Figuras referentes à Revisão Bibliográfica

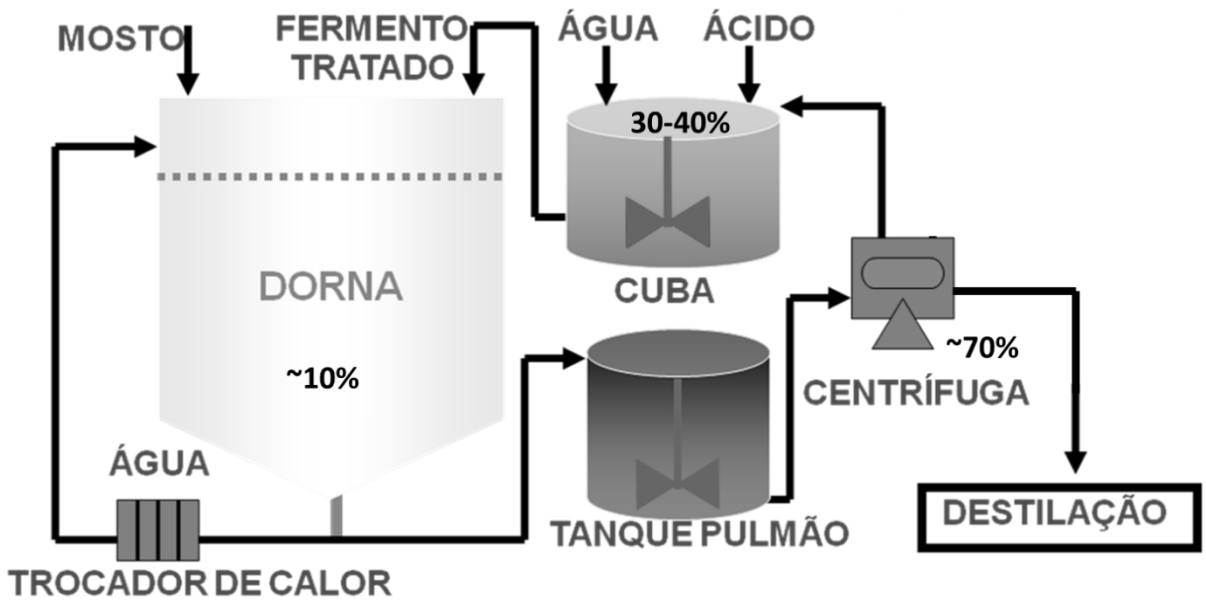

Figura 1 – Esquema do processo de produção de etanol em sistema de batelada com reciclo da levedura. Os valores em destaque correspondem à concentração média de células de leveduras em cada etapa
Fonte: Adaptado de Ferreira (2008)

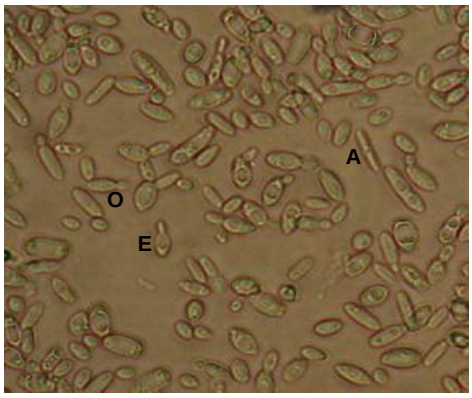

Figura 2 – Morfologia das células da levedura *Dekkera bruxellensis,* apresentando célula alongadas (A), esferoidais (E) e ogivais (O), em meio YPD. Aumento de 400X ao microscópio

Fonte: Arquivo pessoal (Sandra Regina Ceccato-Antonini)

