Top Pressure and Temperature Control the Fusel Alcohol/Ester Ratio through Yeast Growth in Beer Fermentation

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Temperature and top pressure are key factors for maintaining a consistent quality of lager beer. Their influence on yeast growth, CO_2 production, final concentrations of fusel alcohol and ester and production kinetics was analysed under industrial conditions. Fermentations of 12°P lager wort were performed at 10°C or 16°C temperature and 1.05 bars or 1.8 bars top pressure, corresponding to dissolved carbon dioxide concentrations of 1.98 g/litre to 3.65 g/litre. Analysis of variance was performed to test the significance of temperature and dissolved CO_2. Results show that temperature increases fermentation rate and the production ratio and final concentration of fusel alcohol, independently of the top pressure applied. Conversely, dissolved carbon dioxide controls the production rate and final concentration of ester by limiting yeast growth. Relationships between initial or maximum ester production rates and maximal growth rates were shown. Considering the metabolic pathways occurring during anaerobic growth of yeast, a limited production of acetyl CoA was expected in cultures with high concentrations of dissolved carbon dioxide. Also, final ester concentration and biomass produced are linearly correlated. Furthermore, whatever the ester considered, its synthesis is not influenced by corresponding fusel alcohol availability.

It was demonstrated that fermentations performed with a reasoned combination of temperature and top pressure can result in a beer of distinctive aroma without resorting to modification of the initial wort or yeast strain.

Key Words: *Beer fermentation, carbon dioxide, process control, ester, fusel alcohol, yeast.*

INTRODUCTION

In brewing, the primary fermentation is one of the longest stages as well as an important aromatic compound production step. Indeed, fermentation has the main impact on process productivity and product quality. During the last fifty years, brewing process productivity has improved through better control of the various unit operations. In lager beer production, the fermentation time is decreased by increasing the temperature (from 8°C to 13-14°C), but to control the aroma profile characteristic of lager beer, top pressure is also used (in the range of between 1.2 and 1.8 bars). Whereas temperature has a general accelerating effect, the combination of temperature and top pressure mainly control aromatic compound production. It is widely recognized that the real parameter affecting the production of volatiles is dissolved carbon dioxide[12,19], whose concentration depends on the temperature and top pressure applied.

Many components are responsible for determining the flavour of beer. They include fusel alcohols, esters, vicinal diketones and organic sulphur compounds. These last two, present in green beer, are significantly reduced during lagering. The remaining fusel alcohols, and esters in particular, contribute to the quality of finished beer[5].

Isoamyl alcohol (3-methyl butanol) and phenyl ethanol are the most important fusel alcohols in beer. Their final concentrations are generally close to or as high as their odour thresholds (45 ppm and 50 ppm respectively)[14]. The most important esters in beer are ethyl acetate (fruity, solvent-like), isoamyl acetate (isopentyl acetate) (banana aroma), isobutyl acetate

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(pineapple aroma), ethyl caproate (ethyl hexanoate) (apple aroma) and phenyl acetate (honey, fruity). Their odour thresholds are low, ranging from 0.2 ppm for isoamyl acetate to 15-20 ppm for ethyl acetate[25]. Thus, these final ester concentrations are key factors determining the characteristic aroma profile of beer.

Esters are synthesized in yeast through a biochemical pathway that involves ethanol or higher alcohols (isoamyl alcohol, isobutanol, phenyl ethanol), acyl CoA (mainly acetyl CoA) and ester-synthesizing enzymes[17]. To explain the regulation of ester synthesis, many studies have focused on the availability of acetyl CoA because of its central role in yeast metabolism[27,31], but with conflicting results. On the one hand, studies on higher alcohol availability suggest that the concentration of the latter may have a substantial controlling influence on ester levels[2]. On the other hand, recent studies on alcohol acetyl transferase activities, responsible for acetate ester synthesis, also suggest that acetate ester production is mainly controlled by the repression/induction of at least three distinct AATase genes (ATF1, LgATF1 and ATF2) (for review see[4]).

Furthermore, changes in wort composition and/or fermentation conditions affect the regulation of ester synthesis and many attempts have been made to explain this[18]. Unfortunately, top pressures or dissolved CO_2 concentrations are often studied at unusual levels and the focus is generally on final concentrations[19]. Moreover, kinetic aspects have not been extensively studied[15].

In previous reports[28,29], we have developed a flavour model of beer fermentation through the CO_2 production rate which can be measured on-line at the industrial scale. The model is capable of describing the development of flavour active compounds during primary fermentation, and to quantify the simultaneous effects of operating conditions on their dynamics.

In order to complete our understanding of these observed effects, we investigated in this work the biomass and its relationship with aromatic compound production. The results presented here describe the influence of temperature and top pressure, under industrial conditions, on the production and kinetics of biomass, higher alcohols and corresponding esters. The relationship between yeast growth and ester production was studied through the influence of dissolved carbon dioxide. Further evidence is presented to support the view that ethanol and higher alcohol levels do not directly control the production of corresponding esters.

MATERIALS AND METHODS

Fermentation system

Fermentations were carried out successively in a stainless steel fermentor (15-litre capacity – LSL-Biolafitte, France). The fermentor included: (1) a temperature sensor (standardized platinum probe – 100 Ω at 0°C); (2) a volumetric flowmeter (Schlumberger, France) measuring the volume of released CO_2; (3) a pressure sensor (Wika-type 98, Germany); (4) a dissolved oxygen probe (Ingold, Switzerland) connected to a Demca 140 S transmitter. Temperature and top pressure were regulated respectively with an accuracy of ± 0.07°C and ± 10 mbars.

