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Special Issue Article

Biosynthesis of higher alcohol flavour compounds by the yeast *Saccharomyces cerevisiae*: impact of oxygen availability and responses to glucose pulse in minimal growth medium with leucine as sole nitrogen source

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Abbreviations:

AE, aerated condition; NAE, non-aerated condition; 2MP, 2-methylpropanol (isobutanol); 3MB, 3-methylbutanol (isoamyl alcohol); 2MB, 2-methylbutanol (active amyl alcohol); 2PE, 2-phenylethanol.

Abstract

Higher alcohol formation by yeast is of great interest in the field of fermented beverages. Among them, medium-chain alcohols impact greatly the final flavour profile of alcoholic beverages, even at low concentrations. It is widely accepted that amino acid metabolism in yeasts directly influences higher alcohol formation, especially the catabolism of aromatic and branched-chain amino acids. However, it is not clear how the availability of oxygen and glucose metabolism influence the final higher alcohol levels in fermented beverages. Here, using an industrial Brazilian *cachaça* strain of *Saccharomyces cerevisiae*, we investigated the effect of oxygen limitation and glucose pulse on the accumulation of higher alcohol compounds in batch cultures, with glucose (20 g/l) and leucine (9.8 g/l) as the carbon and nitrogen sources, respectively. Fermentative metabolites and CO₂/O₂ balance were analysed in order to correlate the results with physiological data. Our results show that the accumulation of isoamyl alcohol by yeast is independent of oxygen availability in the medium, depending mainly on leucine, α -keto-acids and/or NADH pools. High-availability leucine experiments showed a novel and unexpected accumulation of isobutanol, active amyl alcohol and 2-phenylethanol, which could be attributed to *de novo* biosynthesis of valine, isoleucine and phenylalanine and subsequent outflow of these pathways. In carbon-exhausted conditions, our results also describe, for the first time, the metabolization of isoamyl alcohol, isobutanol, active amyl alcohol but not of 2-phenylethanol, by yeast strains in stationary phase, suggesting a role for these higher alcohols as carbon source for cell maintenance and/or redox homeostasis during this physiological phase. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: amino acid metabolism; flavour compounds; industrial alcoholic fermentation; Ehrlich pathway; alcoholic beverage

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Introduction

Higher alcohols are aliphatic, medium-chain compounds of four or five carbon atoms with branched

or aromatic side-residues. In the fermented beverages industry, it is well established that the initial amino acid content of fermentation media directly influences higher alcohol production by yeast.

These metabolites constitute an important group of organoleptic compounds, due to their low flavour and taste thresholds (Valero *et al.*, 2002; Lachenmeier *et al.*, 2008), that confer an intensification of alcoholic taste and warming character (Boulton and Quain, 2001). Higher alcohols are produced as a result of branched-chain and aromatic amino acid catabolism through the Ehrlich metabolic pathway (Ehrlich, 1907; Hazelwood *et al.*, 2008). Furthermore, the amino acid precursors can be partly transformed into the corresponding branched-chain carboxylic acids, depending on the redox state of the cell (Dickinson and Dawes, 1992). Once produced, higher alcohols cannot be used as auxiliary carbon sources in central metabolism and are secreted into the medium as such, or after esterification with acetyl CoA. In the specific case of leucine catabolism, the transamination, decarboxylation and subsequent dehydrogenation reactions of the Ehrlich pathway result in the assimilation of leucine's amino group and in the production of isoamyl alcohol. It is widely accepted that isoamyl alcohol should be the only higher alcohol accumulated in yeast cultures under non-limiting carbon conditions, if leucine is the sole nitrogen source (Dickinson *et al.*, 1997). However, in a parallel study, we observed that valine, isoleucine, leucine or phenylalanine as sole nitrogen sources led to unexpected accumulation of higher alcohol compounds, which were not possible to explain through the catabolism of the amino acids present in the medium (unpublished results). In such cases, the relationship between central metabolism and the regulatory biosynthetic mechanisms involved in this unexpected production remain unclear. One hypothesis to explain the production of higher alcohols is a complementary feedback mechanism for fine-tuning yeast cellular redox potential (Quain and Duffield, 1985). With the aim of analysing the relationship of higher alcohol production and central respiro-fermentative metabolism in *S. cerevisiae*, we studied the consequences of oxygen availability and glucose pulsing in aerobic cultures with regard to leucine metabolism and isoamyl alcohol production. To achieve this, we conducted aerobic and micro-aerobic fermentations using an industrial *S. cerevisiae* strain named JP1, which is used in production of *cachaça* (a Brazilian spirit), and the laboratory strain CEN.PK122, in YNB medium with leucine as the sole nitrogen source.

Among the various factors that influence the production of higher alcohols, the use of specific branched-chain amino acid concentrations should lead to the production of differentiated beverages with particular flavours and tastes.