Figuras referentes ao Capítulo 1

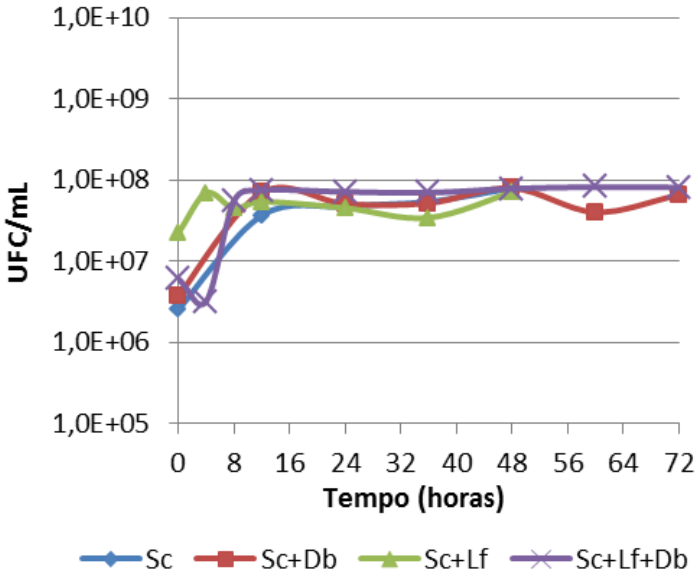

Figura 1 – Crescimento de *S. cerevisiae* (Sc) em meio de caldo de cana, a 30 ºC, 160 rpm, em cultura pura e em co-cultura com *D. bruxellensis* (Db) e ou *L. fermentum* (Lf)

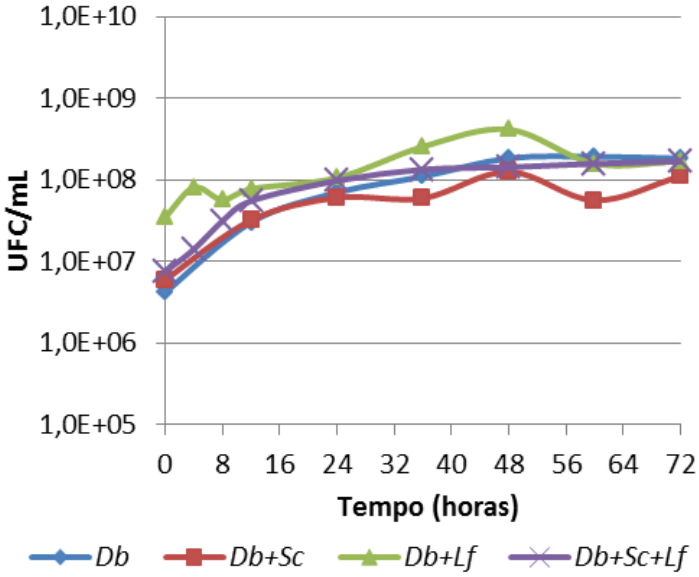

Figura 2 – Crescimento de *D. bruxellensis* (Db) em meio de caldo de cana, a 30 ºC, 160 rpm, em cultura pura e em co-cultura com *S. cerevisiae* (Sc) e ou *L. fermentum* (Lf)

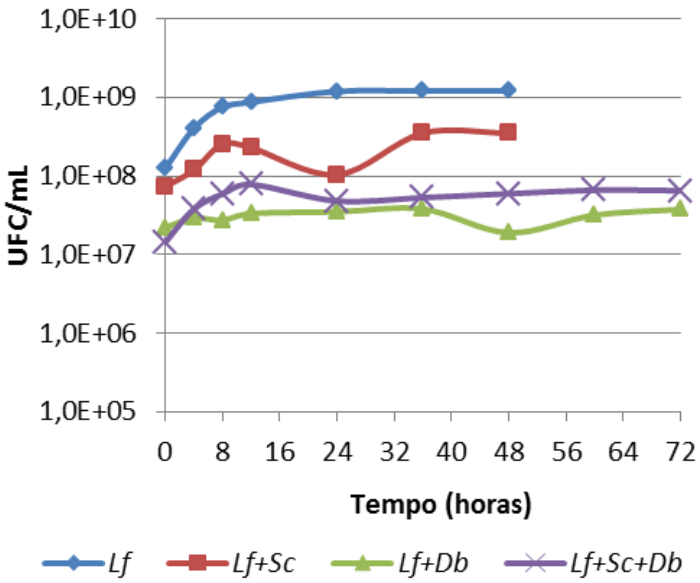

Figura 3 – Crescimento de *L. fermentum* (Lf) em meio de caldo de cana, a 30 ºC, 160 rpm, em cultura pura e em co-cultura com *S. cerevisiae* (Sc) e ou *D. bruxellensis* (Db)