An electronic interface (OPTO 22, France) was used for the analog/digital conversion of the signals. A user-written software program was run on a PC-compatible micro-computer for real time data acquisition.

The correction of the volume of released CO_2 took into account the variations in head space volume and wort volume with sampling. Carbon dioxide production rate was calculated by differentiating the volume of released CO_2 and filtered with a moving average technique so as to avoid noise measurement.

Fermentation conditions

For each experiment, 11 litres of the same lager wort (12°P), provided by the Institut Français de Brasserie et Malterie (IFBM, France), were used. The wort was aerated up to saturation before inoculation. To prevent premature yeast flocculation in the fermentor, agitation was applied (100 rpm) using two Rushton impellors separated by 20 cm in the vertical axis. Samples were removed every 12 hours until the fermentation was complete in order to determine: cell number, wort density, FAN (Free Amino Nitrogen), ethanol, higher alcohol and ester concentrations. To prevent the evaporation of aromatic compounds during fermentation, the released CO_2 collector was equipped with an air condensor maintained at a temperature of 0°C. Furthermore, to prevent the evaporation of aromatic compounds during sampling, the fermentation broth was filtered directly through a kieselguhr filter in a cooled flask flushed with a constant flow of CO_2.

The combination of two temperatures (10°C and 16°C) and two top pressures (1.05 and 1.8 bars) were studied (uppersection of Table I). Dissolved CO_2 concentrations were determined with an abacus used in the brewing industry (Fenart-Bouguet, France). At 16°C and 10°C, top pressure was established after 20 hours and 40 hours of fermentation respectively. After this transition period, dissolved CO_2 concentration in the wort was kept constant.

The fermentations were performed one each, except fermentation C (10°C, 1.05 bars) which was duplicated.

Yeast preculture

An industrial lager-type yeast strain, *Saccharomyces cerevisiae var. uvarum*, provided by IFBM (France), was

TABLE I. Final concentrations and kinetic parameters of fermentations performed at different temperatures and top pressures (sugar and FAN consumption, CO_2, ethanol and fusel alcohol production).

	Experiment	A	C*	B	D
	Temperature (°C)	16	10	16	10
	Top pressure (bars)	1.05	1.05	1.8	1.8
	Dissolved carbon dioxide (g/litre)	1.78	2.13	2.98	3.65
Maximum rates	Sugar consumption rate (g/litre/h)	-1.15	-0.56 ± 0.198	-1.18	-0.59
	FAN consumption rate (g/litre/h)	-1.97	-0.88 ± 0.372	-1.51	-0.68
	CO_2 production rate (litre/litre/h)	0.36	0.16 ± 0.04	0.30	0.14
	Fusel alcohol production rate (mg/litre/h)	2.19	1.25 ± 0.66	2	0.72
Final concentrations	Ethanol (% v/v)	4.9	4.99 ± 1.5	5.1	5.0
	Fusel alcohols (mg/litre)	115	102 ± 25.2	120	103

* results of duplicated fermentations (mean ± $t_{(0.975,n-1)}\ \sigma_{n-1}$; with n=2, t=12.7)

used. The wort was inoculated at 5.10^6 cells/mL. Precultures were carried out at 20°C in 5 litre lager wort over 3 days. Temperature was decreased to fermentation temperature (10°C or 16°C) one day before inoculation. Before inoculation, precultures were centrifuged three times at 3500 g and washed with cold saline. A vitality test was performed with 9 g of the preculture yeasts by using the modified acidification power test[11]. This vitality control of the pitching yeasts indicated healthy yeast: Acidification Power (AP) = 2.6 with a 3% of coefficient of variation.

Cells count

Fermentation samples were enumerated with a Coulter counter ZI (Coultronics, France). The number of yeasts $F(l)$ with a diameter smaller than l was determined for a range of values between 1.5 and 10 µm at 0.5 µm intervals. The volume distribution $f(v)$ of yeast with volume v was obtained with Eq. 1:

$$f(v) = \frac{6\,.\,F(l) - F(l + 0.1)}{\pi \quad (l + 0.1)^3 - l^3} \qquad (1)$$

where $v = \frac{\pi\,.\,l^3}{6}$

The experimental volume distributions were fitted to a log-normal curve[3] with a quasi-Newton algorithm (Matlab® 4.2). The total number of micro-organisms, which is the moment of order zero of the volume distribution curve, was calculated.

The relation between number of yeasts (NY in millions cells/mlitre) and dry matter (DM in g/litre) was established previously using a standard method: DM = 0.09171 x NY (R^2 = 0.96).

Analytical methods

Wort density

The density (d) of filtered and degassed wort was determined with a 10 mlitre pycnometer. The real density value obtained was converted to degrees Plato with the following relationship, established using a reference table[7]:

°P = [(1000 x d) – 999,448] / 4.08745

Free amino nitrogen concentration

Free Amino Nitrogen (FAN) was determined using the standard ninhydrin method[1].

Ethanol concentration

Ethanol content was determined by direct injection using a Carlo Erba 5300 GC fitted with a stainless steel column (2 m x 0.3 mm i.d.) coated with Chromosorb 101 (SGE, USA). Experimental conditions were: injection temperature, 220°C; column temperature, 150°C; detector temperature, 220°C. Helium flow rate was 30 mlitre/min. N-propanol was used as the internal standard.