Materials and methods

Yeast strains

Two strains were studied in this work: (a) the *S. cerevisiae* industrial diploid strain designated JP1 (Silva-Filho *et al.*, 2005; Reis *et al.*, 2012), which is commercialized for distilleries of bioethanol and *cachaça* in Brazil under the name Fermol Distiller[®] (AEB Biochemistry Latin America, Brazil); and (b) the prototrophic diploid laboratory strain MATa/ α CEN.PK122 (CEN.PK) (MATa/MAT α URA3/URA3 LEU2/LEU2 TRP1/TRP1 HIS3/HIS3 MAL2-8c/MAL2-8c SUC2/SUC2) (Nijkamp *et al.*, 2012). Clones of these strains were kept in 30% glycerol at -80°C until use.

Medium

YPD medium (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose) was used for the preparation of the pre-inoculum. Batch cultivations were performed in YNB-leucine minimal medium, consisting of yeast nitrogen base medium (YNB; Difco) containing salts, minerals and vitamins without a nitrogen source at 1.7 g/l, supplemented with glucose at 20 g/l and potassium sulphate at 3.63 g/l. Leucine was used as the sole nitrogen source at a concentration of 9.8 g/l (which corresponded to a nitrogen atom concentration of 1.05 g N/l - or 75 mM -, the equivalent nitrogen content of 5 g/l ammonium sulphate). Concentrated solutions of amino acids and YNB were sterilized by filtration through 0.22 μm membranes and transferred aseptically to fermentation vessels.

Cultivation conditions

Fermentations were performed in 2.5 litre cylindrical glass Biostat[®] B Plus bioreactors (Sartorius) with automatic control of temperature, pH and dissolved oxygen concentration through varying the agitation speed and air inflow. The on-line measurements of these parameters, as well as the

on-line determinations of CO₂ and O₂ concentrations in the gas outlet and inlet (see Off-gas analysis), were recorded and monitored on a computer connected to the bioreactor.

The inoculum for the bioreactor was prepared in two steps: (a) a pre-inoculum culture of one isolated yeast clone was incubated overnight in 5 ml YPD medium at 30 °C and 200 rpm orbital agitation; and (b) the inoculum was prepared by adding the pre-inoculum to 200 ml YNB-leucine medium and incubating for 12 h at 30 °C and 200 rpm orbital agitation. This culture was used to inoculate 2 l YNB-leucine medium in a 2.5 l bioreactor.

The fermentations were carried out with automatic control of temperature (30 °C) and pH (5). Oxygen-limited cultures (NAE, non-aerated), were conducted under agitation (400 rpm) without any aeration. In the oxygen-unlimited condition (AE, aerated), the automatic control of dissolved oxygen was set to a minimum of 40% air saturation in order to ensure aerobic conditions throughout the entire fermentation, and was regulated by varying the agitation speed and air inflow, initially fixed at 400 rpm and 1.0 l/min (0.5 VVM), respectively. In order to prevent evaporation, the fermentor was equipped with a condenser in the output gas stream, operated at 2 °C.

Yeast biomass was monitored by evaluating the absorbance of the culture at 620 nm ($A_{620\text{nm}}$). Glucose pulsing was conducted by injecting directly into the fermentor, after 71 h at the stationary phase of the culture, the necessary volume of a sterile preparation to reach a final glucose concentration of 12 g/l in the reactor. Culture samples were taken aseptically and centrifuged at 5000 rpm at 18 °C for 5 min in a benchtop microcentrifuge. The supernatant was collected and stored in 1.5 ml microcentrifuge tubes for analysis of metabolites by high-performance liquid chromatography (HPLC) and gas chromatography (GC).

Analysis of glucose and fermentation products

The concentrations of glucose, ethanol, acetate and glycerol in the fermentation samples were determined by an HPLC apparatus, comprising an automatic injector, refractive index and UV detectors and an AMINEX HPX-87H cation-exchange column (Bio-Rad, USA), with a micro-Guard precolumn (Bio-Rad). The mobile phase was 5 mM sulphuric acid and it was pumped at a flow rate of 0.5 ml/min. Column temperature was regulated

at 70 °C. The sample injection volume was 10 µl. The compounds were identified by their relative retention times and quantified with external standard calibration curves. Results are the average of at least two technical replicates.

