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Full Length Research Paper

A new manometric method for measuring carbon dioxide production by dairy starter culture: A case of *Leuconostoc mesenteroides*

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This paper describe a technique for carbon dioxide (CO_2) measurement based on the displacement of acidifying liquid in burette at constant pressure. It was adapted to allow the growth of *Leuconostoc* sp. on semi-synthetic media and milk. A highly precise measurement was obtained using the chemical reaction of sodium carbonate with hydrochloric acid. This method has been proven to present an excellent linear response from 0 to 0.895 mM of CO_2 with a high sensitivity. With cultures in milk medium, kinetics of CO_2 evolved by *Leuconostoc mesenteroides* subsp. *dextranicum* L4 were higher than *L. mesenteroides* subsp. *mesenteroides* 19D and *L. mesenteroides* subsp. *cremoris* Ec195 grown on milk.. The use of *L. mesenteroides* subsp. *dextranicum* L4 and their variants *Lac(-)Cit(+)*, *Lac(+)Cit(-)* and *Lac(-)Cit(-)* showed that the CO_2 production correlated to the substrate utilization. The curve of CO_2 measured was close to the amount of CO_2 calculated from lactose and citrate used. The CO_2 production rate was higher with the parental strain (6.5 mM/ h) than with the variants. The amount of CO_2 produced was always close to the theoritical value, and was also correlated to the growth rate and substrates utilization by *L. mesenteroides.* The volume of CO_2 produced by *Leuconostoc* in milk was highly correlated with the D-lactate production (r = 0.995). This method could be used routinely for the evaluation and the selection of bacteria having potential ability for CO_2 production.

Key words: *Leuconostoc* sp., CO_2 production, lactic acid, milk.

INTRODUCTION

Mesophilic lactic acid bacteria are widely used in the manufacture of fermented dairy products and the *Leuconostoc* genus are of particular importance in food grade application (Devoyod et al., 1969; Morisset and Frere, 2002; Carr et al., 2002; Rodas et al., 2003, Lopez-Diaz et al., 2000). *Leuconostoc* species ferment glucose *via* phosphoketolase pathway to equimolar amounts of D(-) lactate, ethanol and CO_2 (De Moss et al., 1951; Desmazeaud, 1983). It has been said that citrate added in glucose or lactose broth stimulates growth and CO_2 production in *Leuconostoc* sp. (Cogan, 1987; Diviès, 1991; Matoh et al., 1994). Several authors have measured the stimulatory effect of accessory growth factors on the *Leuconostoc* by the acid production, turbidity and colony count (Gibson and Abdelmalek, 1945; Speack et al., 1958; Park et al., 2003). The ability of *Leuconostoc* sp. to produce gas was studied by a qualitative method, by which an agar plug was forced up the neck of a volumetric flask measured gas visually (Holmes et al., 1968, Kim et al., 2000). The biochemical activities may vary considerably among the cultures, yielding different extents of gas

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production. Carbon dioxide is an end product of bacterial fermentations and may be desirable or undesirable in dairy products.

Historically the Warburg apparatus has been used to determine accurately the amount of CO_2 generated by microorganisms. Other techniques, titrimetric method, manometric assay, infrared (Burt and Rau, 1994), gravimetric analysis, enzymatic assay and visual evaluated have been described (Umbreit et al., 1957; Garvie, 1980; Rosenberg, 1980; Tseng and Montville, 1990; Smart and Thomas, 1987). Recently, Kneifel and Greitner (1992) described a novel method for the estimation of carbon dioxide produced by mesophilic starter culture under defined conditions. A commercially available drager (Drägerwerk AG., Lübeck, Germany) CO_2 sensitive gas diffusion tube is placed in the bore of rubber stopper covering an Erlenmeyer flask containing pasteurized milk inoculated with the starter culture to be tested. This method cannot measure dissolved CO_2 in culture. Gas chromatographic methods have been developed, but they required 17 to 24 h incubation period, and the sampling procedures neglected the solubility of CO_2 in aqueous solution (Mohr et al., 1993; Girard et Boyaval., 1994; Kim et al., 2000). However, most of this technique either needs a specialized instrumentation or they are lacking accuracy because of subjective interpretation. Data on the kinetics of CO_2 evolved by *Leuconostoc* used in dairy industry of blue cheeses are not available probably because of the difficulties encountered to measure it. Therefore, the first objective of the present study was undertaken to develop a simple method which can to determine exactly the quantity of CO_2 evolved by *Leuconostoc* sp. The second investigation was carried out to clarify the relationship existing between the growth and CO_2 production by *Leuconostoc* sp.