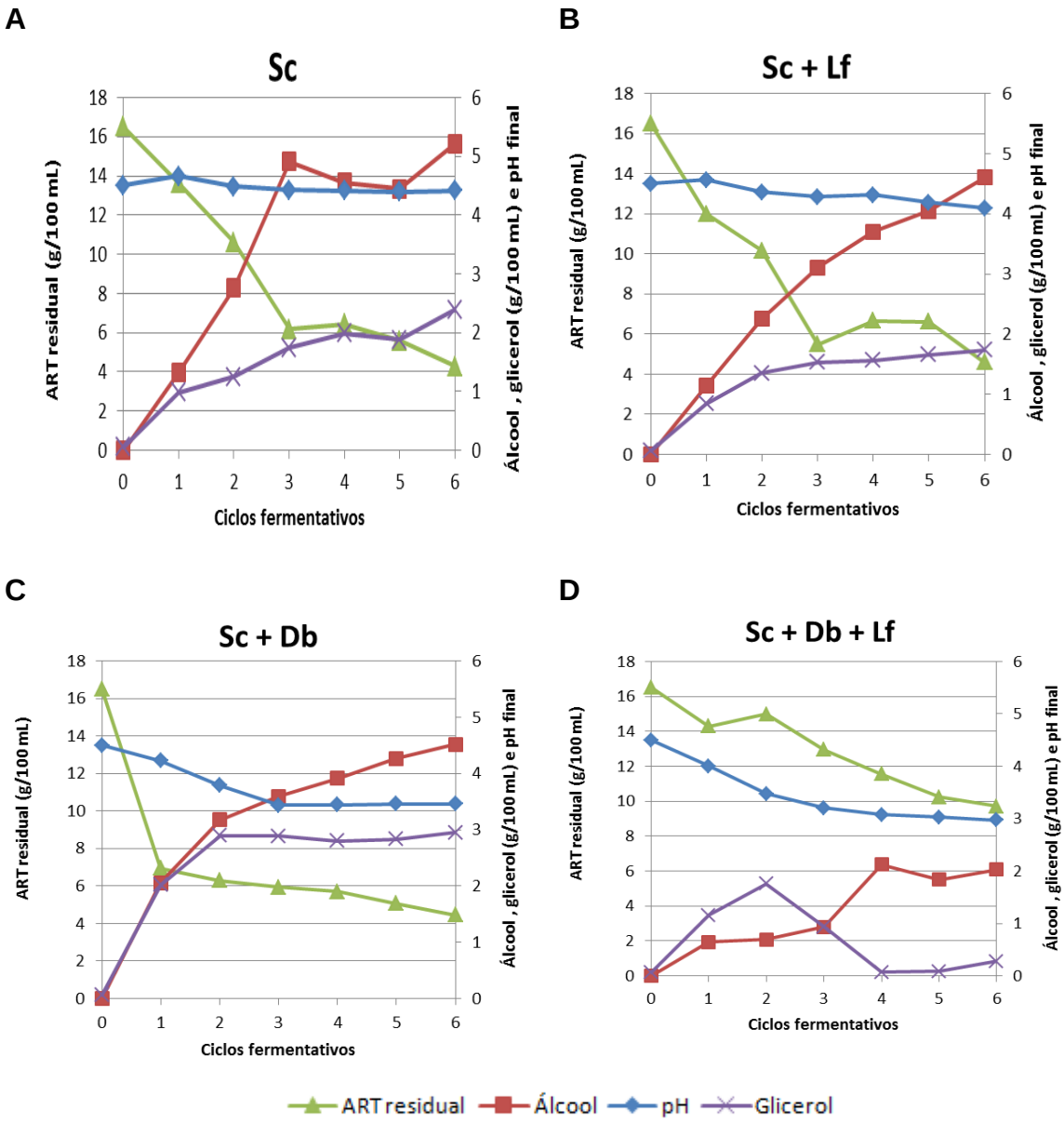

Figura 4 – pH final e concentrações de açúcar redutor total (ART) residual, glicerol e álcool nas fermentações desenvolvidas em caldo de cana, com a cultura pura de *S. cerevisiae* (Sc) e contaminadas com *D. bruxellensis* (Db) e ou *L. fermentum* (Lf), ao longo de seis ciclos fermentativos de 12 horas, a 30 ºC. Ciclo 0 refere-se aos resultados no início do 1º. ciclo fermentativo

