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PII: S0308-8146(97)00218-5

Food Chemistry, Vol. 63, No. 1, pp. 115–123, 1998

Printed in Great Britain
0308-8146/98 $19.00+0.00
Analytical, Nutritional and Clinical Methods Section

Isotopic characterization of the bioconversion of lactose into ethanol

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(Received 23 June 1997; revised version received and accepted 13 September 1997)

In order to compare natural abundance isotopic properties of lactose with those of other carbohydrates such as glucose, fructose, sucrose and starch, the accessibility to ethanol obtained by fermentation, as a common probe, has been investigated. Only strictly reproducible isotopic fractionation will ensure that the fermentation products are always representative of their lactose precursor. An analytical strategy has therefore been designed that avoids discriminating isotope effects in the fermentation of pure lactose or whey permeates by *Kluyveromyces fragilis*. Appropriate experimental conditions ensure nearly full consumption of lactose and a high yield of conversion into ethanol. A lactose concentration of the order of magnitude of that found in whey permeates ($50\,g\,l^{-1}$) is convenient and an inoculum grown for 24 h is selected. Whereas sodium ions exert an inhibitory effect, added peptones optimize the fermentation process without introducing isotopic perturbations. The overall carbon isotope parameter of ethanol, $\delta^{13}C$, is measured by isotope ratio mass spectrometry with a reproducibility of 0.2‰ and the site-specific natural isotope ratios of the methyl, $(D/H)_I$, and methylene, $(D/H)_{II}$, sites of ethanol are obtained with a reproducibility of about 1.5 ppm. These parameters provide useful criteria for comparing various starting materials such as lactoses (α or β) from different commercial producers, milk powders, ordinary or skimmed milks and lactoserum. The methyl parameter, $(D/H)_I$, mainly reflects the deuterium content of the lactose precursor, which is itself expected to provide information on the feeding of the animal and on the metabolism. The methylene isotope ratio is connected to that of the fermentation medium and may be influenced by isotopic fractionation introduced in the exchangeable sites of lactose by the extraction process.

INTRODUCTION

Biosynthesis in natural environments is accompanied by isotope fractionation phenomena which result from kinetic and thermodynamic effects associated with the various steps of the mechanistic pathway and from physico-chemical transformations such as evapotranspiration in plants. The overall molecular isotope ratios of carbon, hydrogen, oxygen, nitrogen, etc., usually measured by isotope ratio mass spectrometry (IRMS), are therefore a rich source of information on the various metabolic processes (Simon, 1983; O'Leary, 1988; Smith and Ziegler, 1990; Yakir, 1992; Raven, 1992). We have shown that the Site-specific Natural Isotope Fractionation can be directly studied by Nuclear Magnetic Resonance (SNIF-NMR) (Martin and Martin, 1981). The very large deviations with respect to a statistical distribution of deuterium exhibited by most natural and synthetic products provide powerful criteria for inferring hydrogen genealogies. Since the carbohydrates, which are key compounds of the photosynthesis, exist under several different chemical structures (various mono-, di- or poly-saccharides) and, in addition, do not produce suitable ^2H-NMR spectra, they are more conveniently compared by resorting to a common derivative, ethanol, obtained by fermentation (Martin *et al.*, 1991). A matrix of isotopic coefficients can be determined, which relates individual sites or clusters of sites in the products (ethanol and water) on one hand and in the reactants (carbohydrates and water) on the other (Martin *et al.*, 1986). When isotopically reproducible conditions are defined, the behavior of the hydrogen isotope ratios of the methyl, $(D/H)_I$, and methylene, $(D/H)_{II}$, sites of ethanol, possibly combined with the overall ^{13}C content, can be analysed in terms of the botanical or geographical origin of the plant precursor for instance (Martin and Martin,

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1990). In the case of animal products, it is known that the overall isotope content of organic matter is primarily representative of the feeding materials but noticeable influences of the trophic chain have also been detected (De Niro and Epstein, 1978; Peterson and Fry, 1987; Kennedy and Krouse, 1990; Wada *et al.*, 1991; Fry, 1991). In order to render the SNIF-NMR method amenable to the comparison of carbohydrates from both animal and plant origins we consider here the accessibility of lactose to the common ethanol probe. The influence of various fermentation conditions on the isotopic parameters is investigated and the isotopic connection between lactose and its fermentation products is analysed. From a practical point of view an isotopic characterization of lactose is of interest since lactose has found a number of utilizations in the food industry (Smart *et al.*, 1991). Moreover, in the dairy, beverage and fuel industries, several metabolites, including ethanol, are produced from whey permeates. A strategy exploiting the isotopic fingerprint is therefore potentially useful for tracing back the origin of the lactose raw material.

MATERIALS AND METHODS

Sugars and milks

Commercial lactose, lactose extracted from milk (farm, sterilized, semi-skimmed, powder, etc.) and whey permeates have been investigated.

The whey permeates were supplied by Dr Boyaval (INRA, Rennes) and have been obtained after cheese whey ultrafiltration (Boyaval *et al.*, 1987).

Lactose has been extracted from different sources of milk. The milk proteins were discarded by precipitation with cold trichloroacetic acid (TCA) (10%) and the lipids were extracted with $CHCl_3/CH_3OH$ 2/1, v/v. The aqueous phase was partially evaporated until a syrup was obtained, which was crystallized with acetone. The lactose crystals were washed with ethanol (25%) and dried.