Higher alcohol and ester concentrations

Isobutanol, amyl alcohol, isoamyl alcohol, phenyl ethanol, ethyl acetate, isoamyl acetate, isobutyl acetate, ethyl hexanoate, and ethyl octanoate concentrations were determined, after CS_2 extraction[24], with a Carlo Erba 6000 Vega series GC with split/splitless injector and flame ionization detector. Capillary HP-FFAP column (50 m, 0.32 mm i.d.) (Hewlett Packard, France) coated with PEG (film thickness 0.52 µm) was used. Experimental conditions were: injection temperature, 220°C; column temperature program, held at 35°C for

3 min; then raised to 60°C at 3°C/min; then raised to 230°C at 9°C/min; then finally held at 230°C for 18 min; detector temperature, 220°C. Helium flow rate was 2 mlitre/min. Splitless injection. Benzylic alcohol was used as the internal standard. Octanol was used as the extraction standard. An Apex integrator (Stang Instrument, France) was coupled with the chromatograph.

Repeatability of analytical methods

Analyses were performed in triplicate above all to determine volatile concentrations (after each extraction). Coefficients of variation were always below 10%.

Data processing

In order to estimate initial and maximal rates, the concentration values of substrates, metabolites and biomass were smoothed over the time by the smooth.spline function of Splus3.2 software (Unix) with an appropriate degree of freedom in the range of 5 to 7. The smoothed results provided the concentration values and the corresponding rates for each hour of fermentation.

Analysis of variance was performed with Statgraphics software using the ANOVA table of experimental design procedure.

RESULTS AND DISCUSSION

Since yeast physiology and the biosynthesis of volatiles are affected by dissolved carbon dioxide[20] and not directly by top pressure, results were analysed taking into consideration the two following parameters: temperature and dissolved CO_2 concentrations.

Temporal events of main variable kinetics depend on temperature

Figures 1 and 2 describe the evolution of the main variables and the associated kinetics of the lager fermentation cycle under laboratory conditions. Tables I and III present the corresponding numerical data and the reproducibility of the data. Coefficients of variation were always below 12% for rates and final concentrations.

Whatever the variables considered, times corresponding to maximum production rates depended on temperature, even though the maximum value was controlled by temperature or by top pressure (Fig. 2).

Maximum growth, CO_2 and fusel alcohol production rates were reached at nearly the same time, and earlier at 16°C. Fusel alcohols were produced during sugar and FAN assimilations which occurred at the same time (Fig. 1). Consequently, the fusel alcohol production rate and the fermentation rate were similar. This suggests

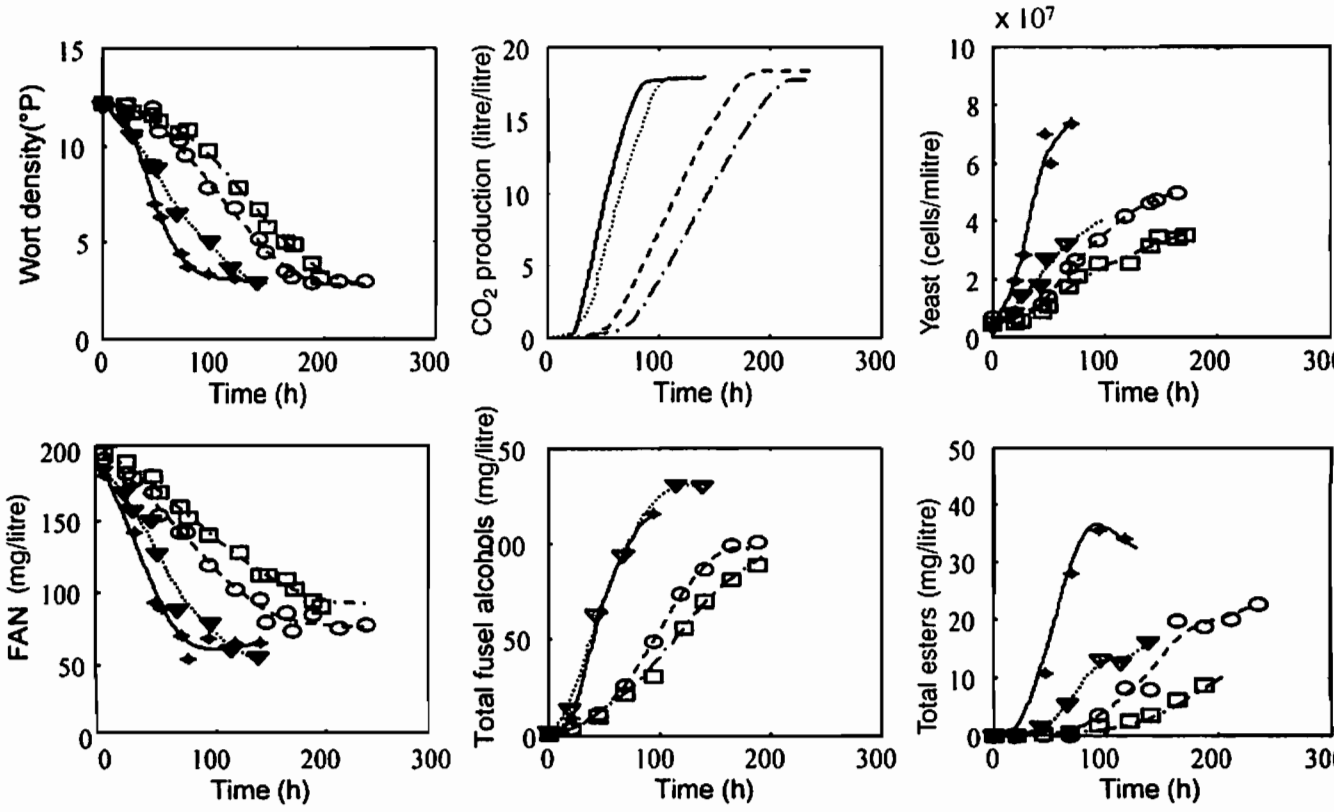

FIG. 1. Influence of temperature and top pressure on evolution over time of wort density (°P), FAN concentration (mg/litre), CO_2 production (litre/litre), yeast concentration (number of cells/mlitre), fusel alcohol and ester concentration (mg/litre).
——•—— experiment A = 16°C, 1.05 bar, [dissolved CO_2] = 1.78 g/litre;
.....▼..... experiment B = 16°C, 1.8 bar, [dissolved CO_2] = 2.98 g/litre;
– – –o– – – experiment C = 10°C, 1.05 bar, [dissolved CO_2] = 2.13 g/litre and
–·–·–□–·–·– experiment D = 10°C, 1.8 bars, [dissolved CO_2] = 3.65 g/litre.

