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Diacetyl Production Mechanism by a Strain of *Lactococcus lactis* spp. *lactis* bv. *diacetylactis*

Study of α-Acetolactic Acid Extracellular Accumulation Under Anaerobiosis

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ABSTRACT

Diacetyl production via α-acetolactic acid (ALA) extracellular decarboxylation in *Lactococcus lactis* spp. *lactis* bv. *diacetylactis SD 933* cultures has been assessed under anaerobiosis both in batch and continuous fermentations at pH 5.5 and 8.0 by studying the effects of α-acetolactate decarboxylase (ADC) addition in the culture broth. This enzyme, favoring the formation of acetoin instead of diacetyl, was added extracellularly and did not disturb diacetyl production. Moreover, oxidation experiments on extracellular culture media did not reveal any increase in diacetyl amount caused by extracellular ALA oxidative decarboxylation. These observations confirm previous assertions concerning the mechanism and localization of diacetyl synthesis by the *SD 933* strain.

Index entries: Lactic acid bacteria; *Lactococcus*; bacterial metabolism; flavor production; diacetyl.

INTRODUCTION

Lactococcus lactis spp. *lactis* bv. *diacetylactis* is a strain used in the dairy industry in starter cultures for acidification and flavor production *(1)*. Its metabolism mainly leads to lactic acid formation, and is associated with an

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interesting range of secondary compounds, such as acetate, acetaldehyde, 2,3-butanediol, acetoin, and, finally, diacetyl, which is one of the most important flavors found in butter *(2)*. Diacetyl is produced from pyruvic acid accumulated as a consequence of lactose and citric acid catabolism. Diacetyl and its reduced derivatives acetoin and 2,3-butanediol (C4 compounds) production pathways act as a pyruvate detoxification mechanism for the cell.

The bioconversion schemes from pyruvic acid to diacetyl and acetoin in *Lactococcus lactis* spp. *lactis* bv. *diacetylactis* involve a common first step, during which a pyruvate decarboxylase catalyzes the formation of an acetaldehyde-thiamin-pyrophosphate (acetaldehyde-TPP) complex *(3–5)*.

Diacetyl can be formed from this complex following two pathways: condensation of acetyl-CoA with the acetaldehyde-TPP complex catalyzed by a diacetyl synthase, directly leading to diacetyl formation *(5,6)* (however, some authors *(7,8)*, although admitting the existence of this pathway, do not consider it as responsible for diacetyl production); acetaldehyde-TPP complex condensation with a molecule of pyruvate through the action of an acetolactate synthase, leading to ALA formation. The ALA formed is then mainly decarboxylated to acetoin by an α-acetolactate decarboxylase, but can also be oxidatively decarboxylated to diacetyl in small proportions *(3–5,9,10)*.

Knowledge about ALA oxidative decarboxylation remains poor because of the lack of characterization of any enzyme performing this reaction. According to ALA chemical instability *(11,12)*, a spontaneous oxydative decarboxylation to diacetyl has been suggested *(13)*. However, the observation of diacetyl production by *Lactococcus lactis* spp. *lactis* bv. *diacetylactis SD 933* under conditions in which chemical oxidative decarboxylation is not prone to occur (because of low redox potential and strict anaerobiosis), led to the study of the ALA reactivities under anaerobiosis in extracellular media *(14)*. These ALA reactivities were shown to be unrelated with diacetyl production rates observed during fermentations in the same conditions, indicating that, with this particular strain, diacetyl production did not seem to proceed via extracellular oxidative decarboxylation of ALA. In order to confirm these observations, an indirect enzymatic method was applied to detect diacetyl extracellular production via ALA extracellular oxidative decarboxylation. Comparative fermentations were carried out in the presence or absence of a huge excess of ADC, an enzyme that performs ALA decarboxylation to acetoin, and consequently prevents ALA from being oxidatively decarboxylated to diacetyl, in order to detect potential disturbances in diacetyl formation kinetics. These comparative fermentations were carried out in both batch and continuous mode, to study the effect of ADC during dynamic reactions or at steady state. A chemical method based on extracellular media chemical oxidation, leading to an increase of the diacetyl

amount in the presence of ALA, was used as a control for this indirect enzymatic method.

METHODS

Bacterial Strain and Experimental Conditions

Comparative batch and continuous fermentations were carried out in order to collect the kinetic data concerning the effect of ADC on culture kinetics.