Yeast

The strain *Kluyveromyces fragilis* was supplied by MUCL (Mycothèque de l'Université Catholique de Louvain). The yeasts were routinely maintained (pH 6.2) on 2% agar slants containing ($g l^{-1}$): yeast extract 3.0; malt extract 3.0; peptone 5.0; dextrose 10.0, and were transferred every 3 months. After incubation ($\simeq$24 h) at 30°C they were stored in a cold room at 4°C.

Culture media

All the vessels and media were sterilized at 120°C for 30 min, except for sugar solutions which were sterilized by filtration through Millipore filters (0.20 mm).

Several kinds of fermentation media denoted M_C, M_1, M_2, M_3, M_4 have been used. Their compositions are as follows ($g l^{-1}$):

M_C: yeast extract 3.0; peptone 5.0
M_1: $(NH_4)_2SO_4$, 4.0; KH_2PO_4, 3.0; $MgSO_4\ 7H_2O$, 1.0
M_2: M_1 + NaCl 1.0
M_3: M_1 + peptone 2.0
M_4: M_1 + NaCl 1.0 + peptone 2.0

All these media were prepared with distilled water and the pH was adjusted to 5.5. Unless otherwise stated, lactose was added at a concentration of 50 $g l^{-1}$.

Growth of culture and fermentation

The cells from the agar slants were reactivated in medium M_C (20 ml) and introduced into 100 ml flasks. After 18–24 h the active cells were transferred into a greater volume of the same medium (M_C) (with a ratio medium/flask 2/5). The amount of cells required for the fermentation was aseptically harvested by centrifugation and subsequently resuspended in the defined fermentation medium.

The fermentation experiments were performed with 300 ml of the appropriate medium contained in a 500 ml flask equipped with a stopper ensuring the evacuation of CO_2. The batch cultures were incubated at 30°C in a water bath with stirring (150 rpm).

Analytical parameters of the fermentation

The fermentation kinetics were followed by measuring the evolution of the biomass, the sugar consumption and the ethanol production in the culture media. Samples were taken off at different reaction times and immediately centrifuged at 3000*g* for 15 min. The supernatants were stored at −20°C for sugar and ethanol analysis. The cell pellets were washed and kept dry for further analysis.

The biomass concentration was followed by measuring the turbidimetry of the fermentation broth at 600 nm with a Bausch and Lomb spectronic 20 spectrophotometer. The optical densities were converted to dry biomass weight per volume with the use of previously established calibration curves.

- The initial and residual sugar concentrations were determined using the phenol–sulfuric acid method according to Dubois *et al.* (1956).
- The ethanol concentration was determined by enzymatic assay using ADH and NAD as described by Bernt and Gutmann (1974). The alcoholic content was also determined by chromic oxidation. Ethanol is first oxidized to acetic acid by a solution of $K_2Cr_2O_7$ and the excess of bichromate is reduced by Mohr salts. Azeotropic ethanol was

recovered by distillation performed on a Cadiot column fitted with a Teflon spinning band. The percentage of water in ethanol was measured by the Karl-Fisher method (±0.05%).

The evolution and yields of the fermentation reaction are characterized by the following parameters:

V_S, V_B and V_E are the reaction rates, respectively, for sugar (S) consumption and for biomass (B) or ethanol (E) production. These parameters are defined as the slopes of the fast evolution period and are expressed in $g\,l^{-1}\,h^{-1}$.

$C_{B/S}$ is the conversion coefficient of sugar into biomass

$$C_{B/S} = \Delta B/\Delta S \quad (1)$$

where ΔB and ΔS are the variations of the concentration of biomass and sugar in the considered reaction.

- $P_{E/B}$ is the quantity of ethanol produced per unit of biomass.
- $Y_{Sf/Si}$ is the per cent of sugar consumption between the initial (i) and final (f) states
- $Y_{E/S}$ is the yield in ethanol expressed with respect to the converted sugar (1 mol of lactose→1 mol of glucose + 1 mol of galactose→4 mol of ethanol = 100%).

$$Y_{E/S}(\%) = \frac{\text{ethanol produced(w/v)}}{\text{sugar converted(w/v)} \times 0.51} \times 100 \quad (2)$$

In order to minimize undesirable isotope fractionation effects due to the extraction process (Moussa *et al.*, 1990), a distillation procedure ensuring an ethanol recovery higher than 95% was carried out.

Isotopic determinations

The isotopic parameters have been measured either by isotope ratio mass spectrometry (IRMS) or by deuterium Nuclear Magnetic Resonance (SNIF-NMR) (Martin and Martin, 1990).

In the case of hydrogen, they are expressed on an absolute scale as the ratio, D/H in ppm, of the numbers of heavy and light isotopes. In the case of carbon and nitrogen the traditional relative scale d‰ has been adopted:

$$\delta‰ = \frac{(H/L)_{sample} - (H/L)_{ref}}{(H/L)_{ref}} 1000 \quad (3)$$

where H and L denote the heavy and light isotopes, respectively. The reference compounds adopted internationally are a carbonate denoted PDB for ^{13}C and atmospheric nitrogen for ^{15}N.

