M*/h under nitrogen to 4.8 m*M*/h for culture under 2 atm of oxygen.

Except for the culture under 2 atm of oxygen, the population reached the maximal mean of 1.5×10^9 cfu/ml (Table 1). The maximal growth rates, determined from the linear portion of the growth curves, were 0.97/h under nitrogen, 0.74/h under 2 atm of oxygen, and 0.88/h when averaged over all conditions of oxygenation.

Effect of Oxygen on Diacetyl and Acetoin Production

Regardless of oxygenation conditions, diacetyl started to accumulate between the 1st and the 3rd h of growth (Figure 2). Production was maximal after 5 or 6 h and coincided with complete depletion of citrate. Diacetyl concentration did not decrease after 24 h of growth (results not shown). The concentration of diacetyl was maximal at 0.015 m*M* under nitrogen and was 0.13 and 0.26 m*M* when the amounts of soluble oxygen were 21 and 50%, respectively. This production reached the maximal values of 0.39 and 0.45 m*M* when the culture was under 1 and 2 atm of oxygen, respectively.

Acetoin production also started within the first hours of growth. The maximal amount of acetoin produced was 2.5 m*M* under nitrogen but was 3.9 and 4.6 m*M*, respectively, under 21 and 50% of soluble oxygen. Slightly lower production occurred under 1 and 2 atm of oxygen; maximal values were 4.1 and 3.6 m*M*, respectively. This decrease was not related to the reduction of acetoin by butanediol dehydrogenase because no production of 2,3-butanediol was observed.

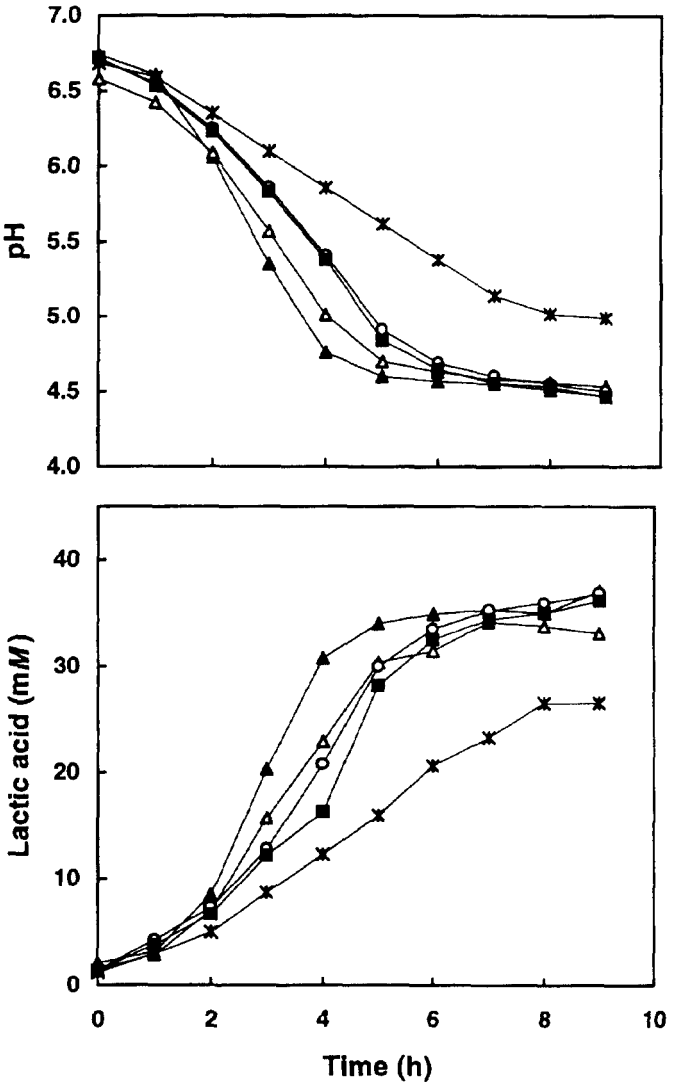

Figure 1. Evolution of pH and lactic acid production by *Lactococcus lactis* ssp. *lactis*. CNRZ 483 cultivated under different conditions of oxygenation: nitrogen (▲), oxygen controlled at 21% (△), oxygen controlled at 50% (○), oxygen pressurized at 1 atm (■), and oxygen pressurized at 2 atm (*).