Bacterial Strain and Conservation

Lactococcus lactis spp. *lactis* bv. *diacetylactis SD 933* was obtained from Visby Laboratories (Tönder, Denmark). The strain was stored at –18°C in a De Man, Rogosa, and Sharpe (MRS) derivative medium *(15)*, and was routinely cultivated in the same medium.

Inoculum and Fermenter Inoculation

The inoculum was prepared by cultivating the bacteria in 200 mL of medium at 30°C for 15 h. The bioreactor already containing 1.4 L of medium was then inoculated with the whole preculture. In order to achieve a strict anaerobiosis, nitrogen was injected for 20 min into the culture medium before inoculation and for 5 min after inoculation.

Cultivation Conditions

Cultures were carried out in a 2-L Applikon (Schiedam, The Neitherlands) bioreactor containing 1.6 L of fermentation broth stirred at 250 rpm and maintained at a temperature of 25°C by thermostated water circulation in the double jacket. The pH was maintained by addition of NaOH 4 *M*. For continuous cultures, two pumps, one for fresh medium feeding (101 U, Watson Marlow, Falmouth, England) and the other one for culture broth removing (501 U, Watson Marlow), were coupled to the bioreactor to ensure a close control of the dilution rate and a constant reaction volume.

Medium Composition

D (+) lactose monohydrate	10 g	Sodium acetate	5 g
Casein peptone	10 g	Citric acid monohydrate	1.5 g
Yeast extract	5 g	$MgSO_4$, 7 H_2O	0.1 g
Milli Q Water	qs. 1 L		

For cultures carried out with ADC, the enzyme addition was performed via sterile injection of a 0.2 μm filtered (Millex GS, Millipore, France) ADC-enriched extract of genetically engineered *Bacillus subtilis* (Maturex L, Novo Nordisk Ferment, Switzerland) in the sterilized bioreac-

tor. The minimum enzymatic activity achieved was 25 ADU/L in the experimental conditions, with one ADU corresponding to the amount of enzyme catalyzing the production of 1 μ*M* of acetoin/min from ALA.

Media Filtration

Cells were removed from the culture media by filtration with 0.2 μm Sartobran (Sartorius) capsules under nitrogen atmosphere to maintain strict anaerobiosis.

Air Oxidation

Oxidation of the extracellular media was achieved by a 30 s injection of air at a flow rate of 1.5 L/min in the bioreactor.

Potassium Permanganate Oxidation

The content of extracellular media was oxidized by sterile injection of 250 mg/L of $KMnO_4$.

Analytical Methods

Biomass Determination

Biomass was estimated by means of absorbance measurements at 650 nm. Optical density was calibrated against cell dry wt. Cell dry wt estimations were made by filtering 25 mL of fermentation broth through tared 0.2 μm filters (Sartorius 11107-50-N, Göttingen, Germany) and drying the filters for 24 h at 105°C.

Diacetyl, Acetaldehyde, and Ethanol Determination

These compounds were measured on-line by gas chromatography coupled to a gas membrane sensor immersed in the fermentation media *(16)*.

2,3-Butanediol and Acetoin Determination

The off-line analyses of 2,3-Butanediol and acetoin were realized by injection of 1-μL filtered samples in a Delsi Nermag (ILS, Lyon, France) DI 200 gas chromatograph fitted with a flame ionization detector and a J&W Scientific (Courtaboeuf, France) 30 m carbowax 20 *M* 0.25 μm capillary column fed by helium with a division of 75 mL/min and a leak of 0.5 mL/min. The column temperature evolution was the following: 90 s at 80°C, a gradient to 160°C at 13°C/min, 30 s at 160°C, another gradient to 165°C at 10°C/min, 15 s at 165°C, and a decrease to 80°C at 15°C/min.

Organic Acids and Lactose Determination

The concentrations of lactose, citric, lactic, pyruvic, and acetic acids were measured by a Waters (Milford, MA) high performance liquid chro-

Table 1
Effect of ADC on Substrate Consumption and Compound Production After 16 h of Batch Fermentations Carried Out at pH 5.5

ADC	–	+
Biomass produced (g/L)	0.9	1.1
Lactose consumed (g/L)	9.8	9.9
Lactate produced (g/L)	9.3	9.4
Citrate consumed (g/L)	1.5	1.5
Acetate produced (g/L)	0.5	0.5
Diacetyl produced (mg/L)[a]	7.3	7.8
Acetoin produced (mg/L)[a]	214	264
2,3-butanediol produced (mg/L)[a]	226	241

[a]Maximum amount reached during the 16 h cultures.

matograph fitted with a 65°C thermostated polypore H column (Brownlee Labs, A.B.I., Santa Clara, CA) fed with 0.04 *N* H_2SO_4 at a flow rate of 0.9 mL/min. Organic acids and lactose were detected, respectively, with a Waters UV lambda max model 481 LC spectrophotometer at 210 nm and a Waters 410 differential refractometer.