Mass spectrometry

The natural abundance ^{13}C and ^{15}N parameters and the total deuterium content of lactose have been measured by Isotope Ratio Mass Spectrometry (IRMS). Since the product is previously burnt into carbon anhydride and water, only overall ^{13}C, ^{15}N or ^{2}H molecular contents are directly accessible.

The ^{13}C and ^{15}N determinations were performed on a Delta E Finnigan instrument interfaced with an elementary analyser NA 1500-1 from Carlo Erba. The spectrometer is fitted with a six-cup multi-element multi-collector, which enables different isotope ratios to be determined without readjusting collector spacings. A few milligrams of sample were introduced into a tin container, which was sealed and loaded in the autosampler of the spectrometer.

The overall deuterium content of lactose and that of water were measured by means of a VG SIRA 9 isotope ratio mass spectrometer. In order to eliminate the contribution of the exchangeable hydroxylic sites, lactose was converted into its pentanitrate derivative. The procedure was adapted from Dunbar and Schmidt (1984). Purified lactose (1 g) was added to a nitrating mixture under stirring for 4 h. The lactose pentanitrate was then precipitated in a mixture of ice and water, rinsed with cold water and dried on a vacuum line. The overall hydrogen isotope ratio of this compound, $(D/H)_{LNE}$, represents that of the non-exchangeable carbon bound positions of lactose. The nitrate was burnt in a Carlo Erba microanalyser to give CO_2 and H_2O. The nitrogen oxides were reduced on a copper catalyst to avoid subsequent poisoning. The combustion water, separated from carbon dioxide by differential cooling, was reduced by Zn at 550°C into hydrogen gas. The standard deviation of the deuterium determination is about 0.5 ppm.

Nuclear magnetic resonance

The Site-specific Natural Isotope Fractionation of hydrogen has been investigated by deuterium Nuclear Magnetic Resonance (SNIF-NMR).

The ^{2}H-NMR spectra of ethanol were recorded at 61.4 MHz on a AM400 Bruker spectrometer equipped with a $^{2}H\{^{1}H\}$ dedicated probe, an external ^{19}F locking device and an automatic sample changer. The acquisition conditions were the same as those described in Vallet *et al.* (1996).

The isotope parameters of the methyl, I, and methylene, II, sites of ethanol are expressed as the ratios of the numbers of deuterium and protium atoms: $(D/H)_I$ and $(D/H)_{II}$. These absolute parameters are obtained from the deuterium spectrum of a precisely weighted mixture of ethanol (taking into account the residual water) and of a secondary reference, tetramethylurea (TMU), the isotope ratio of which has been previously determined by comparison with the international standard, V.SMOW.

A relative parameter R is also defined from the intensities of the methyl and methylene signals

$$R = 3I_{II}/I_I \quad (4)$$

If I_I and I_{II} denote the area of the signals, R represents the number of deuterium atoms in site II in a situation where the methyl position is arbitrarily characterized by the probability factor 3. More precise determinations can be obtained by considering that I_I and I_{II} are signal heights but the empirical factors thus obtained must be corrected for the slight systematic difference in the transverse relaxation times of the I and II nuclei. In all cases the value of R is significantly higher than the theoretical value, 2, which would correspond to a random distribution of deuterium.

Accuracy of the analytical chain

In order to check the reproducibility of the experiments, eight series of fermentation have been carried out at different times in identical conditions (lactose 50 g l^{-1}; medium M_3; age of the inoculum: 24 h; duration of the fermentation 24 h). The mean values of the fermentation parameters are associated with very satisfactory confidence intervals: sugar consumption 98.9 ± 0.32%, biomass production 1.41 ± 0.22 g l^{-1}, ethanol yield 91.9 ± 0.6%. Similarly the yield of the distillation step is very reproducible: 96.1 ± 0.15% (n = 8). Interlaboratory comparisons have shown that the repeatability and reproducibility of the SNIF-NMR determinations of ethanol reaches, respectively, 0.8% and 1% (when the same type of spectrometer is used) (Guillou *et al.*, 1988; Martin *et al.*, 1996). The IRMS determination of the carbon and nitrogen isotope parameters is performed with a precision of ± 0.2‰ on the δ-scale (eqn 3). At least two repetitions of every experiment have been performed.

RESULTS AND DISCUSSION

The yeast species *Kluyveromyces* is known to be efficient for fermenting concentrated whey permeates relatively rapidly (Zakrzewski and Zmarlicki, 1988) and it is used on an industrial scale for alcohol production. In a first step, lactose is hydrolysed into glucose and galactose by the β-galactosidase enzyme. Following the transformation of galactose into glucose-1-phosphate the monomeric sugars enter the glycolytic pathway to give pyruvate, which is subsequently transformed into ethanol. Since this metabolic pathway involves a great number of steps with the possible occurrence of side reactions, many experimental conditions are likely to influence the rates and yields of the overall bioconversion and possibly the isotopic parameters of the required final products, ethanol and water. We have therefore investigated the role of the physiological state of the yeast, of the nature and concentration of the initial sugar, of the fermentation time, and of the composition of the fermentation medium, on the rates and yields of the bioconversion and on the isotopic parameters of the products.

