doi: 10.17221/258/2014-CJFS

Fermentative Activity of Promising Yeasts for Cereal-based Beverages using CO_2 Headspace Analysis

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Abstract

Casanova F.P., Bevilacqua A., Petruzzi L., Sinigaglia M., Corbo M.R. (2015): **Fermentative activity of promising yeasts for cereal-based beverages using CO_2 headspace analysis**. Czech J. Food Sci., 33: 8–12.

This article proposes an approach based on the evaluation of CO_2 produced by *Saccharomyces cerevisiae* var. *boulardii*, *Kluyveromyces lactis* × *Saccharomyces cerevisiae*, *Saccharomyces pastorianus* var. *pastorianus*, *Kazachstania exigua*, as a function of different media (laboratory media with glucose and maltose) and sugars to screen promising yeasts for cereal-based beverages. Data were modelled by the Gompertz equation to estimate the time of metabolic adaptation (λ), the rate of CO_2 production (k_{max}), and the maximum concentration of CO_2 [$(CO_2)_{max}$]. *Kl. lactis* showed the lowest value of $(CO_2)_{max}$, which suggests an "attenuated" metabolic response in the medium containing glucose. *K. exigua* showed a reduced production of CO_2 in the presence of maltose; however, the decrease of $(CO_2)_{max}$ was not related to an increase of λ.

Keywords: carbon dioxide; modelling; metabolic response; Gompertz equation; attenuation

Nowadays, cereals are used for the production of traditional fermented beverages as well as to design new foods with enhanced healthy properties (Blandino *et al.* 2003) for their high content of essential vitamins, dietary fibre, and minerals (Charalampopoulos *et al.* 2002). Unfortunately, the low content of proteins and essential amino acids (lysine), the low starch availability, and anti-nutrients (phytic acid, tannins, and polyphenols) represent a drawback compared to milk and dairy products (Blandino *et al.* 2003). However, fermentation could improve the quality of whole grain and cereal-based products (Gobbetti *et al.* 2010).

A variety of yeasts and bacteria was found in some traditional cereal beverages such as kvass (Wood & Hodge 1985), bouza (Morcos *et al.* 1973), chi-chi (Nicholson 1960), and mahewu (Hesseltine 1979); hereby indigenous microbiota significantly contributes to starch breakdown, acidification, detoxification, and flavour enhancement (Oyedeji *et al.* 2013). Moreover, there is an increasing interest in cereal-based beverages produced by using starter cultures (Zannini *et al.* 2013), thus a focus on the factors that regulate the metabolism of a starter culture is of great concern to optimise the production of this kind of fermented beverages (Blandino *et al.* 2003). An interesting approach relies upon the ability of some microorganisms to produce CO_2 from carbohydrates, as this compound can be assessed in a relatively easy way by some non-destructive and relatively low-cost sensors (Bevilacqua *et al.* 2013). Nowadays the headspace gas analysis is a useful tool for routine analysis in packaged products, such as milk (Bevilacqua *et al.* 2013), sausages (Gøtterup *et al.* 2008), mushrooms (Borchert *et al.* 2014), ready-to-eat salads (Borchert *et al.* 2012), fresh-cut apples (Altisent *et al.* 2014); however no data are available for the production of CO_2 in cereal-based media by potentially beneficial yeasts.

The use of mathematical model is a great challenge in food microbiology to predict and describe microbial growth and inactivation through the use of some primary (cell count over time) and secondary models (effects of pH, temperature, a_w, and other parameters of growth and/or inactivation) (Bevilacqua & Sinigaglia 2010). One of the most important

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growth models is the Gompertz equation, which is based on an assumption that microbial growth follows a sigmoidal trend and could be divided into three phases: lag, exponential, and stationary phases (Zwietering *et al.* 1990). Positive or negative Gompertz function can be also used to model physicochemical parameters (pH, colour, sensory acceptability), thus Gardini *et al.* (1997) used it to model the evolution of CO_2 in the headspace of sealed systems, inoculated with some bacterial pathogens and *Saccharomyces cerevisiae*. This approach was also suitable to model the growth of *Pseudomonas* in laboratory media and in milk (Bevilacqua *et al.* 2013). To the best of our knowledge, little is known about the suitability of this approach to yeasts and to the microorganisms of cereal beverages.

