

Effect of pH control on lactic acid fermentation of starch by *Lactobacillus manihotivorans* LMG 18010^T

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J.P. GUYOT, M. CALDERON AND J. MORLON-GUYOT. 2000. Lactic acid fermentation of starch by *Lactobacillus manihotivorans* LMG 18010^T, a new amyolytic γ (+) lactic acid producer, was investigated and compared with starch fermentation by *Lact. plantarum* A6. At non-controlled pH, growth and lactic acid production from starch by *Lact. manihotivorans* LMG 18010^T lasted 25 h. Specific growth and lactic acid production rates continuously decreased from the onset of the fermentation, unlike *Lact. plantarum* A6 which was able to grow and convert starch product hydrolysis into lactic acid more rapidly and efficiently at a constant rate up to pH 4.5. In spite of complete and rapid starch hydrolysis by *Lact. manihotivorans* LMG 18010^T during the first 6 h, only 45% of starch hydrolysis products were converted to lactic acid. When pH was maintained at 6.0, lactic acid, amylase and final biomass production by *Lact. manihotivorans* LMG 18010^T increased markedly and the fermentation time was reduced by half. Under the same conditions, an increase only in amylase production was observed with *Lact. plantarum* A6. When grown on glucose or starch at pH 6.0, *Lact. manihotivorans* LMG 18010^T had an identical maximum specific growth rate (0.35 h^{-1}), whereas the maximum rate of specific lactic acid production was three times higher with glucose as substrate. *Lactobacillus manihotivorans* LMG 18010^T did not produce amylase when grown on glucose. Based on the differences in the physiology between the two species and other amyolytic lactic acid bacteria, different applications may be expected.

INTRODUCTION

According to Damelin *et al.* (1995), amyolytic lactic acid bacteria (ALAB) may account for 65% of isolates from different types of foods. They are widespread among the non-dairy food environments and different geographical areas. Amyolytic lactic acid bacteria have been isolated from swine and cattle waste-corn fermentations in the USA (*Lactobacillus amylophilus* and *Lact. amylovorus*) (Nakamura and Crowell 1979; Nakamura 1981), fermented cassava roots in Congo and Niger (*Lact. plantarum* strains) (Nwankwo *et al.* 1989; Giraud *et al.* 1991), chicken crops in France (*Lactobacillus* strains) (Champ *et al.* 1983), fish silage in Sweden (*Leu-*

conostoc strains) (Lindgren and Refai 1984), fermented fish and rice food in Japan (*Lact. plantarum*) (Olympia *et al.* 1995) and maize sourdough in Benin (*Lact. fermentum*) (Agati *et al.* 1998). Recently, a new amyolytic lactic acid bacterium, *Lactobacillus manihotivorans*, was isolated during the process of cassava sour starch production in Colombia. This new species is homolactic and produces exclusively γ (+) lactic acid (Morlon-Guyot *et al.* 1998). Most of the known ALAB are DL lactic acid producers with the exception of *Lact. amylophilus* (Nakamura 1981) and *Lact. manihotivorans* (Morlon-Guyot *et al.* 1998). Continuous effort to isolate and characterize non-dairy lactic acid bacteria, such as ALAB, would bring increasing opportunities for selecting and adapting specific starters for non-dairy food applications (Sanni 1993; Vogel 1996; Essers and Nout 1997). Notwithstanding the potential importance of starch fermentation by lactic acid bacteria, very few investigations on the physiology of ALAB

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have been performed to date (Giraud *et al.* 1991; Mercier *et al.* 1992), even though some reports have described possible applications, such as lactic acid production from different types of starches by *Lact. amylophilus* and *Lact. amylovorus* (Zhang and Cheyran 1991; Mercier *et al.* 1992; Yumoto and Ikeda 1995; Xiaodong *et al.* 1997). These two species were compared and it was shown that *Lact. amylovorus* exhibits a higher amylase activity than *Lact. amylophilus* (Pompeyo *et al.* 1993). Starch fermentation by an amylolytic *Lact. plantarum* strain was also investigated (Giraud *et al.* 1991) and an ability to degrade raw starch was demonstrated (Giraud *et al.* 1994).