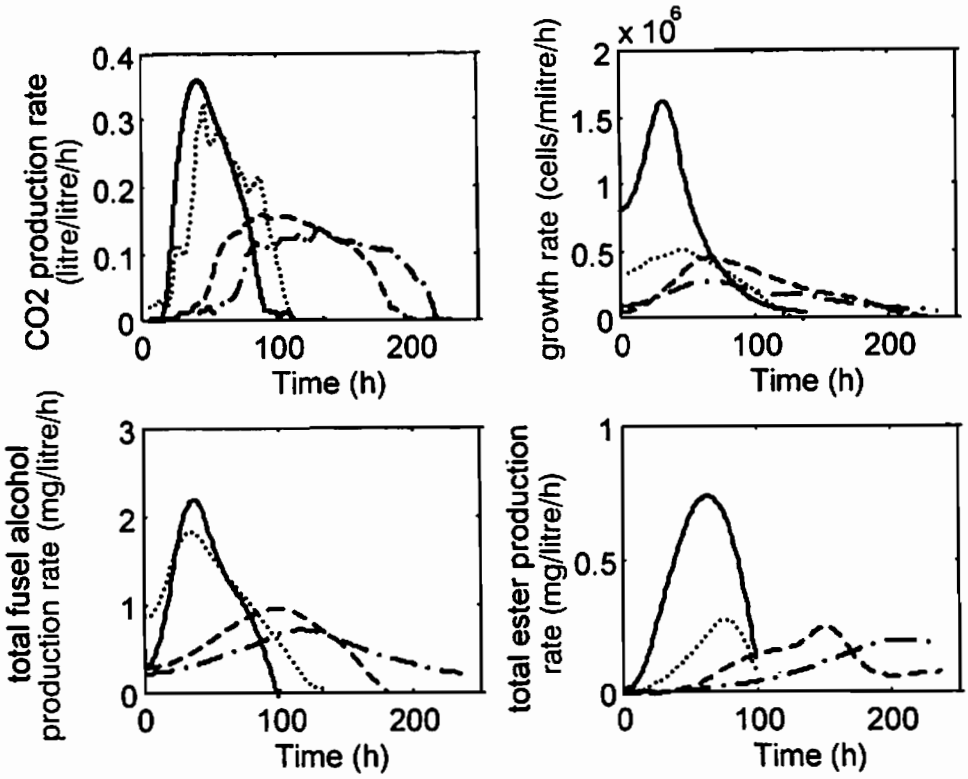

FIG. 2. Influence of temperature and top pressure on the evolution of CO_2 production rate (litre/litre/h), growth rate (number of cells/mlitre/h), fusel alcohol and ester production rate (mg/litre/h) symbols: see Figure 1.

that, in our experiments, FAN concentrations were not a limiting factor for fusel alcohol production[16,21].

Maximum total ester production rates were always reached later than the maximum growth or CO_2 production rates. Ester production rate increased slowly during the first hours and then faster at maximum fermentation rate. A maximum was reached at approximately 80% of total fermentation time, depending consequently on the fermentation temperature.

Whatever the variables considered, the start of production also depended on temperature (Fig. 1). At 16°C, no lagtime was observed for yeast growth, sugar or FAN assimilation and fusel alcohol production whereas, at 10°C, all metabolic reactions were slowed down. The influence of temperature on the start of total ester production is studied in the last section.

Considering maximum or initial rate values and final concentrations, the major effect of temperature on CO_2 and fusel alcohol production and the major effect of dissolved carbon dioxide on growth and ester production, were highlighted. They are examined in the following two sections.

The major effect of temperature on fusel alcohol production

As shown in Table I, maximum fusel alcohol production rates were two fold higher at 16°C than at 10°C. However, final concentrations were only increased 15% by temperature. Analysis of variance confirmed that temperature has a highly significant effect (95% confidence level) on fusel alcohol production rates, through increased sugar and FAN consumption rates, and a significant effect (90% confidence level) on their final concentrations through increased total FAN assimilation (Table II).

Temperature decreased the fermentation period (about 50% less at 16°C) by doubling the CO_2 production rate (Fig. 1 and Fig. 2). The same effect was found for ethanol production and sugar consumption (Table I). Due to a same initial wort density, CO_2 or ethanol versus sugar yields were identical for all experiments (data not shown).

Since fusel alcohols are the product of amino acid and sugar metabolism, it is recognized that parameters such as temperature affecting the assimilation of the latter also control fusel alcohol production[26]. It was observed that the range within which isoamyl alcohol or phenyl ethanol concentrations varied in our study remained above their odour threshold value. The temperature and top pressure studied therefore modified their contribution to beer flavour through a change in the perception of sensory intensity.

It is important to note that the increase in FAN assimilation in experiment B did not lead to an increase in growth. Conflicting results concerning this aspect have been published[9,16]. Moreover, modifications in protein and amino acid content due to different temperatures and CO_2 concentrations have already been described[12,23].

