BIOORGANIC CHEMISTRY **24,** 319–330 (1996)
ARTICLE NO. 0028

Natural Abundance Isotopic Fractionation in the Fermentation Reaction: Influence of the Nature of the Yeast

CLAUDE VALLET, RAJA SAÏD, CLAUDE RABILLER, AND MARYVONNE L. MARTIN

RMN-RC Laboratory, Univertsity of Nantes, URA CNRS 472, 2 rue de la Houssinière, 44072 Nantes cedex 03, France

Received April 29, 1996

Site-specific natural isotope fractionation studied by nuclear magnetic resonance (SNIF-NMR) provides isotopic criteria that characterize a biochemical transformation such as fermentation and enable isotopic ratios measured in end products to be correlated with those of their precursors. In principle, a given set of transfer coefficients applies only to bioconversions performed under strictly identical conditions, a situation that is hardly fulfilled in most usual fermentation processes. In particular, natural raw materials such as fruits frequently involve complex mixtures of various yeast strains present at different concentrations. Series of experiments performed with different yeasts, different concentrations of carbohydrates, and different yields of the transformation have shown that, although glycolysis is associated with overall hydrogen fractionation effects that may exceed 40 ppm, the range of variation in the isotopic ratios of the fermentation products, ethanol and water, does not exceed a few parts per million. Provided that the yield in ethanol reaches values higher than 70%, the nature of the yeast strain has minimal influence on the isotopic ratio of the methyl site of ethanol $(D/H)_I$. In contrast, the isotope ratio of the methylene site, $(D/H)_{II}$, may exhibit significant enhancements, in particular when ethanol is left in contact for a long time with poorly alcohologenic yeasts. These behaviors are consistent with hydrogen transfers from the aqueous medium to the methylene site, and partly to the methyl site, occurring with relatively high kinetic isotope effects. Since water acts as an open pool of hydrogens, however, only small isotopic variations are produced in the course of the fermentation reaction. Moreover, the partial connection between hydrogens from the methyl site of ethanol and hydrogens from glucose operates with relatively small secondary isotopic effects. No significant changes in the percentages of intra- and inter-molecular transfers of hydrogen to the methyl site are observed as a function of the nature of the yeast. These results support the use of the methyl isotopic ratio of ethanol as a probe of the isotopic behavior of carbohydrate precursors, whatever the yeast strains present in natural fermentation media.

INTRODUCTION

At natural abundance the site-specific isotope ratios of fermentation products can be linearly related to those of their carbohydrate precursors, which are themselves a source of information on the photosynthetic pathway (*1*, *2*). On this basis, the isotopic parameters of fermented media have been used for inferring the botanical origin of the plant precursor and the environmental conditions of the photosynthesis of the sugars (*3*). From a general point of view, comparison of isotopic properties of used reactants based on a study of the isotopic parameters of their reaction

0045-2068/96 $18.00

products requires that all possible isotopic fractionation effects associated with the bioconversion can be precisely estimated or remain perfectly reproducible. In this respect the methyl and methylene sites of ethanol obtained in a fermentation reaction exhibit significant deuterium depletion with respect to both the nonexchangeable sites of glucose and the starting water (*1*). These overall fractionation effects are the results of several kinetic and thermodynamic isotopic effects occurring in the course of the glycolytic pathway. The overall transformation can be described by a matrix of isotopic coefficients which relate every site in the products to every site, or group of sites, in the reactants (*2*); however, even when similar technological conditions are implemented, as is the case in wine production for instance, the complexity of the fermentation medium may be the cause of distortions in the isotopic connections. More generally, since the yeast strains and the experimental conditions are not strictly reproducible when fermentation is carried out on natural media, it is necessary to detect and identify possible sources of perturbation in the isotopic transfer matrix describing the biochemical transformation and to estimate the magnitude of the corresponding variations in the isotopic ratios.

In this paper we examine the extent to which the isotopic distribution observed in the fermentation products is influenced by the yeast species. Both the rate of carbohydrate conversion and the yield of the transformation strongly depend on the alcohologenic potential of the yeast. It is therefore necessary to estimate previously the influence of these parameters, to better elucidate the role of the microorganism and to analyze the isotopic information accessible in conditions of incomplete fermentation, as encountered in sweet wines for instance.