Investigation into the physiology of non-dairy lactic acid bacteria, and particularly members of the amylolytic group, is still necessary in order to determine their ecological significance in spontaneous amyloseous crop fermentations, develop new processes and improve existing techniques on a more rational basis by the use of specific starters. In a spontaneous fermentation process (e.g. sour cassava starch production), lactic acid bacteria evolved in non-controlled pH conditions but for other kinds of applications (e.g. lactic acid or biomass production), it may be necessary to achieve pH control. In this work, lactic acid fermentation of starch by *Lact. manihotivorans* LMG 18010^T was studied in batch, either without pH control or with pH maintained at 6.0 (optimal growth pH; Morlon-Guyot *et al.* 1998), and compared with *Lact. plantarum* A6, another amylolytic lactic acid bacterium isolated from a cassava fermentation process.

MATERIALS AND METHODS

Bacterial strains

Lactobacillus manihotivorans LMG 18010^T was isolated during the process of sour starch production in Colombia (Morlon-Guyot *et al.* 1998) and has been deposited at the BCCMTM/LMG culture collection under accession number LMG 18010^T. This strain produced an extracellular α -amylase. *Lactobacillus plantarum* A6 (deposited at the BCCMTM/LMG culture collection under accession number LMG 18053) was used as a reference strain. Both strains were conserved in 40% glycerol at -80 °C.

Culture conditions

MRS medium (deMan *et al.* 1960) was used for cultivation of bacteria. Fermentations were performed at 35 °C in 2 l bioreactors (Biolafitte, France), with pH either controlled or uncontrolled, using soluble starch as substrate or glucose (20 g l⁻¹) when indicated. For pH-controlled fermentations, NaOH (5 N) was used to maintain the pH at 6.0. The growth medium was gently stirred (200 rev min⁻¹) to maintain homogeneity. Bioreactors were inoculated (10% v/v) with 12 h pre-cultures grown on MRS-glucose.

Extracellular amylase activity

Extracellular α -amylase activity was assayed in the supernatant fluid of centrifuged cultures by measurement of the iodine-complexing ability of starch at pH 5.5 and 55 °C as previously described (Giraud *et al.* 1994). One enzyme unit (U) is defined as the amount of enzyme hydrolysing 10 milligrams of starch in 30 minutes.

Biomass estimation and growth parameters

Optical density at 600 nm (O.D.₆₀₀) was measured using a Spectronic 401 spectrophotometer (Milton Roy, Paris, France). Cell cultures were diluted in sterile medium to allow the O.D.₆₀₀ to fall below 0.4 (Koch 1981). Calibration curves between O.D.₆₀₀ and cell dry weight were established for each strain. Specific growth rate (μ) and specific rate of product (lactate) formation (v) were calculated as $(1/X) \times (dX/dt)$ and $(1/X) \times (dP/dt)$. Maximum specific growth rate (μ_{max}) and rate of specific lactate formation (v_p) were determined from the curves (μ vs time) and (v vs time). Growth and lactate yields ($Y_{x/s}$ and $Y_{lactate/s}$, respectively) were calculated as the slope of the linear regressions of either biomass or lactate vs residual substrate (as total sugars) during the exponential growth phase.

Analytical methods

The concentrations of the L(+) and D(-) forms of lactic acid were determined using commercial enzymatic test combinations (Boehringer, Mannheim, Germany). Total lactic acid was determined by high-performance liquid chromatography (LDC Analytical, Roissy, France) as previously described (Giraud *et al.* 1994). Residual starch was determined by measuring the iodine-starch complex colour (Nakamura 1981; Giraud *et al.* 1994). Total sugars and reducing sugars were assayed, respectively, using the method of Dubois *et al.* (Dubois *et al.* 1956) and the DNS method (Miller 1959).

All fermentations were carried out in duplicate.

RESULTS

Starch fermentation without pH control

Starch fermentation by *Lact. manihotivorans* LMG 18010^T was first investigated without pH control. The dynamics of this fermentation are illustrated in Fig. 1. For comparative purposes, similar fermentations were performed with *Lact. plantarum* A6 and parameters are given in Table 1. As shown in Fig. 1, starch was rapidly degraded by *Lact. manihotivorans* LMG 18010^T at a higher rate than lactic acid production during the corresponding period (Fig. 1, Table 1). With both species, starch disappeared during the first 6 h of the fer-