Analysis of higher alcohols

The volatile flavour compounds 3-methylbutanol (3MB) or isoamyl alcohol, 2-methylbutanol (2MB) or active amyl alcohol, 2-methylpropanol (2MP) or isobutanol, and 2-phenylethanol (2PE) were extracted at 4 °C by mixing 1 ml culture supernatant with 0.5 ml diethyl ether/octanol (1000:1). After vigorous vortexing and centrifugation at 1000×g for 5 min, 2 µl organic phase were injected into an Agilent 6890N gas chromatograph equipped with a flame ionization detector (FID) and an Agilent HP5 column (30 m length×320 µm diameter, 0.25 µm film thickness). The injector was operated at 250 °C and with a split ratio of 10:1. The carrier gas was helium at a rate of 1.5 ml/min. Initial oven temperature was 50 °C for 1 min, followed by a ramp of 30 °C/min to 250 °C, and then maintained at 250 °C for 5 min. Detection with FID was performed at 270 °C with 30 ml/min hydrogen and 300 ml/min air flow rates. Data acquisition and processing were performed using the Chromeleon Chromatography Management System (Thermo Scientific Dionex). Compounds were identified by comparison of retention times with those of authentic standards and quantified by calibration curves with octanol as internal standard. Standard solutions were prepared by a 10-fold dilution in ultrapure water of 100 mg/l stock solutions of the higher alcohols in ultrapure water:ethanol (7:3). The results are the average of at least two technical replicates.

Off-gas analysis

The exhaust gas from the fermentor was cooled in a condenser (2 °C) in order to minimize the evaporation of ethanol, flavour compounds and water. After drying through silica-filled flasks, the molecular fraction of CO₂, O₂ and N₂ in the gas inlet (every hour) and outlet (every 6 min) from the reactor was determined using a mass spectrometer (PRIMA 600S, VG Gas, Manchester, UK), with a relative precision of 0.1%. The calibration was done with (a) air and (b) a standard gas composed

of CO₂, O₂ and N₂. These measurements were used to calculate the rates of CO₂ production (r_{CO_2} , mM/h) and O₂ consumption (r_{O_2} , mM/h), taking into account the inlet airflow, pressure, temperature and evolution of the reaction volume. The respiratory quotient RQ was calculated as r_{CO_2}/r_{O_2} .

Statistical analysis

The concentrations of higher alcohols, glucose and fermentation products measured in the different assays were analysed using Assistat software (Silva and Azevedo, 2006). A package of tests was used to analyse the normal distribution of data, and a one-way analysis of variance (ANOVA) in a completely randomized design followed by Tukey's test ($p < 0.01$) was used to determine the significant differences of averages. The coefficient of variation in all cases was $< 10\%$.

Results and discussion

In order to determine the principal metabolic responses to leucine with regard to higher alcohol production, JP1 and CEN.PK122 (CEN.PK) *S. cerevisiae* strains were grown in YNB synthetic medium with leucine as the sole nitrogen source, using automated reactors. The CEN.PK laboratory strain, widely used in research studies, was chosen due to its relatively high fermentation performance (Silva-Filho *et al.*, 2005; Nijkamp *et al.*, 2012).

Effect of oxygen availability on fermentation parameters and higher alcohols accumulation

The industrial yeast strain JP1 is widely used in Brazil for *cachaça* and bioethanol production. In order to evaluate the impact of oxygen availability on the biosynthesis of higher alcohol flavour compounds, and particularly of 3-methylbutanol (3MB), the JP1 strain was cultivated in bioreactors with automated process control under aerated (AE, non-oxygen-unlimited; Figure 1A) and non-aerated (NAE, oxygen-limited; Figure 1B) conditions in YNB synthetic medium with leucine as the sole nitrogen source.

No significant difference was observed among the assays with respect to the time evolution of the reaction volume (average and maximum differences

were 1.2% and 2.6%, respectively) and the final biomass obtained after 28 h of culture, allowing for a direct comparative analysis based on concentrations and rates of consumption/production.

The effect of oxygen availability on glucose metabolism by the JP1 strain was characterized in the AE and NAE conditions through O₂ consumption (r_{O_2}) and CO₂ production (r_{CO_2}) rates, calculated by inlet–outlet gas analysis (Figure 1). The continuous increase of oxygen consumption from 0.9 to 4.1 mM/h during glucose assimilation between 7 and 18 h of culture confirmed that JP1 growth was not limited by oxygen supply in the AE condition (Figure 1A).

All throughout this period, glucose metabolism was respiro-fermentative, as denoted by the respiratory quotient (RQ = r_{CO_2}/r_{O_2}) values that were comprised between 3.5 and 7.1, showing that, as expected for Crabtree-positive yeast, JP1 cells