EXPERIMENTAL

Materials

Glucose. D(+)-Glucose was purchased from commercial sources: Sigma, Janssen, or Prolabo.

Yeast strains. The strains *Saccharomyces cerevisiae* 27817, *Kluyveromyces fragilis* 27774 and 30017, *Kluyveromyces marxianus* 30016, and *Candida utilis* 30091 were obtained from MUCL. Two strains of *S. cerevisiae*, *Saccharomyces bayanus*, *Saccharomyces carlbergensis*, and *Candida tropicalis* were given by Dr. Vincent from ENITIAA (Nantes). Other strains of yeast were provided by Martin-Vialatte, Brocades, or by oenology laboratories.

Except for lyophilized strains of *S. cerevisiae* the yeasts were maintained at 4°C on 2% agar slants containing (g liter^{-1}): yeast extract, 3.0; malt extract, 3.0; peptone, 5.0; dextrose, 10.0 (pH 6.3). They were transferred every 3 months.

Fermentation Experiments

The cells from the agar slants were routinely reactivated in a rich liquid medium (MR) for 18–24 h. Then, the active cells of this culture were inoculated in the same

medium (MR) and grown in a shaker flask until they reached the end of the exponential phase. The amount of cells required for the fermentation was aseptically harvested by centrifugation, resuspended in an appropriate medium (see below), and immediately transferred to the fermentation medium. The fermentation was carried out in batch in a water bath with stirring (150 rpm). Generally, the working temperature was 30°C and the pH of the initial medium was 5.5.

Fermentation media. The composition of the rich medium, MR, was (g liter^{-1}): yeast extract, 3.0; peptone, 5.0; dextrose, 20.0.

Several fermentation media denoted M_1, M_2, and M_3 have been used. Their compositions (in g liter^{-1}) were as follows:

M_1: $(NH_4)_2SO_4$, 4.0; KH_2PO_4, 3.0; $MgSO_4 \cdot 7H_2O$, 1.0; peptone, 2.0.

M_2: NH_4Cl, 2.0; $Na_2H\ PO_4$, 6.0; Na_2SO_4, 0.2; $MgCl_2$, 0.1; NaCl, 3.0; yeast extract, 5.0.

M_3: $(NH_4)_2SO_4$, 2.0; KH_2PO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 0.2; tartric acid, 3.0; malic acid, 6.0; citric acid, 0.3; asparagine, 2.0; and vitamin solution (10 ml) containing (mg ml^{-1}) biotin, 4.0; thiamine, 0.1; pyridoxine, 0.1; nicotinic acid, 0.1; panthothenic acid, 0.1; and myoinositol, 0.1.

Sterilization. Sterilization of the vessels and media was carried out at 110°C for 30 min except for sugar or vitamin solutions, which were sterilized by filtration through 0.2-nm Millipore filters.

Physiological parameters. The fermentation kinetics were followed by measuring evolution of the biomass, sugar consumption, and ethanol production in the culture media. Samples were withdrawn from suspensions at specified times during fermentation. They were centrifuged at 3000*g* for 15 min and the supernatants were stored at −20°C until the determination of the sugar and ethanol concentrations.

Biomass determination. The biomass concentration was measured by turbidimetry at 620 nm using a Spectronic 20 spectrometer (Bausch and Lomb). After centrifugation and washing, the cell pellets were dried at 100°C until they reached a constant weight. An OD of 1.0 is equivalent to between 0.1 and 0.3 g of dry weight depending on the microorganism.

Viable cells were counted by means of a hematocytometer after suitable dilution in blue trypan solution.

In the case of *S. cerevisiae* the initial concentration of cells, [*c*], has an effect on the fermentation rate and therefore on the time, t_c, required for total sugar consumption. Under the considered conditions (glucose, 100 g liter^{-1}; medium M_1), t_c satisfied the empirical law $t_c = 49–36 \ln[c]$.

Sugar and ethanol determination. Glucose was measured by the colorimetric method adapted to the determination of sugars (*4*). Ethanol in the supernatant was determined by enzymatic assay using alcohol dehydrogenase (ADH) and NAD (*5*). The alcoholic content of the fermented medium was also determined by chromic oxidation. Azeotropic ethanol was recovered by distillation and the percentage of water in ethanol was measured by the Karl-Fischer method (±0.05%). The yield of the ethanol extraction was usually greater than 90%.