TABLE II. Analysis of variance for kinetic parameters and final concentrations (sugar and FAN consumption, CO_2 and fusel alcohol production).

		Significant level			Estimated effects*		
	Variable	Temperature	Dissolved CO_2	Interaction	Temperature	Dissolved CO_2	Interaction
Maximum rate	Sugar consumption (g/litre/h)	++	-	-	-0.6200	-0.0600	0.0131
	FAN consumption (mg/litre/h)	++	+	-	-0.8331	0.4569	0.2618
	CO_2 production (litre/litre/h)	++	+	-	0.1579	-0.0621	-0.0316
	Fusel alcohol production (mg/litre/h)	++	+	-	0.9984	-0.4716	0.1747
Final concentrations	FAN (mg/litre)	++	-	+	15.5526	0.5526	7.2510
	Fusel alcohols (mg/litre)	+	-	-	16.8209	4.8209	2.9916

++ = effect with p-values of under 0.05 (95% confidence level)
+ = effect with p-values of under 0.1 (90% confidence level)
- = no significant effect
* effects are in the same unit as the corresponding variable

As shown in Figure 1 and Table II, top pressure or CO_2 concentrations have a slight effect on CO_2 production, fusel alcohol production and related substrate assimilation. Effects of CO_2 pressure on these parameters were generally found for higher pressures than those used in this study[19]. Furthermore, it may depend on the sensitivity of the yeast strain used[12].

The major effect of dissolved CO_2 concentrations on total ester production

The link between ester and biomass production

Figures 1 and 2 show that yeasts and ester concentrations evolved similarly depending on CO_2 concentration levels. The increase in dissolved CO_2 concentration from 1.78 g/litre to 2.13 g/litre decreased maximum growth rates, maximal and initial ester production rates three fold (Table III). Besides, biomass produced and final ester concentrations decreased nearly two fold. Analysis of variance corroborated that dissolved CO_2 concentration has a highly significant negative effect on maximum growth rates, maximal and initial ester production rates compared to the temperature effect (Table IV). Furthermore, dissolved CO_2 concentrations had a significant effect on biomass produced and final ester concentrations, whereas temperature did not have a significant effect (Table IV). Effect of temperature on the initial time of ester production is nevertheless significant (Table IV).

Figure 3 shows that maximal growth and ester production rates were linked and decreased drastically for dissolved CO_2 concentrations of over 2 g/litre. Figure 4 gives the relationship between biomass and total ester concentrations with experimental points and the results of the corresponding smoothed values over time. Final total ester concentrations were linearly correlated to the biomass produced ($r = 0.99$, $p = 0.007$) and they both decreased with an increase in the concentration of dissolved CO_2. However, during fermentation, total ester concentrations increased faster than yeast growth.

Considering that dissolved CO_2 has an inhibitory effect on growth and ester production and a less pronounced effect on ethanol (or CO_2) production, their biosynthetic pathways have to be compared. Since acetyl CoA or acyl CoA are involved in lipid synthesis, and considering their essential nature for yeast growth, it can be assumed that acetyl CoA or acyl CoA are common substrates for yeast growth or ester production. On glucose or maltose substrates, acetyl CoA is produced in the cytosol from pyruvate by the PDH bypass involving three different enzymes: pyruvate decarboxylase (EC 4.1.1.1) (three isoenzymes), acetaldehyde dehydrogenase (EC 1.2.1.4) and acetyl CoA synthetase (EC 6.2.1.1) product of the ASC2 gene[30]. Simultaneously, ethanol is provided from pyruvate by pyruvate decarboxylase (EC 4.1.1.1) and alcohol dehydrogenase (EC 1.1.1.1). If dissolved CO_2 affects acetylCoA synthesis through its enzymic biosynthesis, it could only be through acetaldehyde

TABLE III. Final concentrations and kinetic parameters of fermentations performed at different temperatures and top pressures (growth and ester production).

	Experiment	A	C*	B	D
	Temperature (°C)	16	10	16	10
	Top pressure (bars)	1.05	1.05	1.8	1.8
	Dissolved carbon dioxide (g/litre)	1.78	2.13	2.98	3.65
Maximum rates	Growth rate (g/litre/h)	0.17	0.043 ± 0.042	0.048	0.031
	Ester production rate (mg/litre/h)	0.74	0.24 ± 0.168	0.27	0.29
Initial rate	Ester production rate (mg/litre/h)	0.17	0.041 ± 0.03	0.043	0.022
Final concentrations	Biomass (millions cells /mlitre)	73	47.65 ± 36	40.3	35.5
	Total esters (mg/litre)	41	23.7 ± 22.2	16	15

* results of duplicated fermentations (mean ± $t_{(0.975,n-1)}\,\sigma_{n-1}$; with n=2, t=12.7)

TABLE IV. Analysis of variance for kinetic parameters and final concentrations (growth and ester production).

		Significant level			Estimated effects*		
	Variable	Temperature	Dissolved CO_2	Interaction	Temperature	Dissolved CO_2	Interaction
Maximum rate	Growth rate (g/litre/h)	+	++	+	0.0370	-0.1020	-0.0886
	Ester production (mg/litre/h)	+	++	++	0.1133	-0.3367	-0.3977
Initial rate	Ester production (mg/litre/h)	+	++	+	0.0375	-0.1105	-0.0879
Initial time	Ester production (h)	++	-	-	-32.8651	4.1349	-2.5724
Final concentrations	Biomass (millions cells/mlitre)	-	+	-	4.5446	-32.9554	-18.1383
	Total esters (mg/litre)	-	+	-	-0.6654	-21.6654	-17.3971

++ = effect with p-values of under 0.05 (95% confidence level)
+ = effect with p-values of under 0.1 (90% confidence level)
- = no significant effect
* effects are in the same unit as the corresponding variable

dehydrogenase and/or acetyl CoA synthetase activities or through the lack of availability of NAD(P)+. Although, the effect of dissolved CO_2 has already been studied in brewing fermentation[22] and baking yeast production[12], no information has been reported on inhibitory effects on these two enzymes.