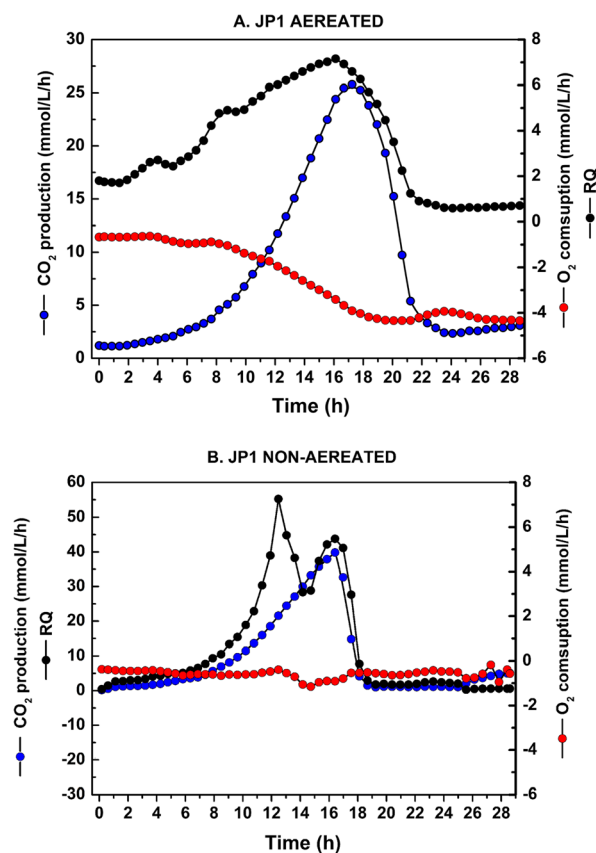


Figure 1. Time evolution of CO₂ production and O₂ consumption rates during aerated (A) and non-aerated (B) batch cultivations of JP1 industrial yeast strain on YNB-leucine medium; RQ, respiratory quotient

preferentially ferment rather than respire glucose, even under strictly aerobic batch-culture conditions. Beyond 22 h the culture entered a respiratory metabolism ($RQ=0.7\pm 0.1$) and started to consume ethanol.

In the NAE assay (Figure 1B), in contrast, r_{O_2} was near zero throughout the entire fermentation (0.6 ± 0.1), validating that the culture was conducted in oxygen-limited conditions. The CO_2 production rate, and consequently the respiratory quotient, reached higher values under NAE conditions ($r_{CO_2 \max}=40$ mm/h; $RQ_{\max}=41\pm 9$, evaluated as mean \pm SD between 12 and 17 h of cultivation) than in the AE culture ($r_{CO_2 \max}=25$ mm/h, $RQ_{\max}=7.1\pm 0.1$).

The time evolutions of glucose, biomass, ethanol, glycerol and acetate during aerated and non-aerated cultures are shown in Figure 2A and B, respectively. After 28 h of cultivation, JP1 yeast

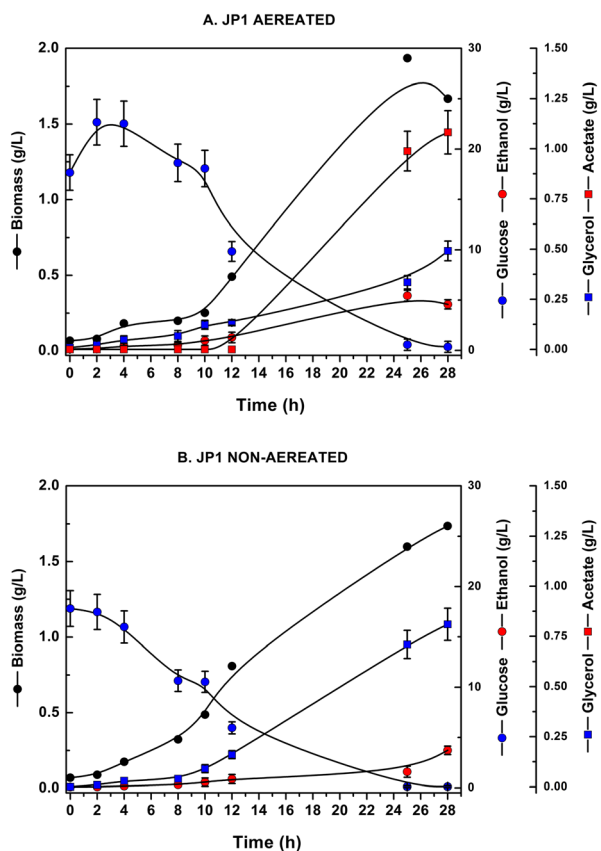


Figure 2. Time evolution of glucose, biomass, ethanol, glycerol and acetate during aerated (A) and non-aerated (B) batch cultivation of JP1 industrial yeast strain on YNB-leucine medium

strain consumed all the initial glucose in both aeration conditions. While no growth lag phase was observed in the NAE condition, the AE culture presented a short initial lag of around 8 h. Although the generation time was 1.7 times higher during the exponential phase of the NAE culture, the final biomass production and overall yield of biomass on glucose were not significantly different between the two assays. The final ethanol concentration was 43% higher in the AE culture, leading to a 50% increase in the overall ethanol yield on glucose, and consequently in the fermentation efficiency as well. Acetate accumulation was only observed under this respiro-fermentative condition, resulting in a final concentration of 1.1 ± 0.1 g/l and an overall yield on glucose of 6%. Glycerol production was coupled to growth in both assays but was strongly favoured in the NAE condition, with a 3.2-fold higher concentration and a 1.7-fold higher yield on glucose. This is in accordance with one of the known major physiological roles of glycerol biosynthesis in *S. cerevisiae*, which is to maintain the redox balance by reoxidizing the NADH produced in oxygen-limited conditions by the synthesis of ethanol, biomass and other secondary metabolites during the fermentative metabolism of glucose (Rodrigues *et al.*, 2006). This role is reduced under aerobic conditions, where respiration is the main NADH-reoxidation process in glucose respiro-fermentative metabolism.