Therefore, the present study was aimed to: (a) investigate CO_2 production by four target strains (*Saccharomyces cerevisiae* var. *boulardii*, *Kluyveromyces lactis* × *Saccharomyces cerevisiae*, *Saccharomyces pastorianus* var. *pastorianus*, *Kazachstania exigua*) in two laboratory media, different in a carbon source (glucose and maltose); (b) evaluate the amount of CO_2 in model systems simulating cereal-based beverages (diluted Malt Extract broth) and model CO_2 through the Gompertz equation to describe this phenomenon by using some simple parameters.

MATERIAL AND METHODS

Strains. Four yeasts were used throughout this research: *Saccharomyces cerevisiae* var. *boulardii* ATCC MYA-796 (GenBank: JQ070086.1) was purchased from the American Type Culture Collection (Manassas, USA), whilst *Kluyveromyces lactis* × *Saccharomyces cerevisiae* DBVPG 6530 (previously known as *Saccharomyces distaticus* and proposed for brewing; Fontana *et al.* 1992), *Saccharomyces pastorianus* var. *pastorianus* DBVPG 6033 (type strain of *Saccharomyces carlsbergensis* E.C. Hansen, isolated from brewery), and *Kazachstania exigua* DBVPG 4384 (previously known as *S. exiguus*, isolated from sea water) were from the Industrial Yeast Collection, University of Perugia (Perugia, Italy).

Media. The following media were used throughout the research: YPG broth (Yeast Peptone Glucose: bacteriological peptone 20 g/l; yeast extract 10 g/l; glucose 20 g/l; all the ingredients were from Oxoid, Milan, Italy); Malt Extract broth (Oxoid), and Malt Extract broth diluted to 15%.

Inoculum preparation. Yeast strains were grown in YPG broth incubated at 25°C for 48–72 h; then, 20 ml of each strain were centrifuged at 1000 *g* for 10 min at 4°C. The supernatant was discarded, and yeast cells were suspended in 2 ml of distilled water (7 log CFU/ml).

CO_2 production. The experiments were performed in glass vials (volume 20 ml; Dani Instruments, Cologno Monzese, Italy) containing 10 ml of media (YPG broth, Malt Extract broth, diluted Malt Extract broth). After yeast inoculation (ca. 5 log CFU/ml), vials were sealed with a butyl cap and a metal ring and stored at 15 and 25°C (for 48–96 h); the content of CO_2 in the headspace (%, v/v) was evaluated through a headspace gas analyser Checkmate II (PBI Dansensor, Ringsted, Denmark). The initial level of yeasts was assessed through spread plating on YPG agar, incubated at 25°C for 72 hours.

The analyses were performed over at least four different batches for each time and sample. CO_2 values were fitted through a positive Gompertz equation, reparameterised by Zwietering *et al.* (1990) and Bevilacqua *et al.* (2013) and cast in the following form:

$$CO_2 = (CO_2)_0 + (CO_2)_{max} \times \exp\left\{-\exp\left\{\left[(k_{max} \times 2.71)\frac{\lambda - time}{(CO_2)_{max}}\right] + 1\right\}\right\}$$

where: $(CO_2)_0$, $(CO_2)_{max}$ (v/v) – initial and the maximum contents of CO_2 in the headspace; k_{max} – rate of CO_2 production in the exponential phase (CO_2/h); λ (h) – time before the beginning of CO_2 production; time – independent variable, i.e. the time of sampling

Statistical analysis. For each parameter, the statistical differences were determined by one- and two-way ANOVA and Tukey's test as the *post-hoc* comparison test ($P < 0.05$). Data analysis and fitting were performed by the STATISTICA software for Windows Ver. 10.0.1011.0 (StatSoft, Inc., Tulsa, USA).