In short, an increased CO_2 concentration probably decreases yeast and ester production rates by affecting the acetyl CoA production rate. Consequently, the relationship found between growth and ester rates may be due to a common limiting acetyl CoA production rate. Furthermore, initial ester production rates and

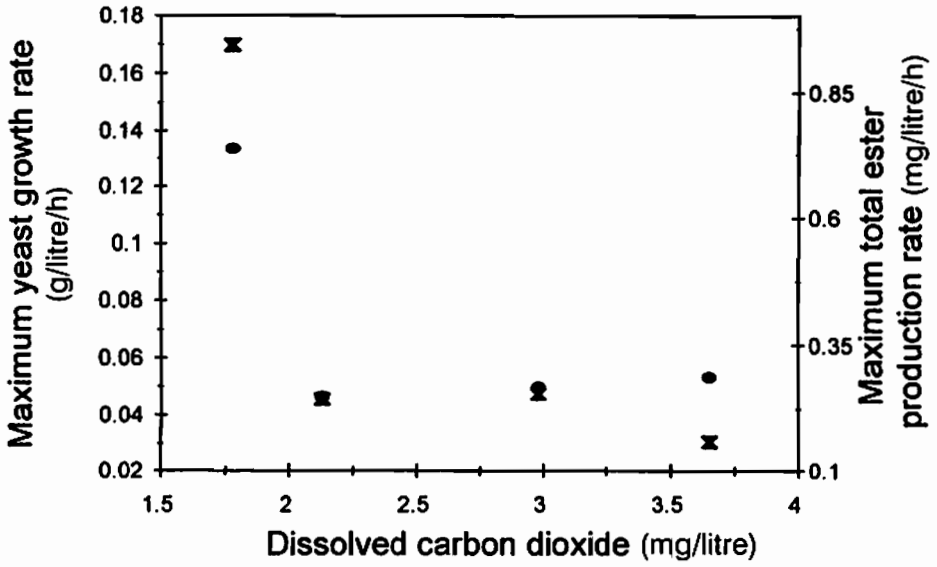

FIG. 3. Decreasing rates with dissolved carbon dioxide concentrations x maximum growth rate (g/litre/h) • maximum ester production rate (mg/litre/h).

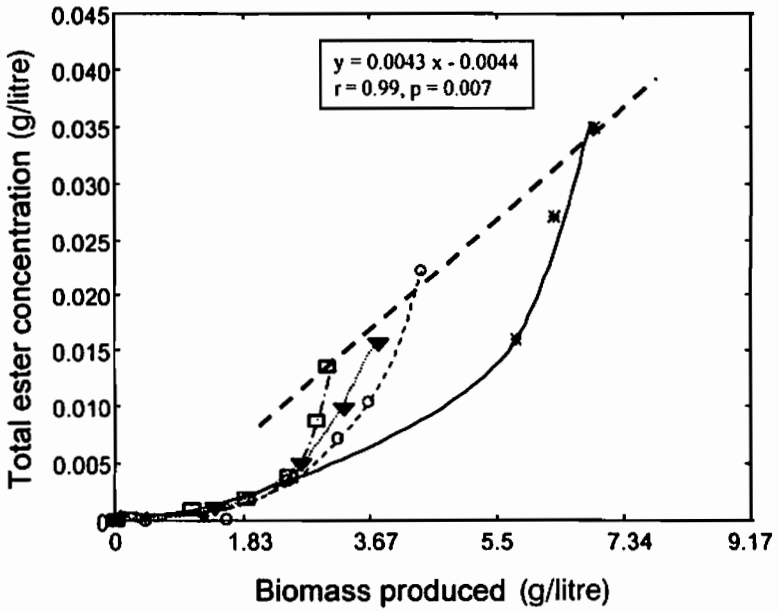

FIG. 4. Variation of total ester and yeast concentrations with time under different fermentation conditions. Relationship between their final concentrations symbols: see Figure 1 ⋆, ▼, O, ❑ : experimental data; – – – smoothed line.

maximum growth rates are correlated (r = 0.999, p = 0.0008) as well as maximum ester production rates and maximum growth rates (r = 0.985, p = 0.0154). Thus, the acetyl CoA production rate would limit the growth and ester rates during the entire process.

A constant yield (0.0043 g/g, with r =0.99, p = 0.007) was found between final ester concentrations and biomass produced (Fig. 4). For 1 g of biomass produced (corresponding to 10.9 million cells produced), 4.3 mg total esters were produced under these experimental conditions. Other fermentations performed at differents pitching values (2.10^7 and 10^7 cells/mlitre) corroborate this result (data not shown). This constant final yield, consistent with previous studies[19], may mean that the total concentration of acetyl CoA produced during the fermentation was split up between esters and biomass at a constant ratio.