The concentrations of higher alcohol flavour compounds showed no significant differences between the aerated and non-aerated cultivations of JP1 yeast strain at the end of glucose fermentation in YNB-leucine medium (Figure 3A, B). The corresponding leucine catabolism product, 3-methylbutanol (also known as isoamyl alcohol or isopentyl alcohol), was the most abundant higher alcohol accumulated by JP1 yeast strain, reaching 532 ± 37 mg/l (6.0 ± 0.4 mM) and 578 ± 40 mg/l (6.6 ± 0.5 mM) after 28 h of cultivation in the AE and NAE conditions, respectively. As shown by these results, the overall production rate of 3MB by leucine degradation through the Ehrlich pathway was not affected by oxygen availability during the fermentation. Moreover, 3MB accumulation paralleled yeast growth and stopped after glucose exhaustion, despite the presence of residual leucine, suggesting that leucine degradation through the Ehrlich pathway was strongly dependent on cytosolic availability of the amino-acceptor α -

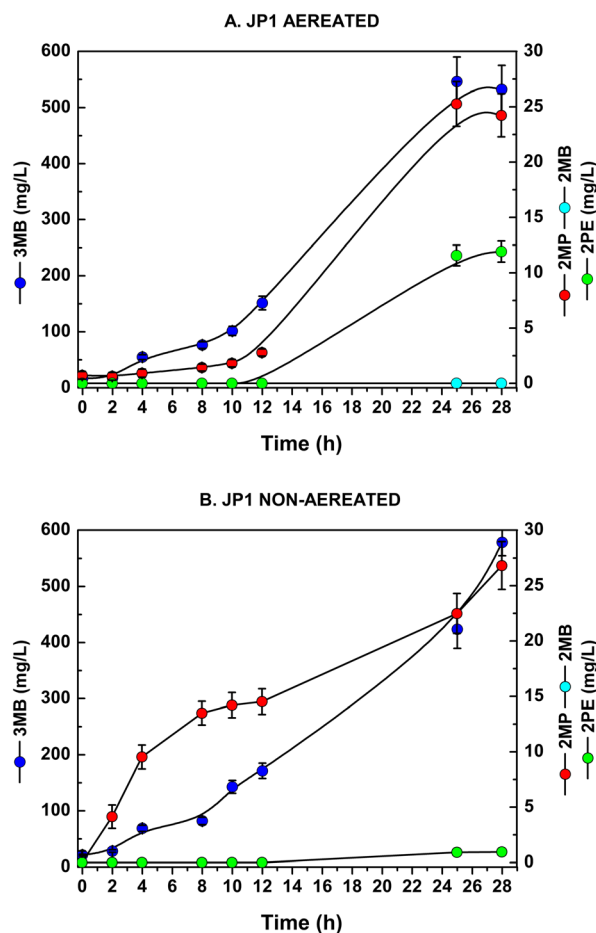


Figure 3. Time course of higher alcohols accumulation by JP1 industrial yeast strain during aerated (A) and non-aerated (B) batch cultures on YNB-leucine medium; 2MP, 2-methylpropanol; 2MB, 2-methylbutanol; 3MB, 3-methylbutanol; 2PE, 2-phenylethanol

ketoglutarate and/or of NADH co-factor (produced mainly by glycolysis during glucose fermentation), which are necessary reactants for the transamination and the reduction steps, respectively.

Although leucine was the sole nitrogen source present in the cultivation medium (metabolized through the catabolic Ehrlich pathway into glutamate and 3-methylbutanol), other unexpected higher alcohols, such as 2-methylpropanol and 2-phenylethanol, accumulated in both conditions, the first compound showing a parallel profile to the biomass/3MB time evolution, and the latter mainly in the aerated culture. In a parallel study, we similarly detected unpredicted levels of higher alcohols that did not derive from the amino acid

present as sole nitrogen source in the cultivation medium (Espinosa Vidal *et al.*, 2014, unpublished results). In this study we suggested that this production could be a result of *de novo* biosynthesis from the glycolysis products phosphoenolpyruvate and pyruvate, through the anabolic pathways of the corresponding amino acids, which could partly go to the higher alcohol from the α -keto-acid branch point. With the aim of testing this hypothesis, and to obtain further insight into the glycolysis dependence of the Ehrlich pathway, we conducted glucose pulse experiments in aerobic long-term batch cultures containing YNB mineral medium with leucine as the sole nitrogen source.