Fermentation Data Treatment

All fermentation data have been submitted to a low-pass filter generating intermediate points and working out instantaneous speeds.

RESULTS

Effect of ADC on Batch Cultures

The effect of ADC on C4 metabolism, and particularly on diacetyl and acetoin productions, was assessed by comparison of batch cultures carried out at pH 5.5 and 8, with and without this enzyme.

At pH 5.5, lactate and acetate production and lactose and citrate consumption were similar with and without ADC. Lactose was fully consumed and catabolized mostly to lactate, with a yield of 0.95 g/g. Citrate too was fully consumed and its consumption was associated with acetate production, with a yield of 0.33 g/g. Only a slight difference could be observed concerning biomass production. All these observations indicate that the enzyme extract has no dramatic influence on the major metabolic pathways (Table 1).

As far as C4 compounds are concerned, the amount of diacetyl produced with ADC was slightly superior to the amount produced without

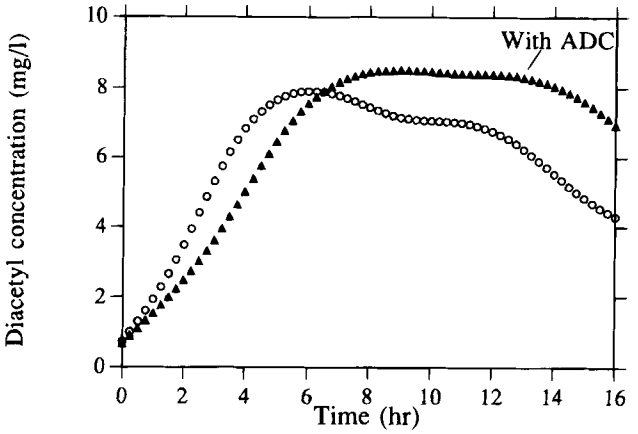

Fig. 1. Effect of ADC on diacetyl production in batch cultures carried out at pH 5.5.

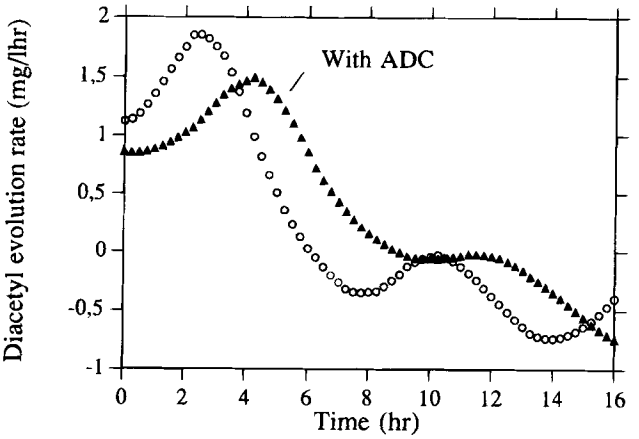

Fig. 2. Effect of ADC on diacetyl production rate in batch cultures carried out at pH 5.5.

ADC (Fig. 1). The production of diacetyl with ADC, compared to that without it began at a slower rate, and also had a lower maximum rate. The evolution rate, however, was higher for a longer period of time (Fig. 2). Acetoin, and, as a consequence, its reduced derivative, 2,3-butanediol, were produced more in the presence of ADC, with a difference of 50 mg/L for the maximum amount of acetoin produced and 15 mg/L of 2,3-butanediol after 16 h of fermentation, corresponding respectively to 23 and 7% of the basic amounts of the same compounds produced without ADC (Figs. 3 and 4). Acetoin production with ADC began immediately at the beginning of the fermentation, as in the culture without ADC, but lasted longer. Consequently, acetoin achieved a higher maximum level with ADC about

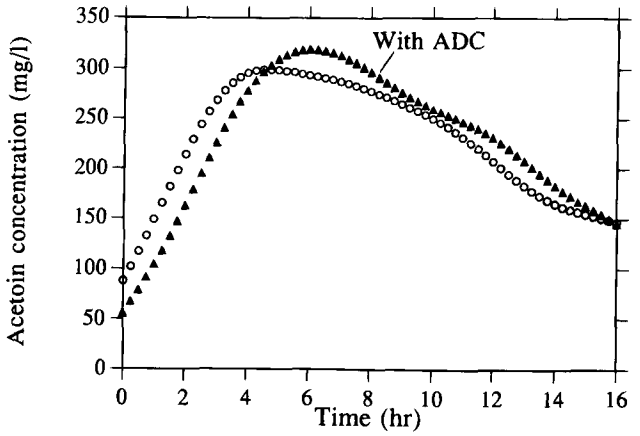

Fig. 3. Effect of ADC on acetoin production in batch cultures carried out at pH 5.5.