RESULTS AND DISCUSSION

CO_2 production in laboratory media. The starting point of this research was the article of Bevilacqua *et al.* (2013); they proposed a headspace gas analysing approach for the evaluation of the level of *Pseudomonas* spp. in milk. The method is based upon the fact that pseudomonads consume O_2 and produce CO_2, and these changes can be easily evaluated. In the present paper, the amount of CO_2 produced by four yeast strains was assessed in two

doi: 10.17221/258/2014-CJFS

Table 1. Production of CO_2 in the headspace of sealed vials, containing YPG broth or Malt Extract broth at 25°C and 15°C (initial inoculum, 5 log CFU/ml)

Strain	25°C				15°C			
	$(CO_2)_{max}$	k_{max}	λ	R^2	$(CO_2)_{max}$	k_{max}	λ	R^2
YPG broth								
A	62.19 ± 2.06^{b}	5.08 ± 0.28^{b}	6.96 ± 0.48^{a,b}	0.996	62.19 ± 1.43^{a}	2.91 ± 0.34^{bc}	12.01 ± 1.71^{a}	0.998
B	36.11 ± 1.64^{a}	2.00 ± 0.07^{a}	7.39 ± 0.46^{b}	0.998	68.55 ± 4.05^{b}	1.39 ± 0.22^{a}	13.11 ± 3.52^{a}	0.993
C	62.02 ± 0.99^{b}	5.21 ± 0.15^{b}	6.47 ± 0.22^{a,b}	0.999	66.08 ± 0.53^{ab}	2.34 ± 0.09^{b}	12.41 ± 0.64^{a}	0.999
D	64.21 ± 1.65^{b}	5.16 ± 0.23^{b}	6.36 ± 0.34^{a}	0.997	66.79 ± 1.10^{ab}	3.19 ± 0.23^{c}	16.53 ± 0.75^{a}	0.999
Malt extract broth								
A	41.78 ± 1.02^{b}	3.18 ± 0.12^{b,c}	7.47 ± 0.33^{b}	0.999	23.32 ± 0.34^{c}	ns	21.24 ± 0.37^{a}	0.999
B	19.08 ± 1.55^{a}	1.12 ± 0.08^{a}	8.41 ± 0.77^{b}	0.996	7.74 ± 0.57^{a}	0.86 ± 0.24^{a}	20.54 ± 1.24^{a}	0.978
C	45.04 ± 1.34^{b}	2.91 ± 0.09^{c}	7.10 ± 0.32^{b}	0.998	14.92 ± 1.32^{b}	0.83 ± 0.99^{a}	–	0.993
D	18.44 ± 0.80^{a}	1.47 ± 0.13^{b}	4.24 ± 0.51^{a}	0.996	13.88 ± 1.04^{b}	0.82 ± 0.17a	–	0.994

Strain: A – *S. cerevisiae* var. *boulardii*; B – *Kl. lactis*; C – *S. pastorianus*; D – *K. exigua*; ns – not significant; Fitting parameters of Gompertz equation ± standard error; $(CO_2)_{max}$ – maximum concentration of CO_2 (%, v/v); k_{max} – maximum rate of CO_2 production (%/h); λ – time before the beginning of the exponential phase in CO_2 trend (h); $^{a–c}$letters indicate significant differences among yeasts (one-way ANOVA and Tukey's test, $P < 0.05$)

different laboratory media (YPG broth containing glucose, and Malt Extract broth with maltose) or in diluted Malt Extract broth, at 15 and 25°C. The target yeasts were selected on the basis of some beneficial effects on human health reported in the literature such as probiotic activity (*S. cerevisiae* var. *boulardii*), improvement of bioavailability of minerals (*S. pastorianus* and *K. exigua*) and folate biofortification (*Kl. lactis*) (Moslehi-Jenabian *et al.* 2010).