MATERIALS AND METHODS

Organisms

Leuconostoc mesenteroides subsp. *dextranicum (L4)*, *L. mesenteroides* subsp. *mesentroides (19D)* and *L. mesenteroides* subsp. *cremoris (Ec 195)* were used. *L. mesenteroides* subsp. *dextranicum* variants, *Lac(-)Cit(+)*, *Lac(+)Cit(-)* and *Lac(-)Cit*(-), were isolated and selected by Huang et al. (1994) and Kihal et al. (1996). All strains were obtained from the culture collection of Laboratoire de Microbiologie-Biotechnologie ENSBANA, Dijon, France.

Experimental procedure

The analytic system presented by Kihal (1996) is illustrated in Figure 1. The principle of the method is based on the pressure created by CO_2 production by the culture in tubes. Evolved CO_2 was trapped and was measured by displacement of acidifying water in the burette (Figure 1). The total amount of CO_2 produced was released by acidifying with 2M HCl. Details of the procedure is as follows:

Quantities of 10 ml of solution test or culture were filled into tubes. The double armed tube with a sterile silicon rubber stopper has a central bore. After incubation at 30°C, the tube contained culture was connected to graduated burette for measurement. 1 ml of 2M HCl was tipped in using a syringe to release any dissolved CO_2 (Holmes et al., 1968). After shaking with vortex, the experiment was terminated 2 min later. Tests were usually performed in tripticates and concomitantly controlled for microbiological contaminations based on the blank tests (base milk without culture added).

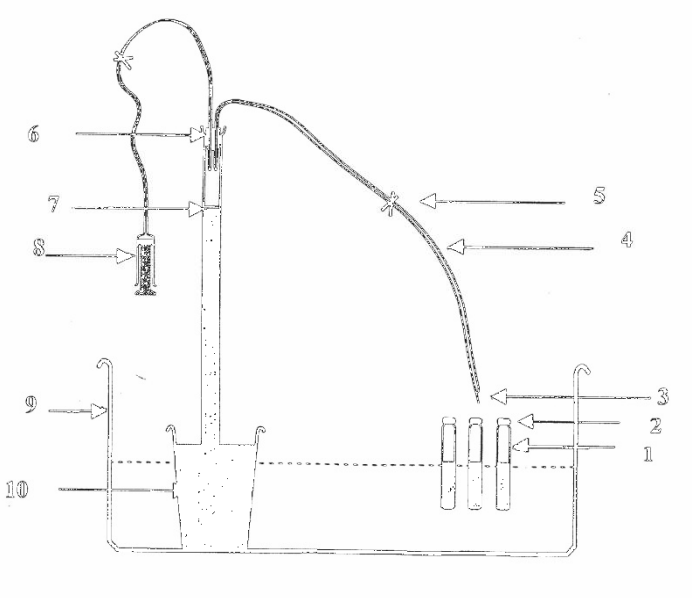

Figure 1. Principal measurement of evolved CO_2 by *L. mesenteroides* by displacement of acidifying liquid in graduated burette in constant pressure. Tube (1), silicon and rubber stopper (2), needle (3), tubing rubber (4), pliers (5), thin rubber stopper (6), graduated burette (7), syringe (8), water bath (9) and acidifying water (10).

Test of accuracy of CO_2 measurement

The accuracy of the measurement of CO_2 production was tested by generating a known quantity of CO_2 using a chemical reaction and measuring the liberated gas using our system (Figure 1). The CO_2 was generated using 10 ml of a solution containing 50 mM of sodium bicarbonate (Mohr et al., 1993). This amount of Na_2CO_3 is expected to release 11.20 ml of CO_2. The solution was placed in the tube having a stopper with a thin rubber. After connecting tube to burette, 1 ml of acid (HCl, 2M) was added to drive off retained CO_2. After shaking tube, total CO_2 produced was measured in a graduated burette. The accuracy of this measurement have been improved by using ten tries which were used in statistical analysis. The nature of Na2CO3 and acid effect on the regeneration of CO_2 were tested in this system. The least significant difference test was performed using statistical analysis.

Statistical analysis according to split pot design was applied to determine the effects of different types of Na_2CO_3 (dissolved or not) on the regeneration of CO_2 (Table I).