The yield in ethanol produced by sugar conversion is referred to as the theoretical percentage (1 mol of glucose → 2 mol of ethanol = 100%):

$$\text{ethanol yield (\%)} = \frac{\text{ethanol content (w/v)}}{\text{sugar content (w/v)} \times 0.51} \times 100.$$

Glucose consumption is described by the decrease in the glucose concentration during fermentation.

Isotopic Determinations

The isotopic parameters are defined on an absolute scale as the ratio, H/L, of the number of heavy isotopes, H, to the number of light isotopes, L. The results are expressed in parts per million with respect to the V. SMOW standard (*6*).

Mass spectrometric determination of the isotope contents. The hydrogen isotope ratio of water, $(D/H)_W$, and the overall isotope ratio of the nonexchangeable sites of glucose, $(\overline{D/H})_{GNE}$, were determined by isotope ratio mass spectrometry (IRMS) on a VG SIRA 9 instrument. To eliminate the contribution of the hydroxylic sites, which are exchanged with water in the medium, glucose is converted into its pentanitrate derivative. The nitrate is then burned in a Carlo-Erba microanalyzer to give CO_2, H_2O, and nitrogen oxides which are reduced on a copper catalyst. The hydrogen isotope ratio is measured on hydrogen gas obtained by reduction of water catalyzed by zinc at 550°C. The standard deviation of the deuterium determination is about 0.5 ppm.

Site-specific natural isotope fractionation studied by NMR (SNIF-NMR). The isotopic parameters were measured on ethanol samples extracted from the fermentation medium by distillation under strictly standardized conditions.

The deuterium NMR spectra of ethanol were recorded at 61.4 MHz with a Bruker AM400 spectrometer equipped with a ^2H $\{^1H\}$ dedicated probe, an internal ^{19}F locking device, and an automatic sample changer. The following acquisition conditions were usually retained; frequency window, 1200 Hz; acquisition time, 6.8 s; broad/band proton decoupling; accumulation of 200 scans. The free induction decays were processed by means of a dedicated system managing a fully automatic curve-fitting software based on a complex least-squares theoretical algorithm (*7*) (SNIF-NMR Concept Core-System and Interliss software from Eurofins). The overall precision is respectively equal to 0.3 and 0.4 ppm for sites I (CH_2D) and II (CHD) of ethanol.

The relative deuterium contents in the methyl, I, and methylene, II, sites of ethanol can also be described by the parameter

$$R = 3S_{II}/S_I, \qquad [1]$$

where S_I and S_{II} denote the area of the deuterium NMR signals of sites I and II, respectively. R represents the number of deuterium atoms in position II in a situation where the methyl position I is arbitrarily given the statistical probability number 3. A random distribution of deuterium would therefore correspond to a value $R = 2$.

The specific isotope ratios of the methyl and methylene positions, $(D/H)_I$ and $(D/H)_{II}$, are accessible by referring the areas of the ethanol signals to that of a reference substance, tetramethylurea, the isotope ratio of which is accurately known (*3*).

TABLE 1
Evolution of the Isotopic Parameters of the Methyl, I, and Methylene, II, Sites of Ethanol and of Water, WQ, in the Course of a Fermentation Reaction of Glucose by *Saccharomyces cerevisiae*

Time (h)	Ethanol yield (%)	$(D/H)_I$ (ppm)	$(D/H)_{II}$ (ppm)	R	$(D/H)^{\S}_W$ (ppm)
8	<5	109.7	118.1	2.15	150.1
12	12	107.6	118.1	2.19	150.3
18	33	107.3	118.5	2.21	151.1
24	38	107.5	117.4	2.18	151.2
33.5	60	108.1	119.3	2.21	152.0
42	75	108.8	121.9	2.24	152.1
94	90	108.5	121.5	2.23	152.9

Note. The isotope ratio of the starting water was $(D/H)^{\S}_W = 149.4$ ppm, and that of the nonexchangeable sites of glucose, $(\overline{D/H})_{GNE}$, was 148 ppm. The parameter R is defined in Eq. [1]. The initial concentration of glucose was 200 g liter^{-1} in the medium described as M_3 under Experimental (temperature, 31°C).