Data were fitted through a common primary model (Gompertz equation) and three fitting parameters were pointed out: lag phase or time of metabolic adaptation (λ), rate of CO_2 production in the exponential phase of metabolism (k_{max}), and the maximum concentration of CO_2 in the headspace [$(CO_2)_{max}$]. The model fitted the experimental data very well, as shown by R^2 values (0.978–0.999) (Table 1); *S. cerevisiae* var. *boulardii*, *S. pastorianus*, and *K. exigua* showed the highest values of $(CO_2)_{max}$ (ca. 60%) in YPG broth at 25°C. Otherwise, the parameters k_{max} (2%/h) and λ (7.39 h), as well as $(CO_2)_{max}$ (36%), suggested an attenuated metabolism in *Kl. lactis*. At 15°C the highest value of $(CO_2)_{max}$ was observed for *Kl. lactis* (68.55%), which also showed the lowest value for k_{max} (1.39%/h). No differences were found for the fitting parameter λ.

S. cerevisiae var. boulardii and *S. pastorianus* showed the highest $(CO_2)_{max}$ (ca. 40%) in Malt Extract broth, although the amount of the gas was lower than in YPG broth. On the other hand, *Kl. lactis* and *K. exigua* experienced lower values of $(CO_2)_{max}$

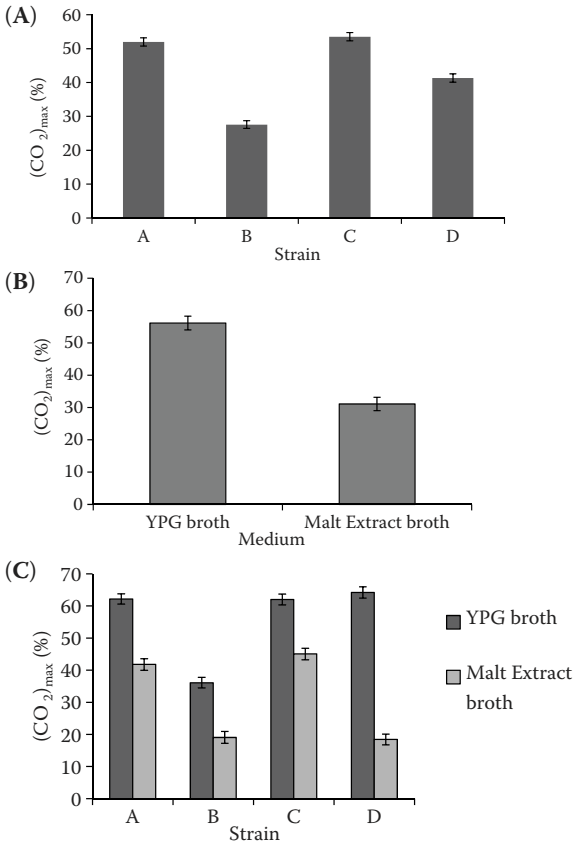

Figure 1. Two-way ANOVA for the effects of strain (**A**), medium (**B**), and strain vs medium (**C**) on $(CO_2)_{max}$ graphs for the decomposition of the effects of the factors; vertical bars denote 95%-confidence; strain A – *S. cerevisiae* var. *boulardii*; B – *Kl. lactis*; C – *S. pastorianus*; D – *K. exigua*