Table 1. Validity of CO_2 measurement regenerated from a known quantity of Na_2CO_3 using a chemical reaction with 2M hydrochloric acid and 2M trichloroacetic acid.

Parameter	Nature of Na_2CO_3				T.V
	50 mM Na_2CO_3		powder	Na_2CO_3	
	10 ml	10 ml	54 mg	54 mg	
Acid used	2M HCl	2M TCA	0.18M HCl	2M HCl	
Volume used (ml)	1	1	11	1	
CO_2 produced (ml)	9.315	8.985	10.47	10.94	11.2
Variance	0.594	0.397	0.358	0.42	
T (calculated)	1.587	2.638	1.615	1.023	2.25
Difference	**N.S**	**S**	**N.S**	**N.S**	

T.V: Theoretical value. S: significant. N.S: not significant.

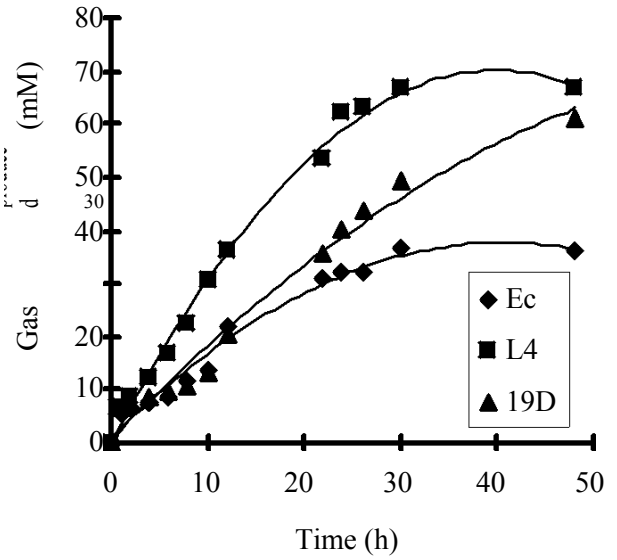

Figure 2. CO_2 production in *L. mesenteroides* subsp. *Dextranicum* L4 ( ), *L. mesenteroides* subsp. *mesenteroides* ( ) and *L. mesenteroides* subsp. *cremoris* (♦) growing on skim milk enriched with 0.3% yeast extratct.

Kinetic comparison of CO_2 production

Tube containing 10 ml skimmed milk with O.1% yeast extract which was inoculated by 10^7 cfu/ml of *Leuconostoc* sp. (*L4, 19D* and *Ec 195*) and incubated at 30°C in a water bath. Evolved CO_2 and viable counts were done each hour in duplicate on MRS agar (Mathot et al., 1994) and were reported as logarithms of colony forming units (Log cfu/ml). Plates were incubated at 30°C for 48 h (Figure 2). Lactose synthetic medium containing the following (g/l): tryptone, 10; yeast extract, 5; Na_3citrate, 5; lactose, 10; K_2HPO_4, 2; KH_2PO_4, 2; $MgSO_4$ $7H_2O$, 0,2; $MnSO_4$, 0,05; pH, 6 (Harvey and Collins, 1962). This media was used to assess the growth of *Leuconostoc (L4)* and their variants; *Lac(-)Cit(+)*, *Lac(+)Cit(-)* and *Lac(-)Cit(-).* Bacterial growth was measured spectrophotometrically at 575 nm (Figure 3).