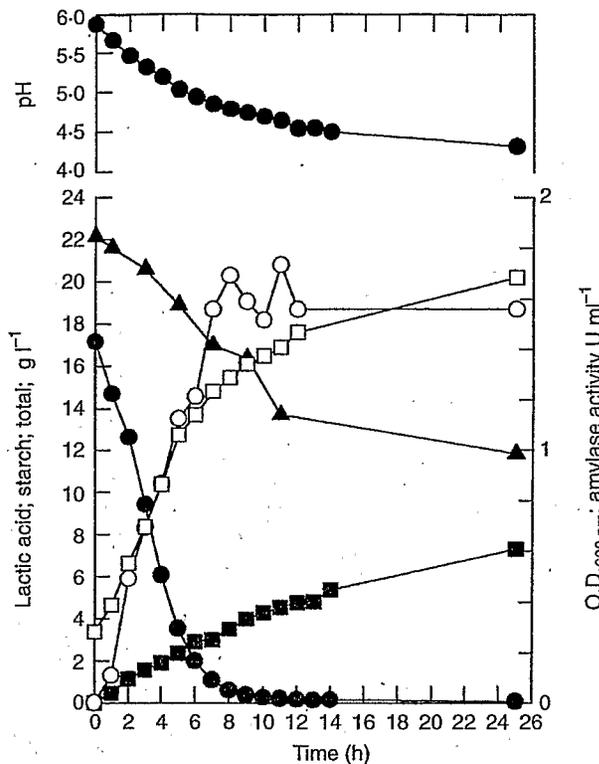


Fig. 1 Batch lactic acid fermentation of starch by *Lactobacillus manihotivorans* LMG18010^T under non-controlled pH. pH (●); total sugars (▲); starch (●); lactic acid (■); O.D. (□); amylase activity (○)

mentation, at maximum degradation rates of 4.1 and 3.0 g l⁻¹ h⁻¹ for strains A6 and LMG 18010^T, respectively. At the end of the fermentation, *Lact. manihotivorans* LMG 18010^T

produced lower amounts of amylase than *Lact. plantarum* A6 (1.7 and 2.8 U ml⁻¹, respectively), but yields of amylase relative to biomass ($Y_{a-amylase/x}$) were similar (Table 1).

Lactobacillus manihotivorans LMG 18010^T produced less lactate and at a lower volumetric rate than *Lact. plantarum* A6 (Table 1), resulting in a lower acidification rate and higher final pH value (Table 1). Unlike *Lact. plantarum* A6 which converted all available starch into lactate (Table 1), lactic acid production from starch by *Lact. manihotivorans* LMG 18010^T was not efficient (at the end of the fermentation, 55% of total sugars were still present in the fermentation broth, Fig. 1). For *Lact. manihotivorans* LMG 18010^T, the specific growth rate and the specific rate of lactate formation decreased rapidly as the pH dropped (Fig. 2). On the other hand, for strain A6, high specific growth and lactate production rates were maintained at pH \geq 4.5 (Table 1, Fig. 2). Growth and lactic acid production levelled off at 12 h with *Lact. plantarum* whereas the fermentation was achieved in 25 h with *Lact. manihotivorans*.

During the course of the fermentation, D- and L-lactic acid isomers were also determined. *Lactobacillus manihotivorans* LMG 18010^T produced exclusively L(+) lactic acid (99%), unlike *Lact. plantarum* A6 which simultaneously produced D(-) and L(+) lactic acid, with the D(-) form representing 64% of total lactic acid throughout the fermentation.

Starch fermentation at pH 6.0

Effect of pH control on amylase production and starch degradation. Controlling the pH allowed amylase production by *Lact. manihotivorans* to increase to a final concentration of 13.2 U ml⁻¹. The same effect was observed for *Lact. plantarum* which produced more amylase (20.0 U ml⁻¹) (Table 1).