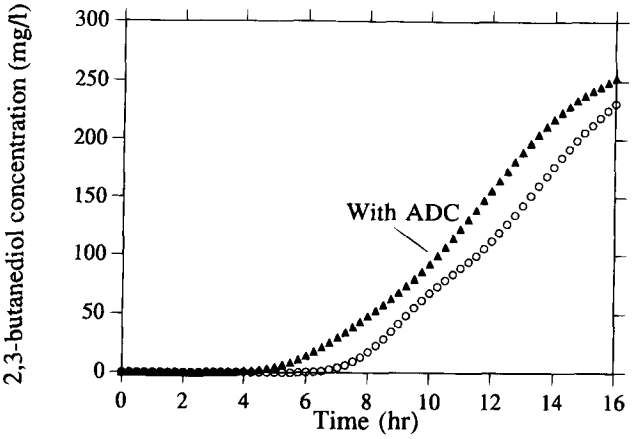

Fig. 4. Effect of ADC on 2,3-butanediol production in batch cultures carried out at pH 5.5.

2 h after the maximal level was observed without the enzyme. 2,3-butanediol production began with approx 2 h of advance with ADC, and production rates were globally equal for the two batches, leading after 16 h of culture to a better 2,3-butanediol production with ADC.

At pH 8.0, no significant difference between cultures carried out with and without ADC could be raised concerning the major substrates and products (Table 2). Lactose was fully catabolized and partially transformed to lactate, with a lower yield than at pH 5.5 (0.80 g/g). Citrate was only slightly consumed, associated with an acetate production. The important amount of acetate produced translates a conversion of both lactose and citrate to this compound. Biomass produced without ADC

Table 2
Effect of ADC on Substrate Consumption and Compound Production After 16 h of Batch Fermentations Carried Out at pH 8.0

ADC	–	+
Biomass produced (g/L)	1.3	1.3
Lactose consumed (g/L)	9.8	9.7
Lactate produced (g/L)	7.9	7.8
Citrate consumed (g/L)	0.1	0.1
Acetate produced (g/L)	0.1	0.1
Diacetyl produced (mg/L)[a]	0.1	0.1
Acetoin reduced (mg/L)	36	30
2,3-butanediol produced (mg/L)[a]	44	42

[a]Maximum amount reached during the 16 h cultures.

was equivalent to that produced with ADC. All these data confirm the lack of effect of the enzyme extract on the strain's primary metabolism at pH 8.0.

Equivalent diacetyl productions, slightly different acetoin decreases, and similar 2,3 butanediol productions were observed with and without ADC. With respect to acetoin, the difference in the reduced amount is probably the consequence of an initially lower level in the fermentation broth with ADC (37 mg/L, compared to 43 mg/L without ADC), the final amount reached at the end of both cultures was identical (7 mg/L).

Batch cultures at pH 5.5 and 8.0 revealed only a small effect of ADC on the primary metabolism of *Lactococcus lactis* spp. *lactis* bv. *diacetylactis SD 933*, with a slight increase of biomass production with ADC at pH 5.5; lactose and citrate consumptions and lactate and acetate productions were equivalent with and without ADC for comparative cultures carried out at pH 5.5 and 8.0. As far as C4 compounds are concerned, the only effect of ADC was an increase of acetoin production at pH 5.5. The enzyme-enriched extract did not modify the production of acetoin at pH 8.0 and the production of 2,3-butanediol at pH 5.5 and 8.0. Moreover, such a large excess of extracellular ADC activity did not succeed in decreasing diacetyl production either at pH 5.5 or 8.0.

Effect of ADC on Continuous Cultures

The effect of ADC on C4 metabolism was also assessed in continuous culture mode at pH 5.5 and 8.0. The dilution rates were roughly chosen from specific growth rates, allowing maximal diacetyl production rates during batch experiments at corresponding pHs. Table 3 indicates the results obtained at steady state during these experiments.