The fermentation reaction is characterized by the rates of sugar consumption, and of biomass and ethanol production, on one hand, and by the conversion rate of lactose and the yield of ethanol on the other (Experimental section). The isotopic parameters considered are the overall ^{13}C content of ethanol (eqn 3), the isotope ratios of the starting, $(D/H)_W^S$, and final, $(D/H)_W^Q$, aqueous media and the site-specific hydrogen isotope ratios of the methyl, $(D/H)_I$, and methylene, $(D/H)_{II}$, sites of ethanol.

This appraisal of the biochemical and isotopic behaviors is necessary to develop an efficient and reproducible strategy that will be applied to the characterization of different kinds of milks.

Influence of the physiological state of the yeast

Since the growth of the micro-organism exhibits several phases, it is necessary to investigate the growth curve of *Kluyveromyces fragilis* in an appropriate medium, M_C (Experimental section). Four points corresponding, respectively, to the acceleration, exponential, slowing down and stationary phases have been selected on this curve (10, 24, 33 and 96 h). Each inoculum has been used to ferment lactose (50 g l^{-1}) from the same pool in medium M_3, for about 30 h.

The biochemical and isotopic parameters measured in the four series of experiments are given in Table 1. A faster development of the biomass at the expense of ethanol production is observed with the youngest inoculum and the incomplete alcohol yield is associated with a noticeable isotope effect on the methyl site of ethanol. With an old inoculum some increase in the isotope ratio of the methylene site of ethanol is observed and the biomass is enriched in the ^{15}N isotope. On the basis of these results we have selected an age of about 24 h, situated at more than one-half of the exponential growth period of the yeast.

Influence of the concentration of lactose and of the fermentation time

Due to the lack of sensitivity of ^2H-NMR detection, relatively large quantities of ethanol (> 3 ml) are required, which therefore means that sufficient amounts of sugar must be fermented.

Two series of experiments have been conducted in the same rich fermentation medium, M_C (Experimental section), with two concentrations of lactose, 50 g l^{-1} and 100 g l^{-1}. The rate of ethanol production, V_E, associated with the concentration of 100 g l^{-1} (1.53 g l^{-1} h^{-1}) is about twice that measured for 50 g l^{-1} (0.82 g l^{-1} h^{-1}) and the amount of ethanol produced per unit of biomass, $P_{E/B}$, is also much higher (13.4 against 9.1). However, both the conversion per cent of lactose, $Y_{Sf/Si}$, and the yield in ethanol, $Y_{E/S}$ (eqn 2) are strongly

Table 1. Influence of the age of the *Kluyveromyces fragilis* inoculum on the reactional and isotopic parameters of the fermentation of lactose (medium M_3). Cells are taken off either during the exponential growth period (A, B, C) or during the stationary phase, D. V_S, V_B, V_E are the initial reaction rates of sugar, biomass and ethanol; $C_{B/S}$ characterizes the production of biomass with respect to lactose (eqn 1), $P_{E/B}$ is the production of ethanol per unit of biomass, $Y_{Sf/Si}$ the per cent of sugar consumption and $Y_{E/S}$ the yield in ethanol expressed with respect to the converted sugar (eqn 2). $(D/H)_I$ and $(D/H)_{II}$ are the site-specific hydrogen isotope ratios of the methyl, I, and methylene, II, sites of ethanol. The relative parameter R is defined in eqn 4. $(D/H)^Q_W$ is the isotope ratio of the fermentation water at the end of the reaction. $\delta^{13}C$ and $\delta^{15}N$ are the carbon and nitrogen isotopic parameters defined in eqn 3

Parameters			Age of the inoculum			
			A 10 h	B 24 h	C 33 h	D 96 h
Biochemical		V_S ($g\,l^{-1}\,h^{-1}$)	3.10	2.75	2.50	2.33
		V_B ($g\,l^{-1}\,h^{-1}$)	0.15	0.10	0.09	0.06
		V_E ($g\,l^{-1}\,h^{-1}$)	1.53	1.50	1.50	0.78
		$C_{B/S}$	0.032	0.029	0.030	0.029
		$P_{E/B}$	10.27	11.42	11.17	11.76
		$Y_{Sf/Si}$ (%)	99.6	99.5	98.9	99.3
		$Y_{E/S}$	88.1	94.4	93.1	96.5
Isotopic	Ethanol	$(D/H)_I$ (ppm)	111.5	113.2	113.6	113.3
		$(D/H)_{II}$ (ppm)	123.9	123.8	124.9	125.4
		R	2.22	2.19	2.20	2.21
		$\delta^{13}C$ (‰)	-27.3	-27.2	-27.5	-27.2
	Water ⟶	$(D/H)^Q_W$ (ppm)	149.9	149.4	149.3	150.3
	Biomass	$\delta^{13}C$(‰)	-26.0	-25.9	-25.9	-26.0
		$\delta^{15}N$(‰)	2.5	2.9	2.9	3.5

degraded in the concentrated medium (Table 2). The fast accumulation of ethanol in this medium is detrimental to cell development and tends to inhibit further ethanol production.