RESULTS AND DISCUSSION

Many peculiarities in the enzymatic equipment and physiological properties of microorganisms may be associated with differences in kinetic and thermodynamic isotope effects at various steps of metabolism. Moreover, since different yeast species exhibit large variations in their tolerance to ethanol, differences in the glucose conversion rate and in the yield in ethanol are expected to affect the isotope ratios of the products.

From an isotopic point of view, it may be considered that the whole set of isotopomers existing at natural abundance in a starting medium composed of glucose and water is competitively transformed into another final set of isotopomeric products, ethanol, carbon dioxide, and water.

Disregarding the case of the isotopomers of glucose monodeuterated at the hydroxylic positions, which are involved in exchange with water, seven isotopomers corresponding to the different monodeuterated carbon-bound positions (GNE) are engaged in a transformation of type

$$C_6H_{12}O_6 + H_2O \rightarrow 2CH_3\,CH_2\,OH + 2CO_2 + H_2O. \quad [2]$$

The overall deuterium content of the glucose skelton, $(\overline{D/H})_{GNE}$, can be measured by IRMS on the pentanitrate derivative, and the site-specific natural isotope fractionation of hydrogen can be studied by nuclear magnetic resonance SNIF-NMR (*8*).

Influence of the Conversion Yield

A typical fermentation reaction of glucose using the reasonably alcohologenic yeast *S. cerevisiae* was investigated to estimate the magnitude of variations in the ethanol and water isotope ratios possibly occurring in the course of the transformation (Table 1). The number of living cells in the medium has been measured in

parallel. In the defined medium, cell growth is relatively fast during the first 24 h and thereafter reaches a plateau. Following some decrease during the period of cell growth, the isotope ratio of the methyl site of ethanol, $(D/H)_I$, undergoes a limited increase, whereas the isotope ratios of both the aqueous medium, $(D/H)_W^Q$, and the methylene site of ethanol, $(D/H)_{II}$, increase regularly. However, although the bioconversion of glucose characterized by an average isotope ratio of the skeleton $(\overline{D/H})_{GNE}$ = 148 ppm, in water with an isotope ratio $(D/H)_W^S \simeq 150$ ppm, is accompanied by a strong overall deuterium depletion (average isotope ratio of the carbon bound hydrogens of ethanol $\simeq$ 113 ppm), the range of variation observed in the course of the reaction is restricted to a few ppm. This behavior is consistent with the fact that a significant part of the methyl hydrogens and most methylene hydrogens of ethanol are derived from water (*2*, *9*, *10*) with relatively high kinetic isotope effects. Since, at the defined levels of dilution, water may be considered as providing an open pool of hydrogens, the isotopic fractionation associated with this hydrogen transfer is not expected to vary significantly in the course of the bioconversion. Moreover it may be suggested that the methyl hydrogens originating from glucose are issued from the relatively depleted 1, 2 and 6,6′ sites with relatively small isotope effects.

The enrichment of the fermentation water can be explained by the progressive incorporation of hydrogens from sites 3, 4, and 5 of glucose, which are characterized by higher deuterium contents than the initial values for water (*2*). This enrichment of the aqueous medium in deuterium can also be an indirect cause for the regular increase in the $(D/H)_{II}$ value, since the methylenic hydrogens of ethanol originate nearly exclusively from water at two steps of the glycolytic pathway: the decarboxylation of pyruvate and the reduction of acetaldehyde (*11–13*). The detailed behavior of the $(D/H)_I$ parameter is relatively complex, since it reflects not only kinetic isotope fractionation effects, which are expected to be highest at the earliest period of the reaction, but also isotopic discrimination possibly associated with some competitive consumption of glucose during the period of cell growth. In the case of the alcohologenic yeast *S. cerevisiae*, however, stopping the fermentation process at a consumption rate of about 70% leads to an underestimate of the $(D/H)_I$ value by less than 1.5 ppm.

Influence of the Yeast Species

When a similar protocol is used for the fermentation of carbohydrate samples of the same origin, but using different kinds of yeasts, noticeable differences in the isotopic values of the fermentation products are observed. Thus the results of two series of fermentation reactions carried out with the same cell populations in the same medium, at the same temperature, are given in Table 2. Glucose from two different origins at different concentrations in different media have been used in the two series of experiments. In the first series similar values of the isotopic parameters are observed for the two species *Saccharomyces uvarum* and *Hansenuela anomala*, whereas the isotopic ratios of ethanol obtained by using the species