Table 3
Effect of ADC on Metabolism of *Lactococcus lactis* spp. *lactis* bv. *diacetylactis* SD 933 Cultivated in Continuous Mode

ADC	−	+	−	+
pH	5.5	5.5	8	8
Dilution rate (h^{-1})	0.29	0.29	0.44	0.44
Biomass (g/L)	0.8	0.6	0.5	0.6
Lactose (g/L)	7.3	7.2	6.1	6.1
Lactate (g/L)	2.4	2.5	3.2	3.2
Citrate (g/L)	0.6	0.6	1.5	1.5
Acetate (g/L)	4.0	4.0	3.7	3.7
Diacetyl (mg/L)	3.8	3.7	0.2	0.2
Acetoin (mg/L)	194	222	39	38
2,3-butanediol (mg/L)	33	32	46	44

At pH 5.5, little influence of ADC could be observed on the main substrates consumption and metabolites production (Table 3), except for biomass. This difference of biomass level can be a result of the higher biofilm formation in the reactor observed with ADC. Similar lactate/lactose (0.89 g/g) and acetate/citrate (0.38 g/g) yields were determined with and without ADC, and these yields were in accordance with those found during batch cultures carried out at pH 5.5. This confirms the lack of effect of the ADC-enriched extract on the strain's primary metabolism already raised in batch at pH 5.5. Concerning the C4 compounds, ADC had virtually no effect on diacetyl and 2,3-butanediol productions, but acetoin production was shown to be increased in the presence of ADC extract, just as it appeared to be in batch.

At pH 8.0, ADC did not have any effect on the primary metabolism of the strain, the only difference raised concerning biomass, with a slight increase of growth in presence of ADC (Table 3). The lactate/lactose yields with and without ADC were similar (0.82 g/g) and corresponded to those found in batch at pH 8.0. No citrate consumption and acetate production were detected in the presence or absence of the enzyme. Comparable diacetyl, acetoin, and 2,3-butanediol levels were determined, translating no effect of extracellular addition of ADC on the C4 metabolism.

At pH 5.5 and 8.0 in continuous culture mode, little influence of ADC could be observed on the main metabolites, except for biomass, with a slight increase of growth in batch mode and the formation of a biofilm in continuous mode in presence of ADC. These observations confirm previous assertions concerning the lack of effect of the ADC extract on the strain primary metabolism following the batch experiments. No significant

decrease in the amount of diacetyl produced in the presence of ADC was observed at either pH 5.5 or 8.0. Acetoin was produced more at pH 5.5 with ADC than in the same conditions without ADC; no modification in concentration was observed at pH 8.0. As was the case in batch cultures, acetoin was shown to be the main C4 compound prone to production variations as a consequence of ADC presence, but this enzyme did not modify the amounts of diacetyl and 2,3-butanediol produced.

Effect of Extracellular Media Oxidation

The observation of increased amounts of acetoin produced in presence of ADC at pH 5.5 led to the use of another method for ALA extracellular accumulation detection. This method is based on the separation of the extracellular medium from the cells by a microfiltration step, and on the rapid transformation of ALA present in this extracellular medium to diacetyl by oxidation. The separation step allows elimination of any effect of the oxidizing reaction on the cells, and consequently limits the occurrence of artifacts.

A batch culture was carried out under anaerobiosis at pH 5.5, and extracellular media were recovered by microfiltration under anaerobic conditions after 3, 6, and 9 h of reaction. The first sample corresponds to the diacetyl synthesis period, the second and third samples correspond, respectively, to the end of the diacetyl production and slow diacetyl concentration decrease phases. These three media were then oxidized by air and $KMnO_4$, and the influence of this treatment on the concentrations of the different components of the media was determined (Table 4).

The filtration step did not prove to have any effect on the chemical content of the culture media. No concentration or dilution after filtration could be observed concerning lactose, lactate, citrate, acetate, acetoin, 2,3-butanediol, and diacetyl (data not shown).

For extracellular media obtained after 3, 6, and 9 h of culture, oxidation by both oxygen and potassium permanganate did not modify the concentrations of the culture media major compounds. No raise in the diacetyl content was observed after oxidation either by air or potassium permanganate. With respect to the global C4 concentration, and particularly to acetoin, no change in concentration was induced by oxidation with either air or $KMnO_4$. These results indicate that there is no detectable extracellular accumulation of ALA, and are in accordance with those obtained with the enzymatic method. The extra production of acetoin observed with ADC consequently seems to be because of an effect of the enzyme extract on the cells. However, the absence of ALA detection by the oxidation method confirms the value of the enzymatic method for ALA extracellular accumulation detection, even if acetoin production is modified.