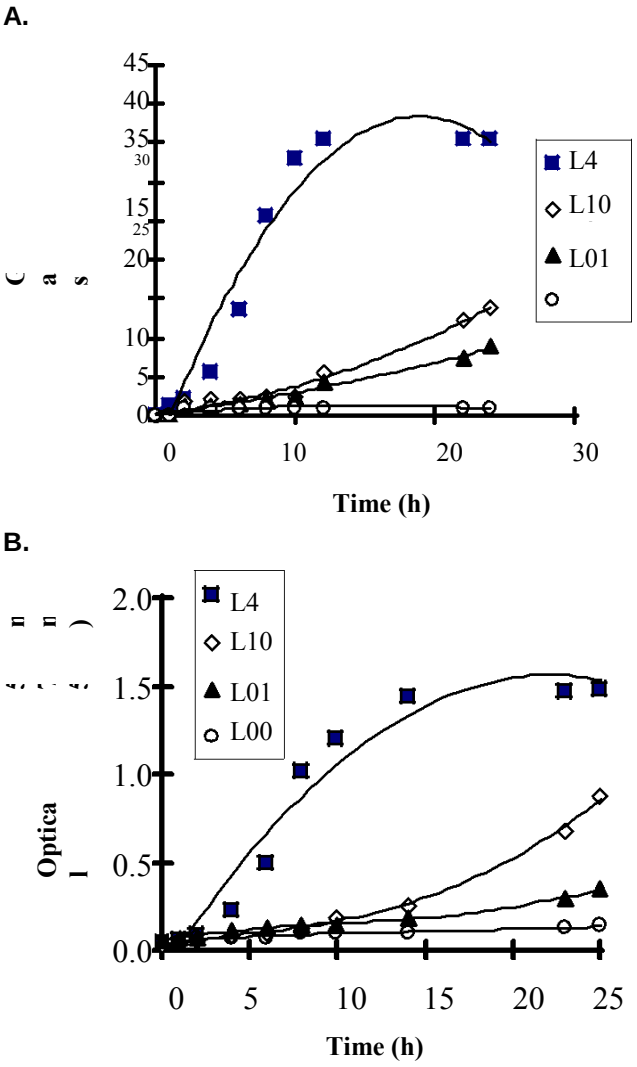

Figure 3. CO_2 production (**A**) and comparative growth (**B**) of *L.mesenteroides* subsp. *Dextranicum L4* ( ) and their variants *Lac(+)cit(-)* ( ), *Lac(-)cit(+)* ( ), and *Lac(-)cit(-)* (o).

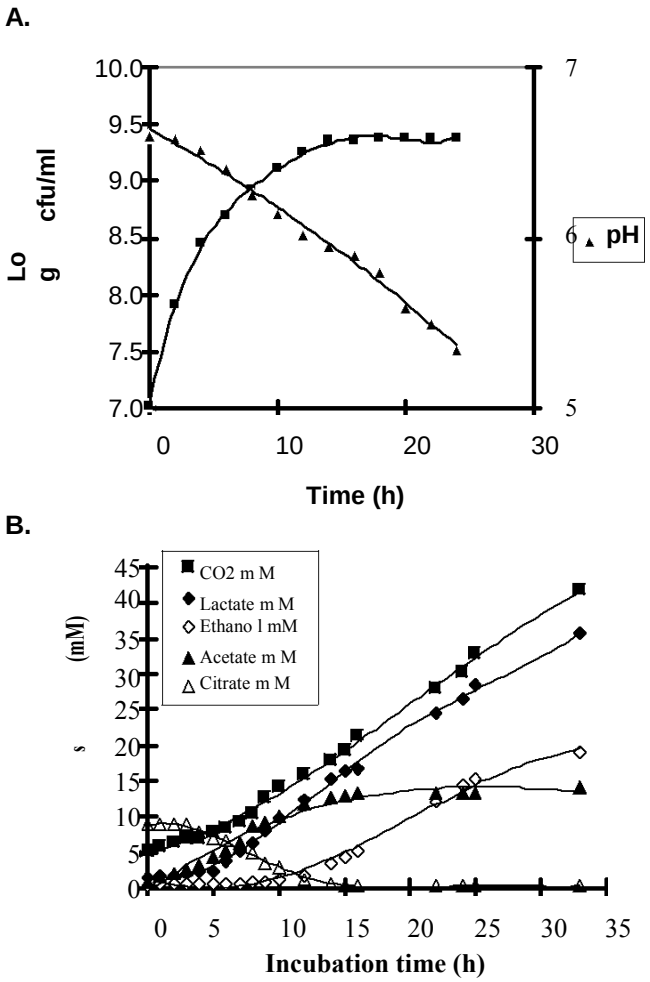

Figure 4. Growth of *L. mesenteroides* subsp *dextranicum L4* in skim milk medium at 30°C: in (**A**) growth( ) and pH evolution( ); in (**B**) Evolved CO_2( ), lactate mM(() and ethanol production mM ( ); citrate utilization mM( ) and acetate production mM ( ) in skim milk

Kinetic of growth and CO_2 production by *L. mesenteroides*

The strain of *Leuconostoc* L4 was inoculated in skim milk (20 10^5 cfu/ml). The culture is spread 10 ml by tubes which are incubated at 30°C. The measure of the produced CO_2, pH, and bacterial count are achieved at an interval regular time. A part of the culture is used to measure the produced lactic acid, acetic acid, and ethanol as well as residual citric acid (Figure 4). This is achieved through enzymatic method Huang et al. (1994).