Glucose pulsing during prolonged culture with leucine as sole nitrogen source

To determine the relationship between 3MB production and central carbon metabolism, glucose pulsing was performed in aerobic-stationary phase cultures. For this, kinetic profiles of industrial strain JP1 were compared with those of the laboratory strain CEN.PK. Figures 4 and 5 show the production of principal metabolites and higher alcohols by these strains during 114 h of fermentation. A glucose pulse was applied to obtain a final concentration in the reactor of 12 g/l during the stationary phase at 71 h of growth. At the beginning of the culture, a first glucose phase of assimilation was observed until 24 h of growth. This corresponded to 75% of total glucose assimilation, resulting in maximal ethanol production of 3.84 g/l for CEN.PK and 5.02 g/l for JP1 in 30 h. Then, subsequent to this first glucose uptake period, a diauxic shift phase was observed for both strains, albeit with different intervals. This diauxic shift was indicated by ethanol assimilation from 24 h to 42 h for the CEN.PK strain (Figure 5A), while for the JP1 strain the diauxic shift interval covered 34 h to 58 h (Figure 4A). The yeast generation rate was 37% faster for the CEN.PK strain than for the JP1 strain during the glucose assimilation phase, and 16% higher during the ethanol assimilation phase.

With regard to the principal metabolites, acetic acid, glycerol and ethanol production were measured for the two strains and some differences were found in terms of concentration. For

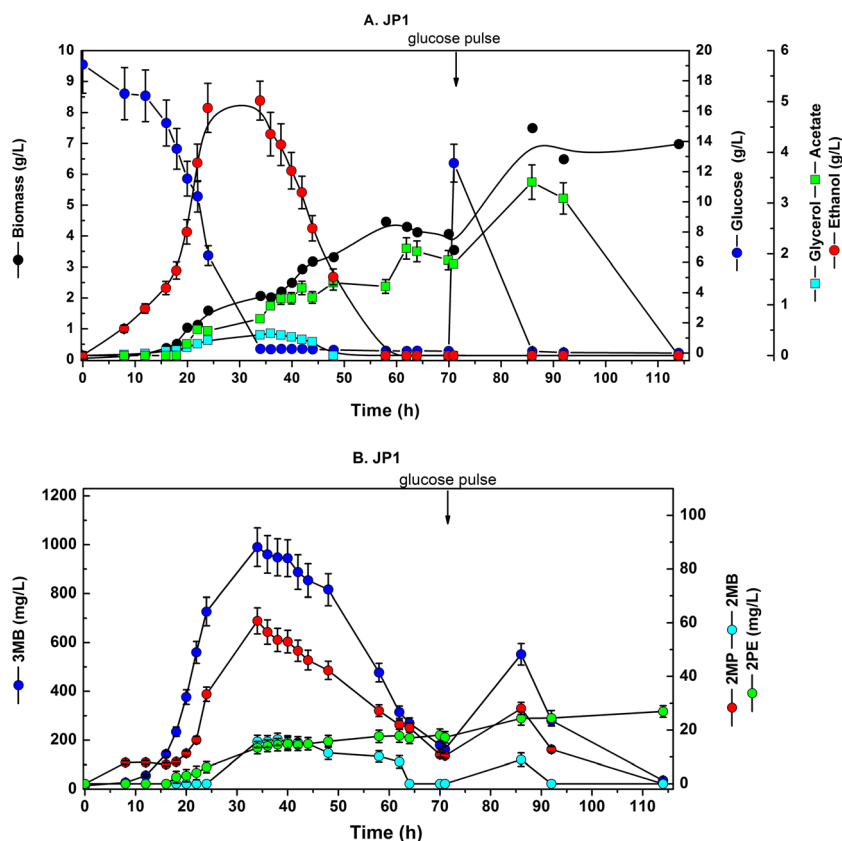


Figure 4. Time evolution of fermentation parameters (A) and higher alcohols accumulation (B) by JP1 industrial yeast strain during aerated long-term batch cultures on YNB-leucine medium. Glucose pulse injection (12 g/l) was performed at $t=71$ h; 2MP, 2-methylpropanol; 2MB, 2-methylbutanol; 3MB, 3-methylbutanol; 2PE, 2-phenylethanol

example, JP1 initiated the acetic acid production at 20 h, and this continued to accompany the biomass curve, with a maximum value of 2.05 g/l at 64 h. In contrast, CEN.PK cell cultures started acetic acid production at 35 h, reaching a maximal production at 42 h, then assimilated it at nearly the same rate as during production (Figure 5A). In general, the acetic acid concentration was higher (and increased constantly) in JP1 than in CEN.PK culture, being most evident between 35 and 64 h of cultivation. The culture of CEN.PK strain showed a third growth phase, spanning from 37 to 47 h, which can be related to acetic acid assimilation (Figures 5A). In relation to the other compounds, the results displayed a higher JP1 culture production, especially in the interval 22 to 44 h, with 5.4 times more glycerol (at 44 h) and five-fold more ethanol produced (at 40 h), respectively.