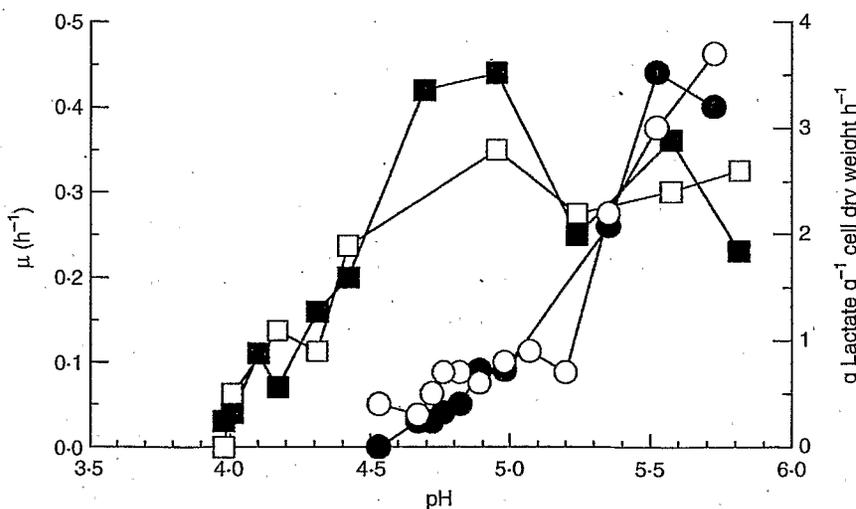


Fig. 2 Changes in specific growth rates and specific lactic acid production rates in relation to pH variations during starch fermentation under non-regulated pH by *Lactobacillus plantarum* A6 and *Lact. manihotivorans* LMG18010^T. Symbols for *Lact. plantarum*: specific lactic acid production rate (□); specific growth rate (■). Symbols for *Lact. manihotivorans*: specific lactic acid production rate (○); specific growth rate (●)

Table 1 Parameters of starch fermentation, with or without pH control, by *Lactobacillus manihotivorans* LMG 18010^T and *Lact. plantarum* strain A6

	<i>Lact. manihotivorans</i> LMG 18010 ^T		<i>Lact. plantarum</i> strain A6	
	Non-controlled pH	pH 6.0	Non-controlled pH	pH 6.0
Initial starch concentration (g l ⁻¹)	17.2	17.5	17.0	17.1
Maximum acidification rate (-dpH/dt) (unit pH h ⁻¹)	0.21	-	0.34	-
Final pH	4.30*	-	3.90†	-
Final OD ₆₀₀	1.65*	5.1†	6.02†	7.6‡
Final lactic acid concentration (g l ⁻¹)	7.6*	12.6†	17.0†	15.2‡
Final amylase concentration (U ml ⁻¹)	1.7*	13.2†	2.8†	20.0‡
Volumetric rate of starch hydrolysis (g l ⁻¹ h ⁻¹)	3.0	3.0	4.1	4.6
Maximal volumetric rate of lactic acid production during the starch hydrolysis step (g l ⁻¹ h ⁻¹)	0.5	0.5	2.0	2.1
Y _{x/s} (g cell dry weight per g of total sugars consumed)	0.09	0.15	nd	0.18
Y _{lactate/s} (g lactate produced per g of total sugars consumed)	0.71	0.67	nd	0.84
Y _{amylase/x} (U/g cell dry weight)	2.4	4.9	2.3	5.7
μ _{max} (h ⁻¹)	see Fig. 2	0.36	0.43	0.41
v _{lactate} (g lactate g ⁻¹ cell dry weight h ⁻¹)	see Fig. 2	1.0	3.1	3.0

*At the 25th hour of fermentation (end of the fermentation for strain LMG 18010^T at non-controlled pH).

†At the 12th hour of fermentation (end of the fermentation).

‡At the 8th hour of fermentation (end of the fermentation for strain A6 at pH 6.0).

nd, Not determined.

The amylase yield (Y_{amylase/x}) also increased but there was no marked difference between the two strains (Table 1). For strains A6 and LMG 18010^T, most of the amylase was produced in great excess after starch exhaustion (77% and 67% of the total amount of amylase, respectively), indicating that much higher starch concentrations could potentially be hydrolysed.

Starch was degraded at the same rate as in the non-pH-controlled fermentation (Table 1). The ratio between starch and total sugar concentrations, and the transient appearance of reducing sugars during starch fermentation by *Lact. manihotivorans*, indicated that products from starch hydrolysis accumulated in the fermentation broth (Fig. 3). The same observation was made for *Lact. plantarum* A6.

Effect of pH control on growth and lactic acid production.