doi: 10.17221/258/2014-CJFS

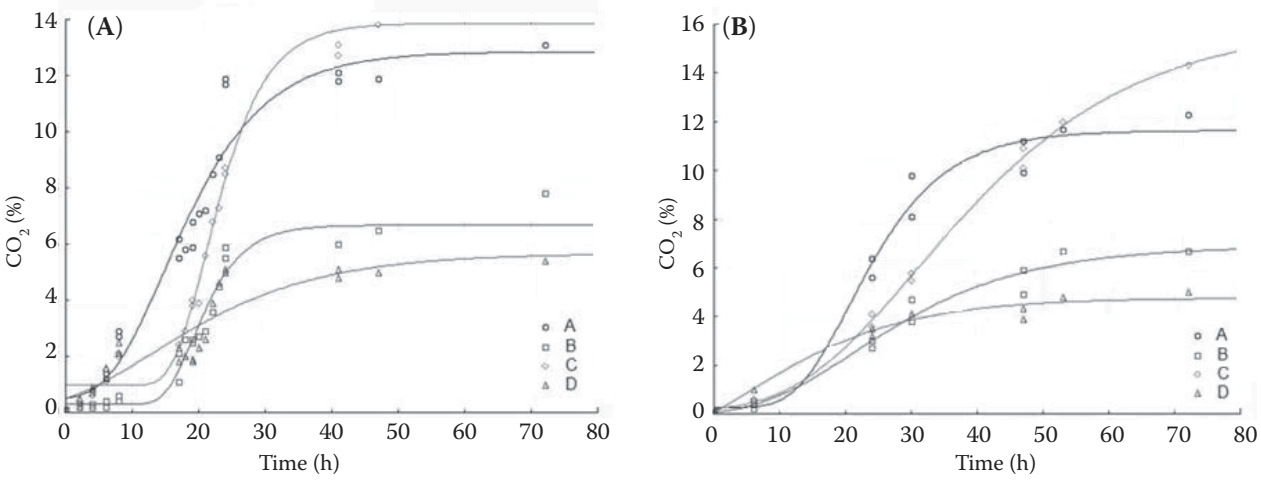

Figure 2. CO_2 production in 15% Malt Extract broth at 25°C (**A**) and 15°C (**B**)

A – *S. cerevisiae* var. *boulardii*; B – *Kl. lactis*; C – *S. pastorianus*; D – *K. exigua*; data point are the mean values of two replicates

(ca. 19%); *K. exigua* showed the lowest value of k_{max} (4.24/h), too.

At 15°C *S. cerevisiae* var. *boulardii* produced the highest concentration of CO_2 (23.32%) followed by *S. pastorianus* and *K. exigua* (14.92–13.88%, respectively), and finally by *Kl. lactis* (7.74%).

The fitting parameter $(CO_2)_{max}$ was analysed by two-way ANOVA and the factor "strain" exerted a strong effect (Figure 1A); moreover maltose caused an attenuation of the metabolic response (Figure 1B), probably related to microbial inability to fully utilise this sugar (Romano *et al.* 2006). Some additional interactive effects of temperature × substrate were also observed (Figure 1C).

CO_2 production in diluted Malt Extract broth. Figure 2 shows the evolution of CO_2 in the headspace of vials containing Malt Extract broth diluted to 15%; this medium was used as a model system to simulate a beverage containing a low amount of sugars. At 25°C the yeasts attained the maximum concentration of CO_2 after ca. 40 h (13% in *S. cerevisiae* var. *boulardii* and *S. pastorianus* and 6% in *Kl. lactis* and *K. exigua*). The lag phase was 6 h for *S. cerevisiae* var. *boulardii* and *K. exigua* and 15 h for *Kl. lactis* and *S. pastorianus*. Yeasts experienced similar trends at 15°C, although the differences for the lag phase (ca. 12–13 h) were not significant ($P > 0.05$).

CONCLUSIONS

This paper proposes a headspace gas analysing approach for the evaluation of CO_2 produced by four yeasts (*S. cerevisiae* var. *boulardii*, *Kl. lactis* × *S. cerevisiae*, *S. pastorianus*, and *K. exigua*) to screen some promising target strains for the production of cereal-based beverages. *Kl. lactis* showed a lower value of $(CO_2)_{max}$ in YPG and Malt Extract broth, whilst *K. exigua* produced a reduced amount of CO_2 in the presence of maltose; however, $(CO_2)_{max}$ was not related to the duration of the lag phase, thus suggesting a partial uncoupling between these parameters.

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Received: 2014–05–13
Accepted after corrections: 2014–09–25

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