The glucose pulse (12 g/l) applied at 71 h of fermentation produced different responses in metabolite profile, depending on the strain under consideration. In both cultures, glucose was almost totally consumed in the next 15 h, with no discernable change in ethanol and glycerol levels (Figures 4A and 5A). In contrast, acetic acid levels increased dramatically in both cultures, reaching similar maximum values in the range of 3.5 g/l during the following 15 h. This is related to the total glucose consumed and was accompanied by an increase in biomass until 92 h.

With respect to the biosynthesis of higher alcohol flavour compounds (Figures 4B and 5B), 3MB accumulation paralleled yeast growth, as previously noted in this study (Figures 2B and 3B). JP1 culture achieved an overall maximum 3MB-specific productivity of $160 \mu\text{M/g dry cell/h}$ at 34 h, while CEN.PK reached $126 \mu\text{M/g dry cell/h}$ at the same time. From this moment, the

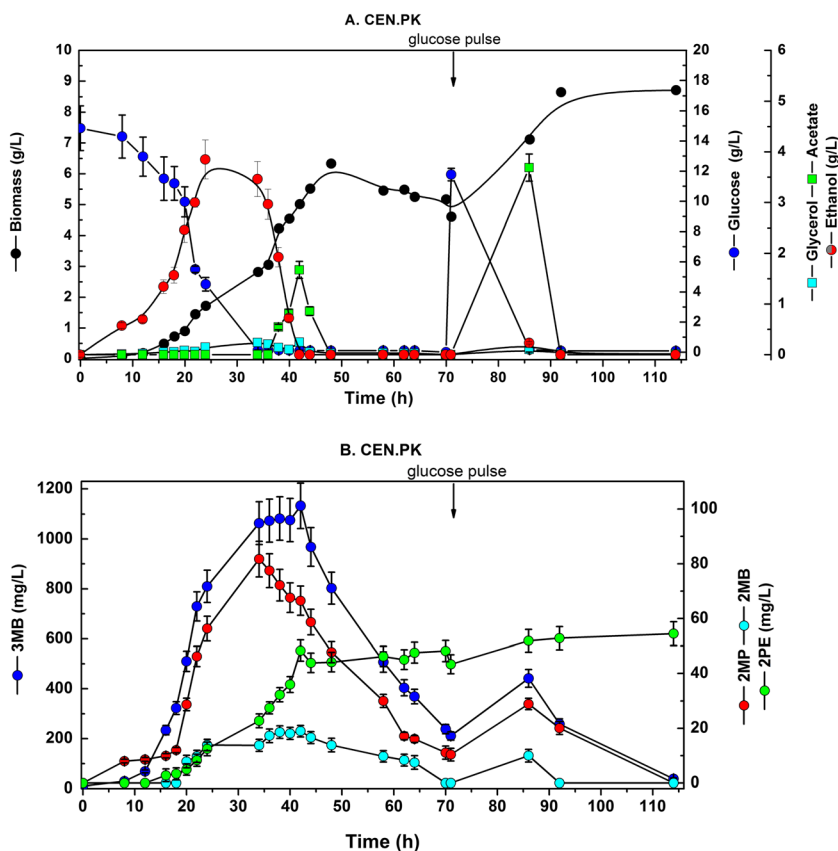


Figure 5. Time evolution of fermentation parameters (A) and higher alcohols accumulation (B) by CEN.PK yeast strain during aerated long-term batch cultures on YNB-leucine medium. Glucose pulse injection (12 g/l) was performed at $t = 71$ h; 2MP, 2-methylpropanol; 2MB, 2-methylbutanol; 3MB, 3-methylbutanol; 2PE, 2-phenylethanol

concentration of higher alcohols, with the exception of 2PE, decreased regularly in one or more phases differentiated by varying reduction rates, depending on the strain and the flavour compound. This phenomenon could not be attributed solely to evaporation, since the exhaust of volatile compounds through the off-gas was strongly limited by the low temperature (2°C) and high efficiency of the condenser in the output gas stream. The results show a maximum net consumption of higher alcohols by yeast metabolism during the stationary phases (58–70 and 86–114 h), with the exception of 2PE, whose accumulation was only reduced during these periods. JP1 strain appeared to metabolize 2MB and 3MB at a slower rate during growth on ethanol (34–48 h) after the diauxic shift, while the concentration of these flavour compounds remained nearly constant in the same growth phase for the CEN.PK culture. 2MP did not show this variation and presented similar

consumption rates for both strains during growth on ethanol (and eventually also on acetate in the case of CEN.PK). As neither growth nor ester formation were observed concomitantly with the depletion of higher alcohols, their metabolism by yeast might play a role in the maintenance of the redox balance through reduction of NAD^+ , while oxidizing the higher alcohols into their corresponding aldehydes in the opposite direction to the final reductive step of the Ehrlich pathway, and could potentially be used as carbon sources after further oxidation to the corresponding acid and assimilation by β -oxidation.