RESULTS

Accuracy test of CO_2 measurement

Ten measurements of CO_2 regenerated from Na_2CO_3 are summarized in (Table 1). Except for the test treated by trichloro-acetic acid (TCA), the statistical analysis of average data was satisfied since the of variation's coefficient ranged from 2.3 to 6.31%. These values obtained were never higher than theoretical value. Remarkably, the variation's coefficient of the sample powder of Na_2CO_3 was excellent (2.3 %). The errors were important in the test with a sample of Na_2CO_3 acidified with TCA. No significant disparity was observed in all the samples treated with hydrochloric acid. Regenerated CO_2 production chemically shows the important effect of the nature of the acid used. Hydrochloric acid give the optimal value of CO_2 regenerated from Na_2CO_3. The various treatment tested were shown in Table 1. This statistical analysis demonstrates clearly the effectiveness of the system for measuring the volume of CO_2. This method has been proven to present an excellent linear response to measure from 0 to 20 ml (0.892 mM) with a high sensitivity.

Comparison of evolved CO_2 in *Leuconostoc* sp.

Figure 2 shows the evolved CO_2 from the strains *L4, 19D* and *Ec 195* growing in skimmed milk with 0.1% yeast extract. All strains produced CO_2 in the milk. The amount of CO_2 produced by *L4* was higher than other strains and this difference was observed after 4 h of incubation. A lower amount of CO_2 production was observed in *Ec 195*. Maximum CO_2 production rate was 3.3 mM/h in *L4*, 3 mM/h in *19D* and 2.1 mM/h in *Ec 195*. A final volume of CO_2 production was 66.56, 61.21 and 34.95 mM in *L4*, *19D*, and *Ec 195*, respectively. The growth rate was high in *L. mesenteroides* subsp. *dextranicum* and the production rate of CO_2 was characterized by two picks.

Comparison of evolved CO_2 and growth

The growth rate and evolved CO_2 were measured for *L. mesenteroides* subsp. *dextranicum L4* and their variants; *Lac(-)Cit(+)*, *Lac(+)Cit(-)* and *Lac(-)Cit(-)*. The comparative curves were observed between growth rate and evolved CO_2 (Figures 3A and B). After 4 h of incubation time, the parental strain gave higher amount of CO_2 production and growth rate. The poor growth of variants on the synthetic medium was confirmed by their feeble amount of CO_2 production. The quantity of CO_2 production was never higher than 10 mM during 14 h of incubation, whereas the parental strain produced 34 mM at the same time. After 25 h of incubation, the growth of variant *Lac(+)Cit(-)* was observed and the amount of CO_2 production was 50% less than the parental strain (Figure 3). The pH value decreased quickly in the parental strain, and there was no change of pH in Lac (-) Cit (-).

Lactose was consumed after 14 h in the parental strain and 25 h was needed in the Lac (+) Cit (-) mutant. Disappearance of citric acid was obtained after 8 h in the parental strain and more than 45 h in the *Lac(-)Cit(+)*

mutant. Analysis of results obtained by parental strain, to compare the CO_2 measured and theoretical CO_2 calculated from lactose and citric acid consumption was tested. High correlation was obtained between CO_2 measured and CO_2 calculated from lactose and citric acid consumption. The maximum CO_2 production rate (6.5 mM/h) and lactose utilization rate (3 mM/h) were obtained after 8 h of incubation. The maximum citric acid utilization rate (4 mM/h) was obtained after 6 h. Analysis of experimental data involved the use of statistical techniques to test the precision and efficiency of the quantity of CO_2 measured by this process.

Growth knetic and CO_2 production by *L. mesenteroides* L4

Figure 4 shows the development of the strain growth on skim milk with the metabolisms products CO_2, lactic acid, acetic acid, ethanol and pH evolution and consumed citric acid. The maximum growth value is reached after 13 h of incubation and presents 18 x 10^8 cfu/ml. The observed correlation in the development of CO_2 and D-lactic acid production is confirmed by the calculated correlation coefficient, r = 0.9961. Whereas, the correlation coefficient is r = -0.994 between citric acid consumption and acetic acid production. After 9 h of incubation, 92.96% of citric acid is consumed. Maximum acetic acid production is reached after 11 h of incubation. The evolution of ethanol production differs from the other metabolism products; its production is accentuated after the exhaustion citric acid.