In spite of the significant differences observed in the biochemical parameters neither the fermentation time at which the sample is investigated, nor the concentration of sugar, exert a significant influence on the $\delta^{13}C$ and $(D/H)_I$ parameters, provided that a high ethanol yield is reached. These results corroborate the fundamental stability of these parameters, which are tightly connected with the isotopic contents of the carbohydrate skeleton (Martin *et al.*, 1986). However, when the reaction is far from completion (time $t = 15$ h) slightly lower values of both $(D/H)_I$ and $(D/H)_{II}$ are observed. Moreover, a significant increase in $(D/H)_{II}$ occurs for long fermentation times as already discussed in the case of slow glucose fermentation by *Saccharomyces cerevisiae* or of poorly alcohologenic yeasts (Vallet *et al.*, 1996).

A concentration of $50\,g\,l^{-1}$, which is of the same order of magnitude as that of milk permeates, has been retained in the analytical procedure and a fermentation time situated at the beginning of the plateau in the ethanol concentration curve (threshold point ≃24 h) will be adopted.

Influence of the fermentation medium

From a practical point of view, it is desirable to define a strategy based on the direct fermentation of whey permeates. In order to compare the fermentation of purified lactose in a synthetic medium to that of whey permeates we have first investigated the influence of the composition of the reaction medium with a view to optimizing the rate and yield of the bioconversion. In a second step it is necessary to appraise possible discriminating effects of the medium on the isotopic parameters of the products.

The evolution of fermentation reactions conducted in different media (M_1 to M_4, described in the Experimental section), is illustrated in Fig. 1 and the corresponding reaction parameters are given in Table 3. With respect to the simplest medium M_1, the addition of peptones (M_3) has a strong activating effect on both the rates and yields of the bioconversion by *Kluyveromyces fragilis*. A nearly complete consumption of lactose is obtained in a reasonable time with a high conversion yield in ethanol (>90%). In contrast, adding sodium ions is strongly detrimental to the rate and yield of the bioconversion. This behavior is probably explained by an inhibitory effect on the β-galactosidase activity necessary for the hydrolysis of lactose. More efficient conditions are restored in medium M_4 prepared from

Table 2. Biochemical and isotopic parameters describing the evolution of the fermentation reaction of lactose at two concentrations $50\,g\,l^{-1}$ and $100\,g\,l^{-1}$ in the fermentation medium M_C (Experimental section). The parameters are defined in the legend to Table 1

Lactose concentration ($g\,l^{-1}$)	Fermentation time (h)	Conversion rate of lactose $Y_{Sf/Si}$ (%)	Ethanol yield $Y_{E/S}$ (%)	Isotopic parameters			
				$(D/H)_I$ (ppm)	$(D/H)_{II}$ (ppm)	R	$\delta^{13}C$ (‰)
50	15	57.8	82.1	112.1	121.9	2.18	−25.8
	24	92.6	95.7	112.7	125.2	2.22	−25.8
	48	98.5	93.4	112.2	126.0	2.24	−25.7
	72	98.4	95.2	112.6	128.0	2.28	−25.5
	96	98.7	92.2	112.3	129.9	2.31	−25.9
100	15	32.6	89.1	112.5	119.3	2.12	−26.2
	24	52.1	91.1	113.5	123.5	2.18	−25.9
	48	60.0	93.4	113.3	127.1	2.24	−26.2
	72	68.4	86.0	113.4	125.5	2.22	−25.0

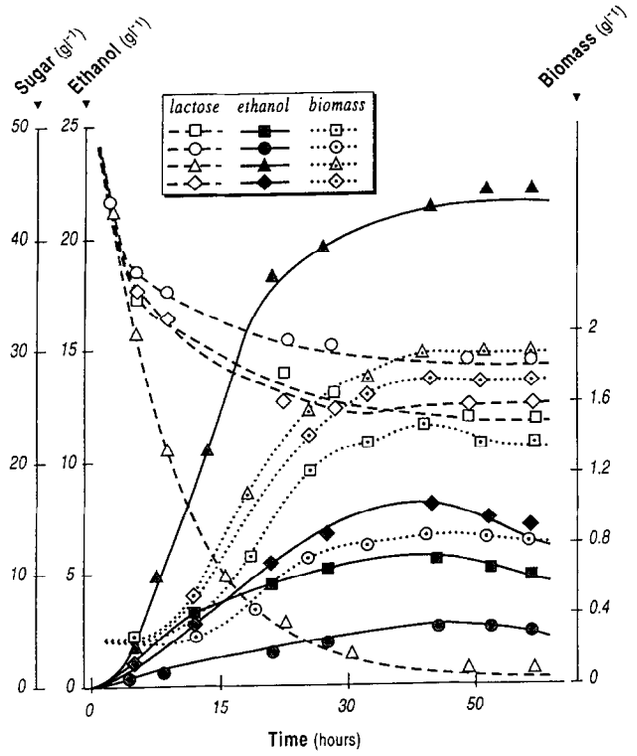

Fig. 1. Influence of the medium on the evolution of the concentrations of lactose, ethanol and biomass in a fermentation reaction by *Kluyveromyces fragilis*. The initial concentration of lactose is $50\,g\,l^{-1}$. The composition of the different media is described in the experimental section and is represented by the following symbols: square: M_1; round: M_2; triangle: M_3; rhomb: M_4.