In terms of the different esters, dissolved CO_2 concentration had a similar inhibitory effect on the evolution of ethyl acetate, isoamyl acetate and ethyl caproate concentrations over time (Fig. 5). Moreover, a final ethyl acetate/isoamyl acetate concentration ratio of 10 was found in all experiments. Furthermore, it is important to note that, for the same yeast strain, the final

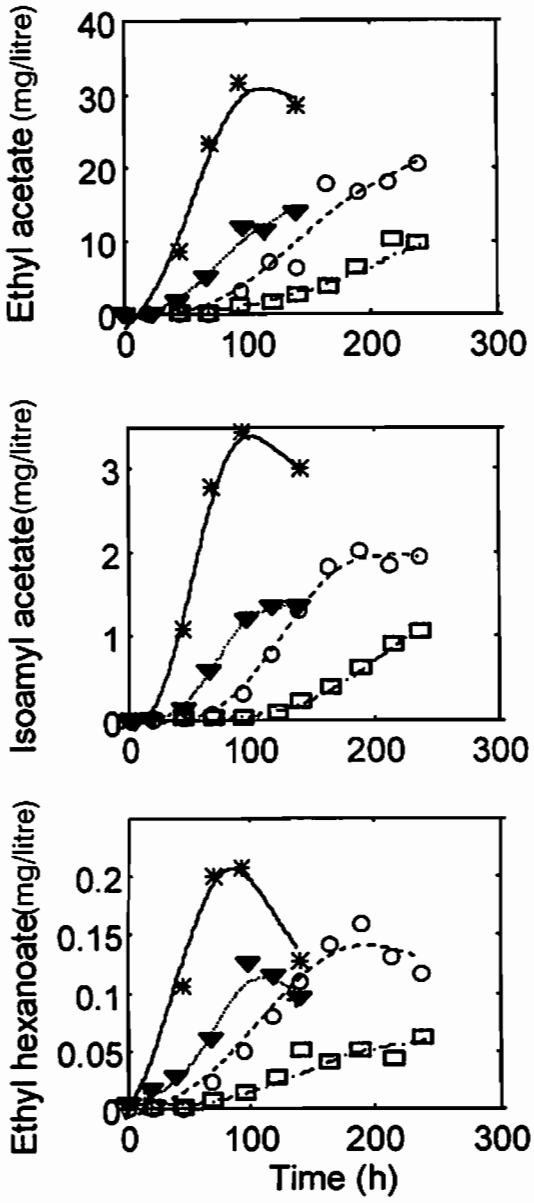

FIG. 5. Variation of ethyl acetate, isoamyl acetate and ethyl hexanoate concentrations with time under different fermentation conditions. symbols : see Figure 1

isoamyl acetate concentration was increased three-fold by a single temperature/top pressure ratio difference.

Ethyl hexanoate exhibited typical behaviour since its concentration showed a maximum value followed by a decreasing phase at the end of the cultures. Suomalainen[25] suggested a higher rate of hydrolysis by esterases for ethyl hexanoate and ethyl octanoate compared to acetate esters.

The link between esters and corresponding fusel alcohols

The relationship between each ester and the corresponding alcohol precursor was considered. Considering ester concentrations against corresponding alcohol precursor concentrations, three phases can be identified: a first phase where alcohol accumulates alone; a second phase where both alcohol and ester accumulate and a third phase where ester accumulates alone. To simplify the analysis, a linear relationship between ester and alcohol was found including second and third phases. Results showed that ester concentration versus precursor alcohol concentration yield varied for each experiment (Fig. 6). These yields were below the dissolved CO_2 concentration negative control (Fig. 7): at the same alcohol concentration, the higher the dissolved CO_2 concentration the lower the ester concentration.

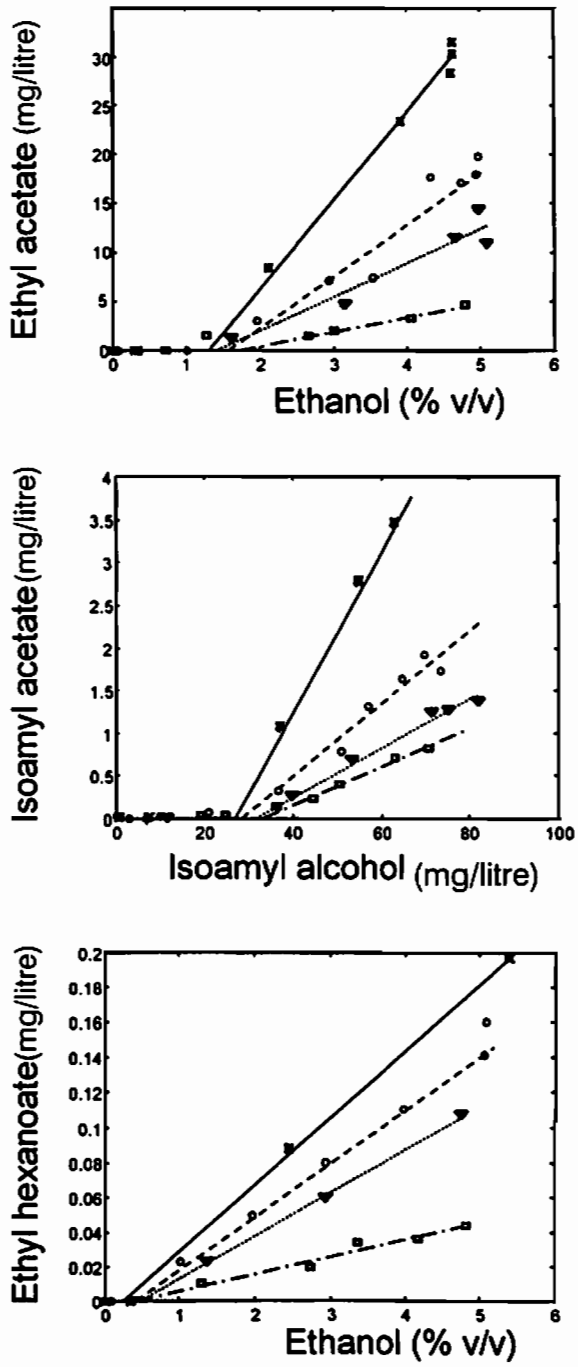

FIG. 6. Relationships between ethyl acetate and ethanol concentration, isoamyl acetate and isoamyl alcohol concentration and ethyl hexanoate and ethanol concentration for increasing levels of dissolved CO_2 concentrations. symbols : see Figure 1