While all higher alcohol flavour compounds were consumed by JP1 and CEN.PK during the first stationary phase (58–70 h), with the exception of 2PE, whose accumulation was only reduced during this period, glucose addition to the cultures at $t = 71$ h induced a rapid accumulation of these secondary metabolites (Figures 4B and 5B) by

both yeast strains. Owing to the residual leucine concentration, glucose metabolism re-established 3MB biosynthesis through the Ehrlich catabolic pathway, thanks to the recycling of the amino acceptor α -ketoglutarate, necessary for the transamination reaction, and/or recycling of NADH co-factor, required for the reduction step. With respect to 2MP, 2MB and 2PE, after the consumption of 2MP and 2MB during the first stationary phase, the glucose pulse induced their positive accumulation as well as a significant increase in 2PE biosynthesis. These results suggest that these flavour metabolites were essentially biosynthesized *de novo* as leakages from the anabolic pathways of valine, isoleucine and phenylalanine, respectively, at the α -keto-acid branch point. As in the first part of the culture, the exhaustion of the carbon sources after the glucose pulse was followed by growth arrest and consumption of the higher alcohols 2MP, 2MB and 3MB, but not of 2PE, whose accumulation continued at a lower rate; the biosynthesis of the latter compound could be originated by Ehrlich catabolism of free phenylalanine released through yeast autolysis. The fact that only 2PE accumulation was observed during the stationary phases might be explained by low NADH co-factor availability for the reduction step of the Ehrlich pathway, as well as by leucine induction of *ARO10* (Boer *et al.*, 2007), together with the higher biocatalytic efficiency for phenylpyruvate of Aro10p decarboxylase. This efficiency is around 30, 70 and 160 times lower for α -keto-isocaproate, α -keto-3-methylvalerate and α -keto-isovalerate, being the α -keto-acid precursors of 3MB, 2MB and 2MP, respectively (Romagnoli *et al.*, 2012).

Conclusion

Higher alcohol flavour compounds quantified in aerated and non-aerated cultivations of JP1 yeast strain in YNB-leucine medium showed no significant differences in terms of concentration at the end of glucose fermentation, indicating that the Ehrlich pathway was not affected by oxygen availability during cell growth. The corresponding leucine catabolism product, 3MB, was the main higher alcohol produced by this strain; its accumulation paralleled yeast growth and stopped after glucose exhaustion, despite the presence of

residual leucine, suggesting that leucine degradation through the Ehrlich pathway was strongly dependent on cytosolic availability of NADH and/or of the amino acceptor α -ketoglutarate. This was confirmed by glucose pulse experiments, performed in stationary phase, that showed a rapid accumulation of the higher alcohol flavour metabolites 2MP, 2MB, 3MB and 2PE by both JP1 and CEN.PK yeast strains. Due to the residual leucine concentration, glucose metabolism re-established 3MB biosynthesis through the Ehrlich catabolic pathway by recycling of NADH co-factor and/or of the amino acceptor α -ketoglutarate. After consumption of 2MP and 2MB during the stationary phase preceding the glucose pulse, this latter induced their positive accumulation, as well as a significant increase in 2PE biosynthesis. This suggests that 2MP, 2MB and 2PE were essentially biosynthesized *de novo* as outflows from the valine, isoleucine and phenylalanine anabolic pathways, respectively.

Moreover, our results describe for the first time the metabolization by JP1 and CEN.PK yeast strains of 2MP, 2MB and 3MB flavour compounds. This conversion was determined during stationary phase, after exhaustion of glucose, ethanol or acetate carbon sources in long-term batch cultures, using minimal medium with glucose as the initial carbon source and leucine as the sole nitrogen source. This was not the case of 2PE, whose accumulation rate was only reduced during this period. As neither growth nor ester formation were observed concomitantly with the depletion of 2MP, 2MB and 3MB, their metabolism by yeast might serve as carbon source and/or play a role in the maintenance of the redox balance. Further studies are needed to fully understand the underlying mechanisms in order to control and optimize the accumulation of higher alcohol flavour compounds in the frame of industrial applications exploiting microorganisms such as yeasts as cellular biocatalysts.

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