M_2 by adding peptones, which seem to induce a protecting effect against the inhibiting influence of the sodium ions. However, since the best performance is obtained with M_3, this medium, which mimics that of whey permeates, has been selected for all the fermentations of lactose which require a synthetic medium.

The fermentation of purified lactose in medium M_3 has then been compared to the direct fermentation of whey permeates from both the biochemical (Table 3) and isotopic (Table 4) points of view. The rate and yields of the bioconversions are nearly identical and the evolution of the isotopic parameters in the course of the reaction are also very similar. Moreover, at high yield of the transformation, the ^{13}C content remains nearly constant as a function of time and differs only slightly from that of the lactose precursor. As in the case of glucose fermentation, the glycolytic pathway is responsible for a strong deuterium depletion since a decrease of about 44 ppm is reached when going from $(D/H)_{LNE}$ to $(D/H)_I$ (Table 4). However, a very good stability of the $(D/H)_I$ parameter is observed. In contrast, $(D/H)_{II}$ regularly increases as a function of time. This behavior may probably be explained, as in the case of glucose fermentation by *Saccharomyces cerevisiae*, by enzymatically mediated exchange reactions (Zhang *et al.*, 1995). In order to avoid discriminating perturbations it is therefore suitable to sample the fermentation products at the threshold point defined on the kinetic curves (Fig. 1) as the beginning of the conversion plateau (Vallet *et al.*, 1996). In such conditions reliable isotopic comparisons can be performed on samples obtained either from the direct fermentation of whey permeates or from that of purified lactose dissolved in medium M_3.

Influence of the technological processes on the isotopic parameters

Since lactose is contained in raw materials with different compositions, it is necessary to appraise the magnitude of isotope fractionation effects possibly introduced by partial or total extraction of lactose before fermentation. Therefore, using the same bulk milk sample, the reactional and isotopic parameters have been determined in experiments carried out either on pure lactose extracted by successive steps of deproteination, delipidation and crystallization or directly on the milk permeate simply obtained by ultrafiltration. Very similar fermentation performances and isotopic values are observed in these experiments. The yield of the fermentation $Y_{E/S}$ reaches about 90% in both cases and the isotopic values are nearly equal: $(D/H)_I = 115.1$ ppm, $(D/H)_{II} = 130.7$ ppm and $\delta^{13}C = 11.7‰$ for the fermentation directly conducted on the permeate and $(D/H)_I = 115.2$ ppm, $(D/H)_{II} = 130.0$ ppm, $\delta^{13}C = -12.3‰$ for the fermentation of the extracted lactose. However, since it could be desirable to avoid the time-consuming step of lactose crystallization, a series of experiments

Table 3. Influence of the medium on the reaction parameters for the fermentation of lactose ($50\,g\,l^{-1}$) or whey permeate by *Kluyveromyces fragilis*. The rate and conversion parameters are defined in the caption to Table 1 and the composition of the media M_1 to M_4 is described in the Experimental section. Two series of experiments (a and b) have been carried out

Fermentation medium		Rate parameters			Conversion parameters		
		V_S ($g\,l^{-1}h^{-1}$)	V_E ($g\,l^{-1}h^{-1}$)	V_B ($g\,l^{-1}h^{-1}$)	$Y_{E/S}$ (%)	$C_{B/S}$	$P_{E/B}$
a	M_1	1.58	0.21	0.09	51	0.07	3.21
	M_2	1.44	0.05	0.05	33	0.04	3.09
	M_3	3.10	0.61	0.12	92	0.04	10.89
	M_4	1.87	0.24	0.10	64	0.07	3.89
b	M_3	2.58	1.14	0.11	94	0.03	11.08
	Whey permeate	2.71	1.08	0.10	94	0.04	10.46

Table 4. Comparison of the isotopic parameters and of their evolution in the course of the fermentation for pure lactoses 1 and 2, and whey permeates 3 and 4. The commercial lactose, 1, and lactose, 2, extracted from whey permeate, 4, are fermented in the 'synthetic' medium M_3 (Experimental section) elaborated from Nantes tap water characterized by an isotope ratio $(D/H)^S_W = 149.8$ ppm. The aqueous medium of the whey permeates 3 and 4 is characterized by isotope ratios $(D/H)^S_W = 151$ ppm and 152.4 ppm, respectively. The carbon-bound hydrogens of lactose have an overall isotope ratio $(D/H)_{LNE} = 156.7$ ppm for 3 and 158.0 ppm for 4. The considered parameters are defined in the legend to Table 1. The reaction parameters, V ($g\,l^{-1}\,h^{-1}$), C and P of fermentations 1 and 3 are given in Table 3. The carbon isotope parameter of the biomass in experiments 2 and 4 is $\delta^{13}C = -25.1\%$ at the beginning and $-18.0‰$ at the end of the reaction