TABLE 1. Growth and acidification parameters of *Lactococcus lactis* ssp. *lactis* CNRZ 483 cultivated under different conditions of oxygenation.

		Conditions of oxygenation			
	Nitrogen (1 atm)	Oxygen controlled at 21%	Oxygen controlled at 50%	Oxygen (1 atm)	Oxygen (2 atm)
Maximal population, cfu/ml	1.4×10^9	1.4×10^9	1.8×10^9	1.6×10^9	8.9×10^8
Maximal growth rate, /h[1]	0.97	0.86	0.90	0.86	0.74
pH at 9 h	4.47	4.53	4.50	4.46	5.02
Maximal rate of acidification, pH unit/h	0.77	0.55	0.49	0.50	0.27
Maximal concentration of lactic acid, m*M*	35.0	33.8	36.7	35.0	24.1
Lactic acid maximal production rate, m*M*/h	13.1	8.4	9.4	7.9	4.8

[1]The maximal growth rate was determined by the slope of a semi-logarithmic plot of colony-forming units versus time.

Effect of Oxygen on Citrate Consumption and Acetic Acid Production

Lactococcus lactis ssp. *lactis* CNRZ 483 required 4 to 6 h to consume all of the citrate with or without oxygen (Figure 2). However, maximal rate of citrate consumption decreased slightly under high concentrations of soluble oxygen. Citrate consumption was about 1.8 m*M*/h under 2 atm of oxygen and 2.8 m*M*/h under nitrogen. Acetic acid production started when citrate consumption started. Maximal concentrations varied between 11.9 and 13.2 m*M*, regardless of oxygenation conditions.

Effect of Oxygen on Enzyme Activities

Table 2 shows enzyme activities in the cell-free extract of *L. lactis* ssp. *lactis* CNRZ 483. Oxygen had little effect on the specific activities of acetoin and butanediol dehydrogenase. However, the presence of oxygen elevated the specific activity of NADH oxidase to 0.098 ± 0.010 U/mg. The specific activity of LDH was 49.6 ± 2 U/mg under nitrogen and 14 ± 1.2 U/mg under 2 atm of oxygen. At 21 and 50% of soluble oxygen, the specific activity of ALS was, respectively, 2.6- and 2.9-fold higher than under nitrogen. However, higher concentrations of oxygen (1 and 2 atm) reduced activity.

DISCUSSION

Culture of *L. lactis* ssp. *lactis* CNRZ 483 under 2 atm of pure oxygen produced less lactic acid and exhibited a lower maximal rate of acidification than did growth under nitrogen. Those results agree with previous observations (2) of this species grown in flasks that had been hermetically sealed after initial saturation of the medium with oxygen. In the presence of oxygen, some lactic acid bacteria have been totally or partially inhibited (1), primarily because of hydrogen peroxide production during the reoxidation of NADH with NADH oxidase (22). However, Bruhn and Collins (4) showed that Cit$^+$ lactococci oxidized the NADH to NAD without production of hydrogen peroxide. The inhibition on acidification observed during this study was probably due to the metabolic shift in the presence of oxygen. When oxygen concentration increased, maximal growth rate

TABLE 2. Specific enzymatic activities of cell-free extracts of *Lactococcus lactic* ssp. *lactis* CNRZ 483 grown in a whey medium.[1]