Table 4
Effect of Oxidation on Content of Extracellular Medium of Batch Culture Carried Out at pH 5.5

Time (h)	3			6			9		
Oxidation	None	O_2	$KMnO_4$	None	O_2	$KMnO_4$	None	O_2	$KMnO_4$
Lactose (g/L)	8.9	8.8	8.9	8.0	7.9	7.9	5.6	5.6	5.6
Lactate (g/L)	0.8	0.8	0.8	1.7	1.8	1.8	4.0	3.9	3.9
Citrate (g/L)	0.4	0.3	0.3	0.1	0.1	0.1	0	0	0
Acetate (g/L)	4.1	4.1	4.1	4.2	4.1	4.2	4.2	4.2	4.2
Diacetyl (mg/L)	6.2	6.2	6.2	7.2	7.1	7.2	7.1	7.1	7.1
Acetoin (mg/L)	131	133	129	165	163	170	265	260	268
2,3-butanediol (mg/L)	28	25	27	27	27	29	25	32	27

DISCUSSION

In batch and continuous culture modes, at both pH 5.5 and 8.0, the addition of the ADC-enriched cellular extract did not prove to have a measurable effect on the main metabolic pathways (for lactate and acetate production and lactose and citrate consumption), except for biomass production mainly in continuous cultures in which biofilm formation could have perturbed cell growth evaluation.

ADC added in large amounts widely exceeding diacetyl or acetoin production rates (25 ADU corresponding to an acetoin production rate of 130 mg/L/h) did not prove to be able to prevent diacetyl production, either in batch or continuous modes, at both pH 5.5 and 8.0. If the diacetyl production mechanism by this strain was proceeding via ALA extracellular spontaneous oxidative decarboxylation, the excess of ADC would have prevented ALA from being transformed to diacetyl by competition between enzymatic decarboxylation (at least 197 mg ALA transformed/L/h) and oxidative decarboxylation (approx 2 mg/L/hr). Consequently, it can be assumed that there is no, or very low, ALA accumulation in the culture medium during fermentations carried out at pH 5.5 and 8.0. These results are confirmed by those of oxygen and $KMnO_4$ extracellular media oxidation at pH 5.5, which did not show any increase in the diacetyl content, and consequently did not reveal any significant accumulation of ALA in the extracellular media.

Because previous experiments *(14)* showed only limited ALA extracellular reactivity compared with diacetyl biological production rates, and because ALA does not accumulate in detectable amounts in the medium, the extracellular oxidative decarboxylation of ALA can be dismissed as a major diacetyl production mechanism by *Lactococcus lactis* spp. *lactis* bv. *diacetylactis SD 933* cultivated under anaerobiosis. Accordingly, this production may occur via intracellular enzymatic or nonenzymatic *(17)* oxidative decarboxylation, or via the Speckman and Collins pathway. One way of determining the pathway followed should be the study of ALA oxidative decarboxylation rates on *Lactococcus* cellular extracts.

Concerning the increase of acetoin production in the presence of ADC in both batch and continuous cultures at pH 5.5, and given the results obtained with extracellular media oxidation experiments, it can be assumed that this phenomenon is an artifact characteristic of the enzymatic method. Two major hypotheses may be raised concerning its origin: presence in the ADC extract of an ADC activator able to act intracellularly, and to promote acetoin production; and rapid transformation of low amounts of parietal or extracellular ALA by ADC to acetoin, inducing a modification of the ALA intra/extracellular equilibrium, leading to an increased ALA excretion, and consequently to an increased acetoin production, but without diacetyl production modification.

SUMMARY

Research of ALA extracellular accumulation during *Lactococcus lactis* spp. *lactis* bv. *diacetylactis SD 933* anaerobic batch and continuous cultures at pH 5.5 and 8.0 has been accomplished through the intermediary of two methods, one enzymatic and the other one chemical, translating ALA extracellular presence in a variation of diacetyl concentration, precisely evaluated by gas–gas chromatography coupled with a gas membrane sensor *(16)*. The results obtained showed no extracellular accumulation of ALA by this strain under the different conditions evaluated in this study. This, taken together with the low extracellular ALA oxidative decarboxylation rates *(14)*, allows the conclusion that this reaction at the extracellular level is not the dominant mechanism of diacetyl production by this strain under anaerobiosis.

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