For *Lact. manihotivorans*, controlling the pH at 6.0 allowed the final biomass and lactic acid concentration to increase markedly (Fig. 3, Table 1). Lactate yield (Y_{lactate/s}) did not vary significantly whereas growth yield (Y_{x/s}) was improved, suggesting a better coupling between energy generation and cell synthesis. The maximum specific growth and lactic acid production rates were 0.36 h⁻¹ and 1.0 g lactate g⁻¹ cell dry

weight h⁻¹, respectively. To determine whether any differences result from the fermentation of either starch product hydrolysis or glucose, these parameters were compared with those obtained during glucose fermentation under the same conditions (Table 2). With glucose as substrate, specific growth rate did not vary, but specific rate of lactate production and yield of lactate were much higher (Table 2). Furthermore, in the presence of glucose, no amylase was produced.

For *Lact. plantarum*, controlling pH during starch fermentation did not change the maximum specific growth and

Table 2 Parameters of lactic acid fermentation of glucose at pH 6.0 by *Lactobacillus manihotivorans* LMG 18010^T

μ _{max} (h ⁻¹)	0.35
v _{lactate} (g lactate g ⁻¹ cell dry weight h ⁻¹)	3.2
Y _{x/s} (g cell dry weight per g of total sugars consumed)	0.12
Y _{lactate/s} (g lactate produced per g of total sugars consumed)	0.90

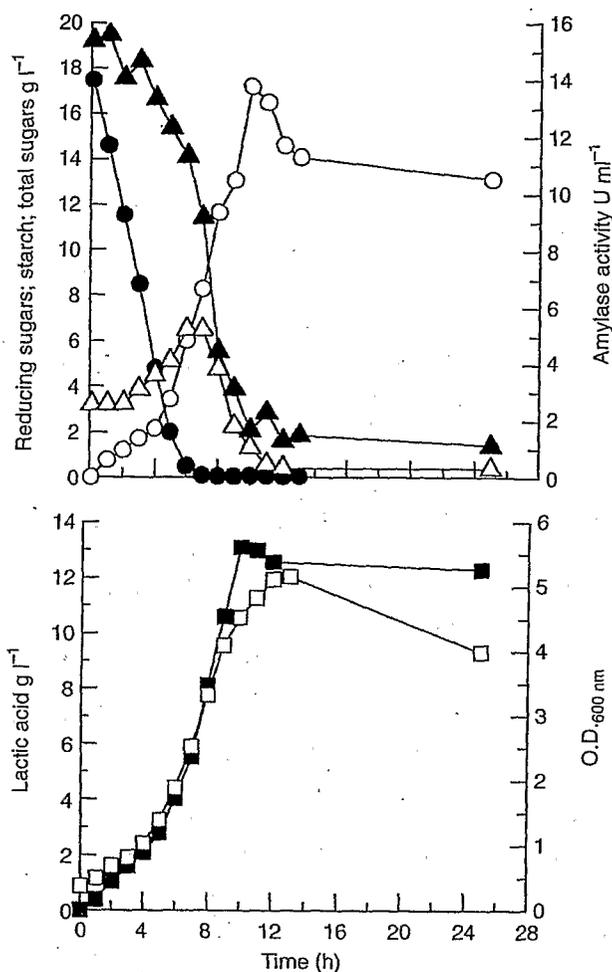


Fig. 3 Batch lactic acid fermentation of starch by *Lactobacillus manihotivorans* LMG18010^T at pH 6.0. Total sugars (▲); reducing sugars (△); starch (●); lactic acid (■); O.D. (□); amylase activity (○)

lactic acid production rates (Table 1). Furthermore, only slight variations in the final amount of biomass and lactic acid were observed; final O.D. increased from 6.02 to 7.6, whereas final lactic acid concentration decreased from 17.0 to 15.2 g l⁻¹ (Table 1) with non-controlled pH and pH 6.0, respectively.

DISCUSSION

The present study showed that starch hydrolysis does not limit lactic acid production and that intermediary products (measured as total and reducing sugars) appear during the course of the fermentation. The small amount of transiently produced reducing sugars and higher concentration of total sugars, detected at the same time (Fig. 3), suggested that starch was not directly converted into reducing sugars by

Lact. manihotivorans LMG 18010^T. This may explain why, during starch fermentation, lactic acid specific production rate was lower than that obtained from glucose fermentation (Tables 1 and 2); intermediary products of starch degradation (e.g. dextrans) have to be first transformed before entering cell metabolism, limiting lactic acid production compared with direct glucose fermentation. Nevertheless, in spite of an increased lactic acid specific production rate in the presence of glucose, the specific growth rate remained unchanged, suggesting that the additional energy flux obtained from direct fermentation of glucose was used for functions other than growth.