Conditions of oxygenation	Specific activities									
	NADH Oxidase		Acetoin dehydrogenase		Butanediol dehydrogenase		Lactate dehydrogenase		α-Acetolactate synthase	
	(U/mg of protein)									
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
Nitrogen (1 atm)	0.031	0.001	0.113	0.016	0.045	0.016	49.6	2.0	1.06	0.18
Oxygen controlled at 21%	0.098	0.004	0.134	0.036	0.020	0.005	34.3	1.3	2.76	0.74
Oxygen controlled at 50%	0.095	0.017	0.106	0.001	0.029	0.002	23.8	1.5	3.05	0.64
Oxygen (1 atm)	0.095	0.004	0.127	0.007	0.034	0.001	22.9	1.2	1.35	0.06
Oxygen (2 atm)	0.098	0.010	0.105	0.011	0.027	0.004	14.0	1.2	0.96	0.10

[1]Cells were harvested at pH 5.2.

of the strain and maximal acidification rate decreased.

Regulation of the soluble oxygen concentration at 21 and 50% and increased oxygen solubility in the medium by pressurization promoted diacetyl production. These results confirm the positive effect of oxygen on diacetyl production that had been observed previously (2, 11, 19). Diacetyl synthesis has been attributed to diacetyl synthase activity (25). Kaneko et al. (13) attributed the enhancement of diacetyl production with oxygen to the increase of diacetyl synthase activity. However, recent work (11) indicated that this enzyme did not contribute to diacetyl production by Cit$^+$ *L. lactis* ssp. *lactis*.

When growing anaerobically, lactic acid bacteria mainly dehydrogenate the NADH produced during glycolysis via LDH activity. Our results confirm that oxygen increases NADH oxidase activity (7), which causes NADH reoxidation to the detriment of LDH, butanediol dehydrogenase, and acetoin dehydrogenase activities (2). Then, excess pyruvate, which is toxic for the cell, is eliminated partially