Medium		Lactose	Fermentation time	Conversion rate	Ethanol yield	Isotopic parameters				
						Ethanol				Water
		$\delta^{13}C$(‰)	(h)	Y_{S_f/S_i} (%)	$Y_{E/S}$ (%)	$(D/H)_I$ (ppm)	$(D/H)_{II}$ (ppm)	R	$\delta^{13}C$ (‰)	$(D/H)^Q_W$ (ppm)
			24	97.8	93.0	113.8	126.5	2.23	−26.1	150.2
M_3	1	-26.7	48	99.0	93.5	113.6	127.2	2.24	−26.4	150.2
			72	99.0	93.5	113.9	127.9	2.25	−26.2	150.2
			96	99.0	94.6	113.4	129.2	2.28	−26.3	150.2
	2	−13.4			90.8	115.2	130.0	2.26	−12.3	—
Whey permeate			12	98.1	97.0	115.0	124.5	2.16	−19.0	151.4
			24	98.1	96.6	114.8	127.3	2.22	−19.2	152.0
		−20.7	45	98.1	96.5	114.8	127.2	2.22	−18.9	152.2
	3		69	98.0	95.4	114.1	127.4	2.23	−19.1	151.9
			93	98.0	97.9	115.7	130.4	2.25	−18.9	152.0
	4	−13.6			89.8	115.1	130.7	2.27	-11.70	154.3

has been performed in order to further estimate the influence of the treatment on the isotopic parameters. Both the yields and the hydrogen isotope ratios of ethanol have been compared for reactions involving the following conditions (Table 5): pure commercial lactose (1); lactose + trichloroacetic acid (TCA is used in the deproteination step) (2); lactose + TCA, with recrystallization (3); lactose + TCA + delipidation solvents, with recrystallization (4). Similarly fermentation reactions have been conducted either on pure lactose extracted from milk powder or directly on the aqueous phase resulting from the deproteination (presence of TCA) and delipidation of the same bulk milk powder sample (Table 5). On condition that crystallized lactose is used, the isotopic values of the fermentation products are nearly independent of the treatments applied for recovering the pure carbohydrate. In contrast, erroneous results are obtained when the fermentation is performed directly on the aqueous phase resulting from deproteination and delipidation. The yield of the fermentation is only 65% and depletions of more than 10 ppm may be exhibited by the hydrogen isotope ratios of ethanol. This behavior can be explained by the presence of residual trichloroacetic acid (TCA) in the

Table 5. Influence of the lactose treatment on the fermentation and isotopic parameters. Either pure commercial lactose (first series of experiments) or milk powder have been used (second series). In a control experiment (1) the pure lactose has been fermented in medium M_3. In (2) the aqueous phase obtained after treatment by TCA has been directly fermented. In (3) lactose treated as in (2) has been recrystallized before fermentation. In (4) lactose having undergone the full treatment corresponding to deproteination (TCA) and delipidation (extraction by $CHCl_3/CH_3OH$) has been recrystallized before fermentation. In the case of milk powder the results corresponding to the fermentation of extracted pure lactose (5) are compared to those of a direct fermentation of the aqueous phase (6) resulting from the deproteination (TCA) and delipidation steps

Starting material	Treatment	Fermentation parameters			Isotopic parameters		
		Y_{S_f/S_i} (%)	$Y_{E/S}$ (%)	$C_{B/S}$	$(D/H)_I$ (ppm)	$(D/H)_{II}$ (ppm)	R
Commercial lactose	1 Pure lactose	98.7	91.8	1.21	112.4	127.8	2.27
	2 + TCA	97.5	64.1	0.97	100.7	111.5	2.22
	3 + TCA + Cryst.	98.9	89.8	1.35	113.1	128.1	2.26
	4 + TCA + Solvents + Cryst	98.9	92.2	1.28	113.9	129.3	2.27
Milk powder	5 Cryst. lactose	97.9	94.3	1.31	124.1	140.8	2.25
	6 + TCA + Solvents	98.3	65.9	1.25	81.5	94.9	2.32

Table 6. Fermentation parameters and isotopic characterization of lactoses contained in different media. Ten commercial samples of pure lactose have been investigated. Samples 1–8 are α-lactoses from Aldrich (1), Sigma (2, 3), Janssen (4), Fluka (5), Prolabo (6, 7), Melun (8) and the two β-lactoses are from Aldrich (9) and Sigma (10). The milk powders are from different producers: five are skimmed milks, 11–15, and one is semi-skimmed, 16. Among the three different milks, one is semi-skimmed 17 and 18, 19 have been obtained directly from the farm (morning 18, evening 19). The two lactoserums 20 and 21 are from commercial origin. The initial concentration of biomass is 0.62 (+0.04) g l^{-1}. The hydrogen isotope ratio of the starting water, $(D/H)^S_W$, is 149.6 ± 0.5 ppm