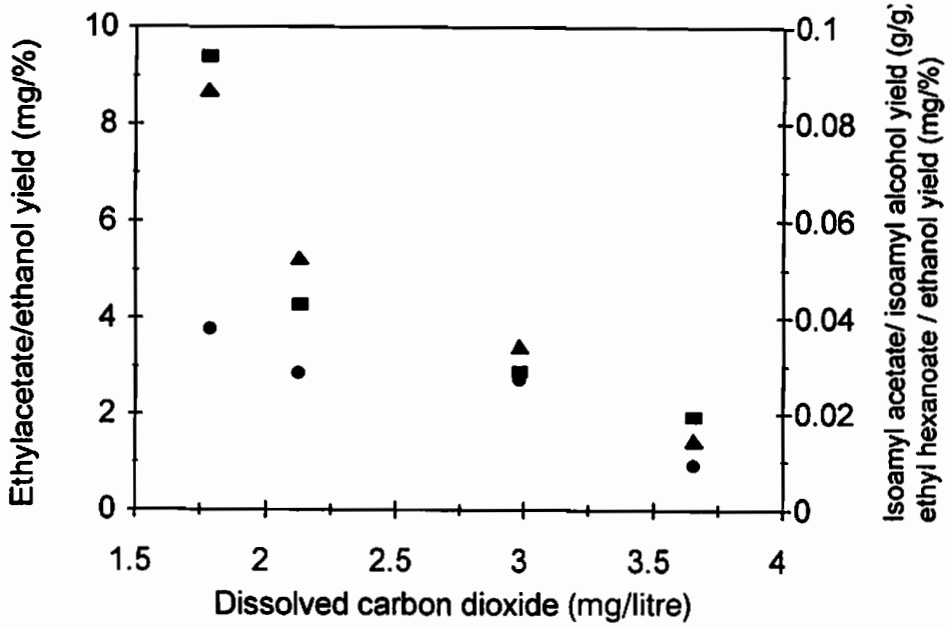

FIG. 7. Decreasing yield ester/alcohol with dissolved carbon dioxide concentrations.
■ ethyl acetate/ethanol yield
+ isoamyl acetate/isoamyl alcohol yield
• ethyl hexanoate/ethanol yield

These results contradict the idea that alcohol concentration may have controlled ester synthesis under these experimental conditions.

The most relevant result was that under limiting CO_2 concentration and in real brewing fermentation conditions, the ester production rate was not controlled by fusel alcohol production rate. Indeed, no correlation was found between ester production rate and fusel alcohol production rate ($r = 0.614$, $p = 0.3862$). Consequently, CO_2 concentrations control yeast growth and the fusel alcohol/ester ratio.

at the same time, while ethyl hexanoate production began earlier. A higher affinity of alcohol acetyl transferase for hexanoyl CoA than for acetyl CoA has already been described[2] which may explain this result. Furthermore, it may be due to a different alcohol acyltransferase enzymes, even if a similar mechanism is involved for acetyl and acyl ester synthesis[13].

Considering that the induction of acetate ester synthesis depends on alcohol acetyl transferase activities[4], these enzyme activities or the repression/induction of corresponding genes could be modified by

TABLE V. Initial time of production for each ester.

	Experiment	A	C	B	D
	Temperature (°C)	16	10	16	10
	Top pressure (bars)	1.05	1.05	1.8	1.8
	Dissolved carbon dioxide (g/litre)	1.78	2.13	2.98	3.65
Initial time of production (h)	Ethyl acetate	20	70 ± 8.5	20	70
	Isoamyl acetate	20	70 ± 7.9	20	70
	Ethyl hexanoate	10	45 ± 9.3	10	45

* results of duplicated fermentations (mean ± $t_{(0.975,n-1)}\ \sigma_{n-1}$; with n=2, t=12.7)

Initial time of ester synthesis

Table V gives the initial time of each ester production. Whatever the ester considered, the initial time of production depended on temperature. Production of ethyl acetate and isoamyl acetate began approximately

temperature more than by dissolved CO_2. Published results have shown that yeast cells change their intracellular fatty acid composition according to environmental conditions, such as incubation temperature, and that AAT activities and genes expression are known to be affected by unsaturated fatty acids[6,10].

CONCLUSION

Previous modelling results[28,29] have emphasized the effect of temperature and dissolved carbon dioxide on aromatic compound production (higher alcohols and esters). Moreover, dissolved carbon dioxide is known to influence microbial growth[19]. Consequently, the main objective of this work was to study the relationship between yeast growth and higher alcohol and ester production. The results obtained underline clearly that if temperature has a general accelerating effect, dissolved carbon dioxide has a negative effect on biomass production as well as on ester production (rates and final concentrations). Furthermore, it was highlighted that dissolved CO_2 has an inhibitory effect on ester production independently of ethanol or higher alcohol production. Consequently, we hypothezise that dissolved CO_2 limits acetyl CoA production through acetaldehyde dehydrogenase and/or acetyl CoA synthetase activities or through the concentration of NAD(P)$^+$. This aspect should be investigated in future works.

Finally, we emphasize that, even on the same wort and with the same yeast strain, beers with a distinctive flavour profil could be produced using an appropiate combination of temperature and top pressure.

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