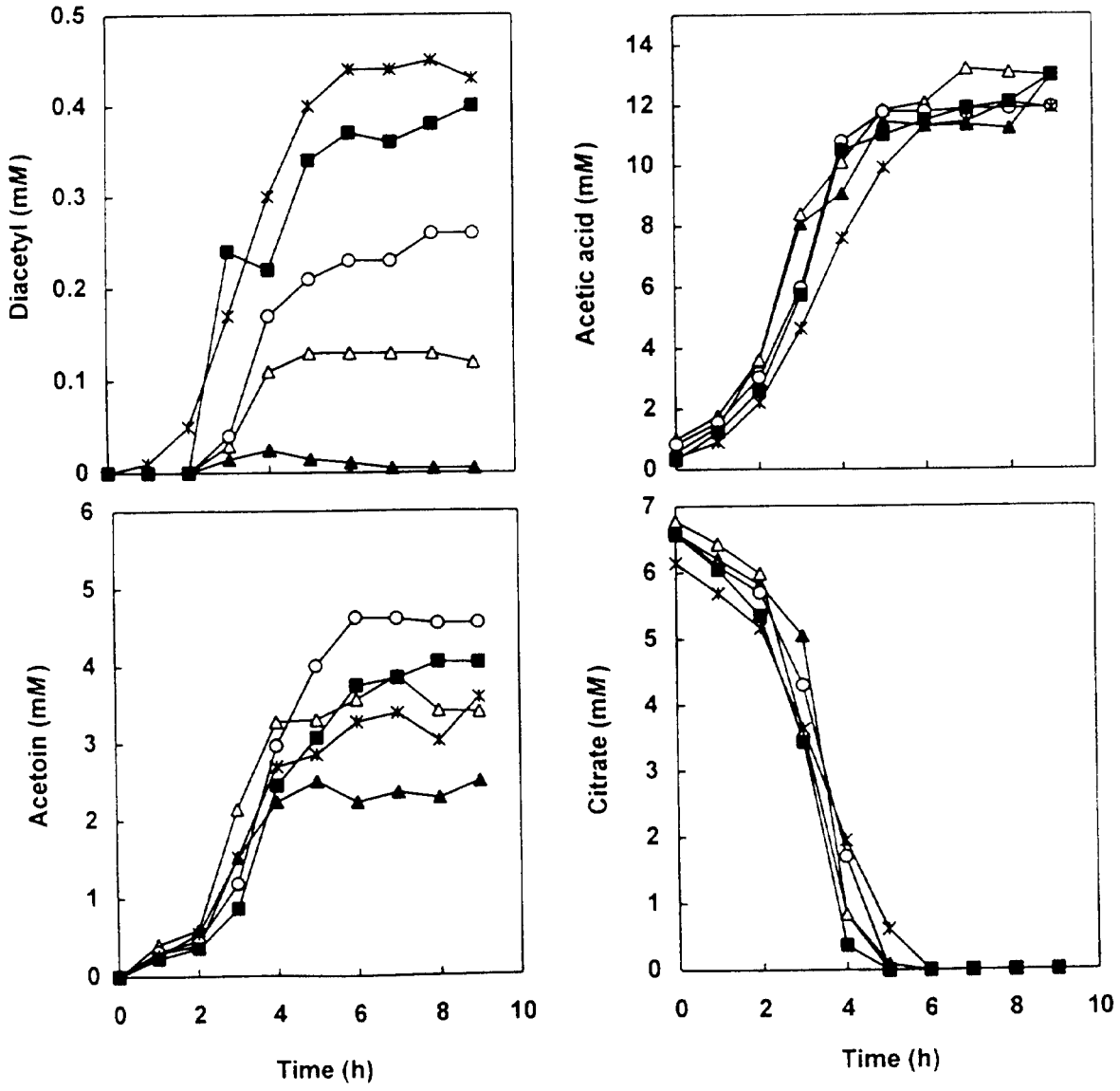

Figure 2. Diacetyl, acetoin, and acetate production and citrate consumption by *Lactococcus lactis* ssp. *lactis* CNRZ 483 cultivated under different conditions of oxygenation: nitrogen (▲), oxygen controlled at 21% (△), oxygen controlled at 50% (○), oxygen pressurized at 1 atm (■), and oxygen pressurized at 2 atm (*).

through acetolactate production, which increases production of diacetyl and acetoin. Increased concentrations of intracellular pyruvate might have resulted also from the reduction in specific activity of LDH when oxygen concentrations increased, which was likely associated with a lower intracellular concentration of fructose-1,6-diphosphate, the principle activator of LDH, caused by the higher oxygen concentrations (23). The increase in pyruvate concentration results in acetolactate formation and, consequently, acetoin and diacetyl formation, but only when the intracellular concentration of pyruvate is high because the ALS of *L. lactis* ssp. *lactic* has a very low affinity for pyruvate (Michaelis constant is about 50 m*M*) as shown by Snoep et al. (24).

According to Bassit et al. (2), the specific activity of ALS increased with oxygen. However, in our study, ALS activity decreased at high concentrations of oxygen (1 and 2 atm). This result suggested that ALS was not responsible for the improvement of diacetyl production by *L. lactis* ssp. *lactis* CNRZ 483 at 1 and 2 atm of oxygen. Nevertheless, the high concentrations of diacetyl that were observed during aerobiosis by some researchers (2, 6) resulted principally from oxygen activation of the ALS. Our results support the conclusions of Hugenholtz and Starrenburg (11) and Monnet et al. (18), who suggested that diacetyl production is a chemical phenomenon mainly caused by the oxidative decarboxylation of α-acetolactate. No significant effect of oxygen on acetic acid production was observed.

In summary, this work showed that proper oxygenation of the culture medium promoted diacetyl production by *L. lactis* ssp. *lactis* CNRZ 483, probably by decreasing the activity of LDH and favoring the chemical oxidative decarboxylation of α-acetolactate. The application of these conditions to the dairy industry would be interesting. Oxygen pressure appears to be more advantageous than oxygen regulation because of the ease of execution and the increased diacetyl obtained.

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