Raw material		Fermentation parameters			Isotopic parameters							
		Y_{S_f/S_i} (%)	$Y_{E/S}$ (%)	Biomass produced (g l^{-1} h^{-1})	Starting materials		Final products					
					Lactose	Biomass	Ethanol			Biomass	Water	
					$\delta^{13}C$ (‰)	$\delta^{13}C$ (‰)	$\delta^{13}C$ (‰)	$(D/H)_I$ (ppm)	$(D/H)_{II}$ (ppm)	R	$\delta^{13}C$ (‰)	$(D/H)^Q_W$ (ppm)
Commerical α-lactose	1	99.2	91.7	1.3	−26.6	−24.1	−25.2	113.4	128.9	2.27	-25.4	150.6
	2	98.6	92.0	1.7	−18.7		−17.2	110.8	126.0	2.27	−18.3	153.2
	3	98.3	92.0	1.5	−18.7		−17.0	111.3	128.1	2.30	−18.7	151.2
	4	99.1	89.6	1.2	−28.3	−24.6	−26.6	113.6	129.7	2.28	−26.3	150.4
	5	98.8	91.4	1.3	−27.6	−24.3	−26.2	114.2	129.7	2.27	−26.2	150.4
	6	97.8	93.0		−26.7		−26.1	113.8	126.5	2.23		
	7	99.5	94.4	1.5	−28.1	−25.1	−27.2	113.2	123.8	2.19	−26.6	150.2
	8	99.0	92.1	1.6	−26.7		−24.9	115.3	130.0	2.26	−25.3	150.5
Commercial β-lactose	9	99.3	90.6	1.4	−18.6	−18.8	−17.0	120.7	140.1	2.32	−18.4	150.6
	10	98.8	92.2	1.3	−19.1	−18.9	−17.7	105.2	121.6	2.31	−18.5	150.3
Milk powder	11	97.9	94.3	1.3	−25.4	−25.0	−24.7	124.9	140.8	2.26	−25.2	151.2
	12	98.2		1.4	−23.9		−22.3	112.4	125.0	2.22	−23.5	
	13	98.4	89.2		−23.6		−22.4	116.5	130.0	2.23	−23.5	
	14	98.8	88.6		−19.8		−18.8	115.9	130.1	2.25	−20.9	
	15	98.1	93.9	1.7	−21.0		−20.1	111.3	128.1	2.30	−22.0	
	16	98.3	94.3	1.7	−24.2		−23.2	114.9	129.8	2.26	−24.0	
Milk	17	98.8	89.2	1.9	-26.8	−25.0	-25.4	116.4	130.6	2.25	-25.5	
	18	98.6	90.5	1.5	-23.9	-25.0	-22.8	117.8	133.8	2.27	-23.4	
	19	98.2	90.4	1.5	-23.0	-25.0	-21.8	114.8	131.5	2.29	-23.4	
Lactoserum	20	97.4		1.6	-23.5	-24.5	-22.2	113.4	126.9	2.24	-23.1	
	21	98.5		1.4	−23.1		-22.0	114.6	128.8	2.25	-23.1	

fermentation medium. A competition between the decarboxylation of TCA and pyruvate intervenes at the expense of the second one. Instead of evolving towards the formation of acetaldehyde the reaction produces chloroform, which can be observed in the ^{1}H-NMR spectrum of the fermentation medium. Inhibitory effects on the production of biomass and ethanol are then observed.

Discriminating potential of the isotopic parameters

The optimized strategy defined above has been used for comparing the isotopic characteristics of lactoses and milks from different commercial origins. Isotopic characterization of lactose and milks, pure commercial α- and β-lactoses, lactoses extracted from milk powders or liquid milk, and milk permeates has been investigated (Table 6). Very similar performances of the fermentation by *Kluyveromyces fragilis* have been achieved. Full consumption of glucose is reached and yields in ethanol of about 92% (±2) are obtained. In all cases the biomass concentration has grown by a factor of about 2. The behavior of the isotopic parameters is also quite similar. A systematic increase in $\delta^{13}C$ of about 1‰ accompanies the transformation of lactose into ethanol. Moreover, the deuterium content of the aqueous medium is slightly enhanced. This behaviour may be explained by transfer to the aqueous medium of relatively enriched carbon-bound hydrogens of lactose (cf. the $(D/H)_{LNE}$ values quoted in Table 4). The overall ^{13}C content and the methyl isotope ratio of ethanol, $(D/H)_I$, may be considered as faithful witnesses of the precursor materials. Since the methylenic hydrogens mainly originate from water at two steps of the glycolytic pathway the isotope ratio, $(D/H)_{II}$, may indirectly reflect fractionation effects introduced into the exchangeable sites of lactose by the technological process of extraction. In fact, due to both kinetic and

thermodynamic effects, significant variations in the isotope ratio of the hydroxylic hydrogens are likely to occur. To some extent these variations are subsequently transferred to the methylenic position via exchange with the aqueous environment.

CONCLUSION

An efficient strategy has been defined that leads to ethanol, which is isotopically representative of its lactose precursor. Possible sources of isotope fractionation intervening in the preparation of lactose or in the fermentation reaction have been appraised. It may be concluded that the isotopic fractionation phenomena that accompany the fermentation of lactose by *Kluyveromyces fragilis* are very similar to those which characterize the fermentation of glucose, fructose, sucrose, etc., by *Saccharomyces cerevisiae*. These bioconversions provide a common probe, ethanol, which enables the different kinds of carbohydrate precursors to be compared. Thus the variation in the $(D/H)_I$ parameter of ethanol obtained from lactose is expected to offer a new criterion for inferring the origin of the raw material. In the case of cow's milk, for instance, it will be used to further characterize the type of feeding products given to the animal. More generally the present approach offers a new isotopic strategy for investigating various metabolic aspects of lactose biosynthesis.

ACKNOWLEDGEMENTS

The authors are grateful to Dr Boyaval (INRA, Rennes) for providing them with authenticated samples of milk and to Mr M. Berry and Mrs F. Mabon for their helpful contribution to the IRMS and NMR experiments.

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