The most significant effect of pH regulation was the increase in amylase production by *Lact. manihotivorans* LMG 18010^T and *Lact. plantarum* A6. Furthermore, a marked increase in biomass and lactic acid production was observed with *Lact. manihotivorans* LMG 18010^T.

The capacity of *Lact. plantarum* A6 to maintain a constant specific growth rate and lactic acid production rate during acidification could be related to the ability of *Lact. plantarum* strains to maintain a pH gradient at high lactic acid concentrations (McDonald *et al.* 1990). Studies on *Lact. plantarum* WSO shown that growth stopped when the intracellular pH (pH_{in}) dropped from 6.0 (pH_{out} = 6.5) to 4.5 (pH_{out} neared 3.0) (McDonald *et al.* 1990). As, for strain A6, (i) amylase production significantly increased at pH 6.0 whereas lactate production and growth did not markedly vary with the pH conditions, and (ii) it has been reported that pH_{in} can regulate different metabolic functions (Hutkins and Nannen 1993), it is possible that the decrease in intracellular pH negatively interferes with amylase synthesis or secretion.

In contrast with *Lact. plantarum* A6, *Lact. manihotivorans* LMG 18010^T metabolism was affected by acidification, as indicated by decreasing specific rates of growth and lactate production under uncontrolled pH. In spontaneous food fermentation, this pH sensitivity may limit the extent of its activity to the first hours, allowing the development of more pH-resistant strains, but this hypothesis will have to be further investigated. For strain LMG 18010^T, the slight increase in Y_{x/s} at pH 6.0 suggests that controlling the pH would improve the coupling between energy production and biomass synthesis. Furthermore, the effect of pH on Y_{x/s} is similar to a previous observation with *Streptococcus cremoris* which demonstrated that cell yield increased with increasing pH (Brink *et al.* 1985).

The kinetics of growth and lactic acid production of *Lact. manihotivorans* LMG 18010^T were different from those of *Lact. plantarum* A6, and *Lact. manihotivorans* LMG 18010^T produced less amylase than *Lact. plantarum* A6. In spite of these differences, both strains shared the ability to produce a great excess of amylase and to hydrolyse starch rapidly, whatever the pH. This means that whatever the metabolic features related to lactic acid production, the strains would have the

ability to interact with the amylaceous fraction of the food matrix with or without efficient lactic acid production.

Lactobacillus manihotivorans 18010^T and *Lact. plantarum* A6 were both isolated from cassava fermented products, but on a physiological basis, *Lact. manihotivorans* is more closely related to *Lact. amylophilus*, a homofermentative $\iota(+)$ -lactic acid producer, than to *Lact. plantarum* (a facultative heterolactic *Lactobacillus*) (Morlon-Guyot *et al.* 1998). A comparison of the fermentation dynamics of *Lact. manihotivorans* with published data regarding *Lact. amylophilus* (Nakamura and Crowell 1979; Mercier *et al.* 1992; Yumoto and Ikeda 1995) indicates that growth and lactic acid fermentation from either starch or glucose are faster with *Lact. manihotivorans* than with *Lact. amylophilus*. For instance, fermentation of glucose (20 g l⁻¹), at pH 6.0, by *Lact. amylophilus* lasted 25 h (Mercier *et al.* 1992) whereas under similar conditions, *Lact. manihotivorans* fermented glucose or starch in less than 12 h. Additionally, the conversion yield of soluble starch into lactic acid by *Lact. amylophilus* (53.4–60%) reported by Yumoto and Ikeda (1995) is lower than that obtained with *Lact. manihotivorans* which yielded 71% (calculated from Table 1). Unlike *Lact. amylophilus*, the ability of *Lact. manihotivorans* to use the α -galactosides (raffinose, melibiose) (Morlon-Guyot *et al.* 1998) contained in numerous plants and causing digestive disorders (flatulence), combined with its higher starch conversion efficiency, may increase its potential in vegetable food processing.