DISCUSSION

The method described here is simple, rapid and reliable to screen a large number of bacterial cultures and select the culture having potential ability for evolved CO_2. In addition, it allows the assessment of their relative abilities for CO_2 production by different strains from different growth media. The results obtained by this method shows that no significant disparity was observed between all test acidifying by hydrochloric acid and the theorical value when Na_2CO_3 was used as source of CO_2. This method has been proven to present an excellent linear response from 0 to 0.892 mM with a high sensitivity. Gibson and Abdelmalek (1945) demonstrated that the amount of CO_2 forming during fermentation of sugar depends on several factors such as sugar (nature and concentration) and buffering capacity of the medium.

The amount of CO_2 increased with increasing initial citrate and lactose concentration. It is also possible that the main tenance energy from citrate serves to detoxify the cells from end products when high citrate concentrations are used (Harvey and Collins, 1962; Dols *et* al., 1997; Debs-Louka et al., 1999). In *Leuconostoc*, all citrate was used and lactose consumption increased with increasing initial citrate concentration, correlated with increase of dry cell weight (Brown et al., 1977; Schmitt et al., 1990). The growth stimulation of *Leuconostoc* sp. in the presence of citrate can be explained by the action of citrate as an external electron acceptor, resulting in more acetate (and ATP) production and less ethanol production during the heterofermentative lactose conversion (Starrenburg and Hugenholtz, 1991; Amanatidou et al., 1999). Comparison of growth and CO_2 production from *L. mesenteroides.* (*L4, 19D* and *Ec195*) were tested on skim milk. *L. mesenteroides* subsp. *dextranicum* L4 produce a larger amount of CO_2 than 19D and Ec 195. The later strain was the slower. Evolution of growth curves corresponded to the curves of evolved CO_2 and decrease in pH value for each strain.

CO_2 production from *L. mesenteroides* subsp. *dextranicum* and their variants *Lac(-)* and *Cit(-)* are shown in (Figure 3). The results indicate that the parental strain of *L4* was a better producer of CO_2 than their variants. These results were further confirmed by cell growth estimation and pH evolution. Thus strain growth was directly proportional to substrates used. Similar observations were obtained by (Schmitt et al., 199; Metaxopoulos et al., 2002). The method revealed another correlation between CO_2 production and growth. The quantity of CO_2 produced was proportional to the amount of substrate used (lactose or citrate). The CO_2 production rate and substrate utilization rate were always higher in the parental strain. The absence of -galactosidase and citrate permease can explain this comportment (Huang et al., 1994).

The study of production rate of substances in the milk culture of *L.mesenteroides* subsp *dextranicum* shows a narrow correlation between the CO_2 production rate and D-lactic acid production rate. The maximum CO_2 production rate is observed after 5 h and lasts 3 h. The maximum rate of citric acid consumption (0.8 mM/h) and acetic acid (1.5 mM/h) are obtained after 4 and 5 h of incubation, respectively. The rapport of the two rates confirms the stoïchiometric relation between citric acid conversions to acetic acid. Samelis and Georgiadou (2000) have observed a high production of acetic acid aerobically, whereas anaerobically the D-lactate was formed in high amount in lactic acid bacteria. Inhibitory interactions were observed between a number of metabolites when it used in the the same media (Gill and Holley, 2003).

The ethanol production curve presents a latency phase of 8 h, then its speed increases and reaches a maximum production rate (0.87 mM/h) after 11 h of incubation. The ethanol production comes essentially from lactose metabolism. The maximum ethanol production rate obtained after 11 h of incubation. The specific rate decrease according to time. At first, the slowing down of ethanol production is caused by partial inhibition of sugar assimilation by the presence of citric acid. Citric acid

exhaustion in the culture and anaerobic conditions stimulate the ethanol production. The specific rate decrease largely up to 7 h of incubation, thus proving the CO_2 production during the first hours of incubation in dairy products. Tavaria et al. (2002) have observed that the wild strains of lactic acid bacteria offer a great potential for flavor generation, which might justify their inclusion in a tentative starter culture for that and similar fermented dairy products.

The method described for CO_2 measurement is simple and reliable for screening large numbers of bacterial cultures having potential ability for evolved CO_2. In addition, it allows the assessment of their relative abilities for CO_2 production.

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