The starch fermentation characteristics of *Lact. manihotivorans* suggest that it has a variety of potential applications. Incomplete starch degradation at non-controlled pH by strain LMG 18010^T may indicate that functional properties linked to the structure of starch may be modified, i.e. for decreasing the consistency of gruels prepared with a high energy density as starchy weaning foods (FAO/WHO 1995; Trèche 1995). Furthermore, for this type of application, $\iota(+)$ lactic acid production by *Lact. manihotivorans* LMG 18010^T is beneficial in the controlled production of fermented weaning foods for young children in tropical countries, as neither D nor DL-lactate should be added to food for very young children (for adults, 100 mg kg⁻¹ body weight is an acceptable daily intake) (FAO/WHO 1967, 1995; Yusof *et al.* 1993).

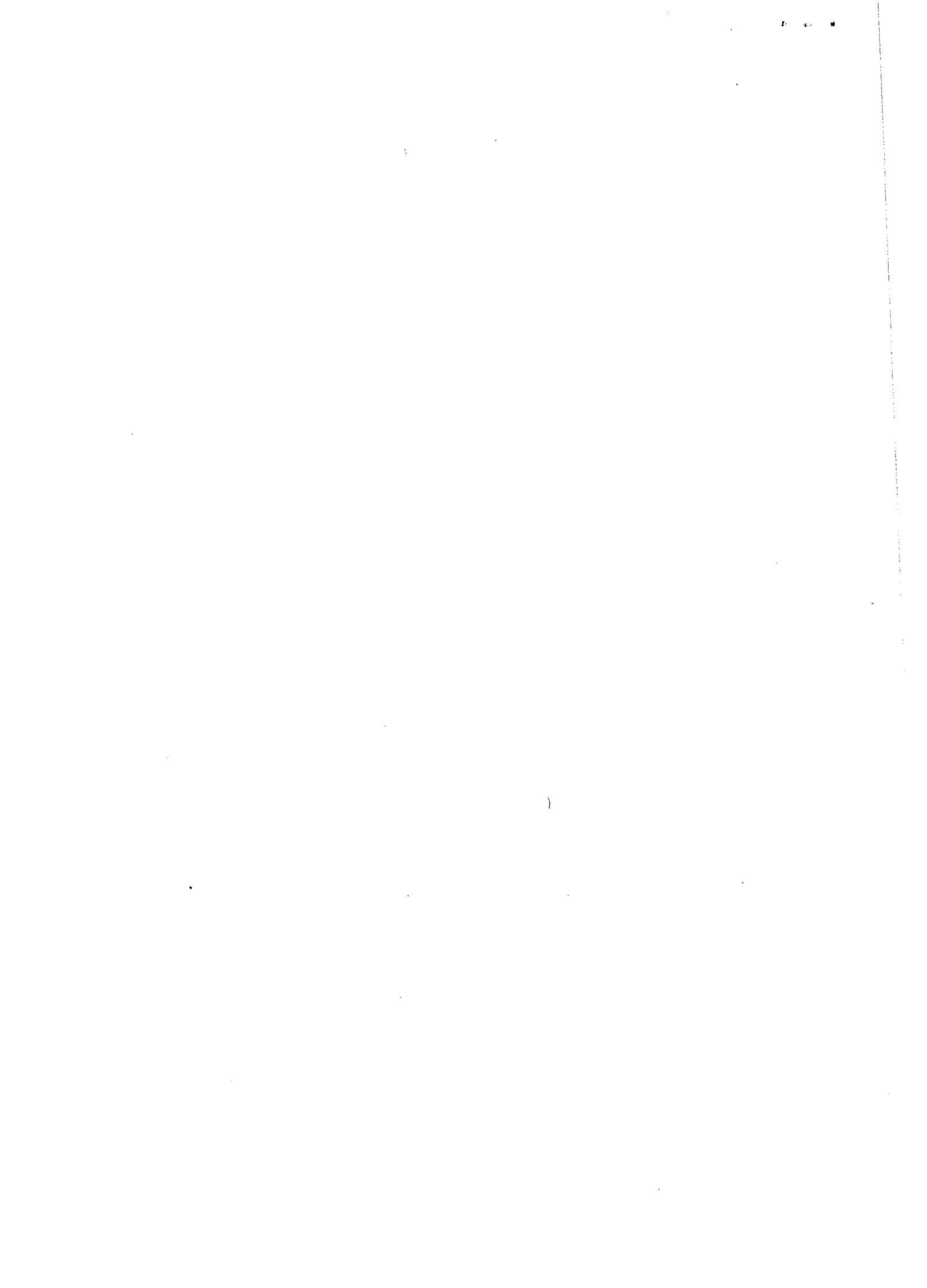
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