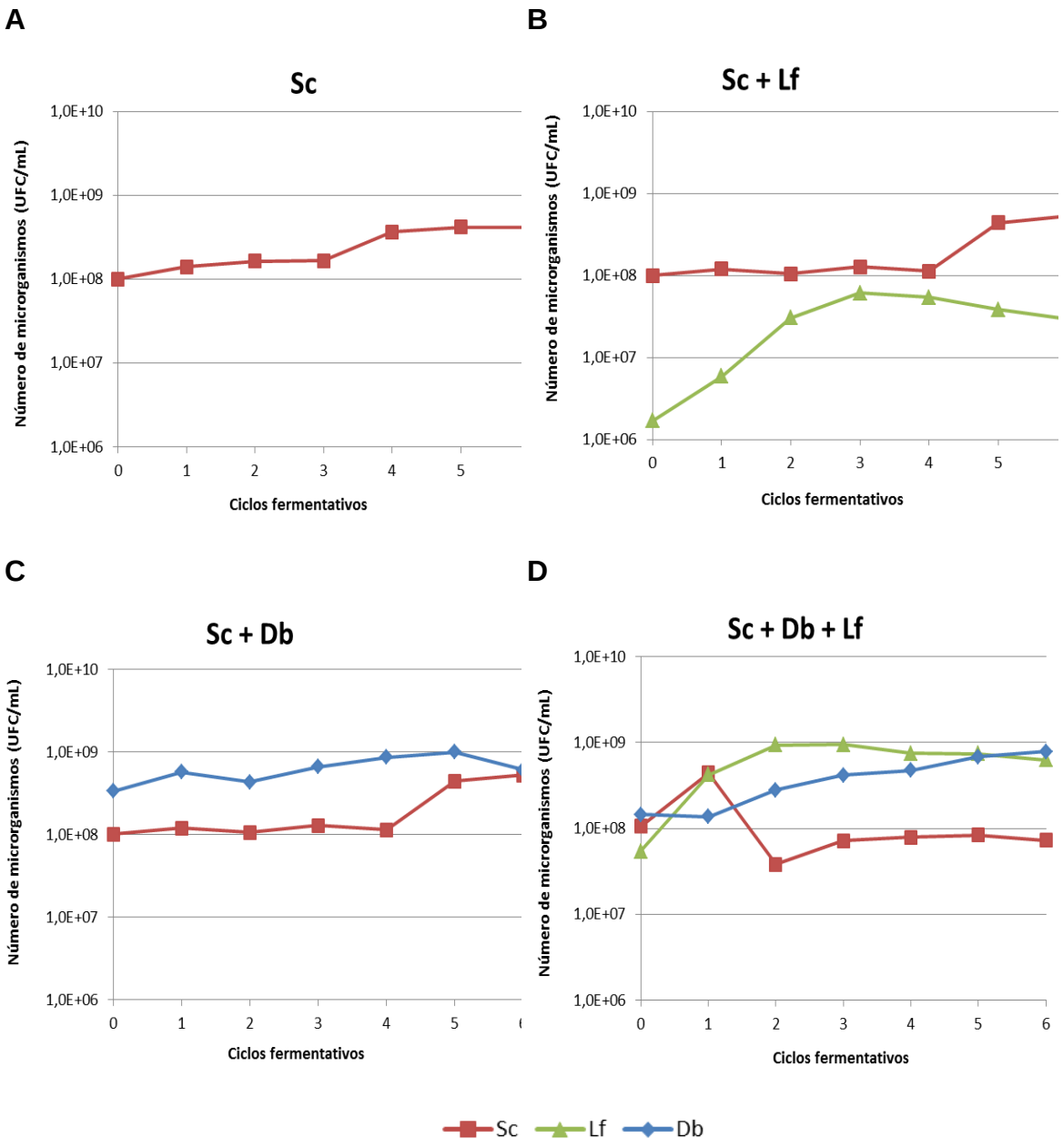

Figura 5 – Número de microrganismos (UFC/mL) nas fermentações desenvolvidas em caldo de cana, com a cultura pura de *S. cerevisiae* (Sc) e contaminadas com *D. bruxellensis* (Db) e ou *L. fermentum* (Lf), ao longo de seis ciclos fermentativos de 12 horas, a 30 ºC. Ciclo 0 refere-se aos resultados no início do 1º. ciclo fermentativo

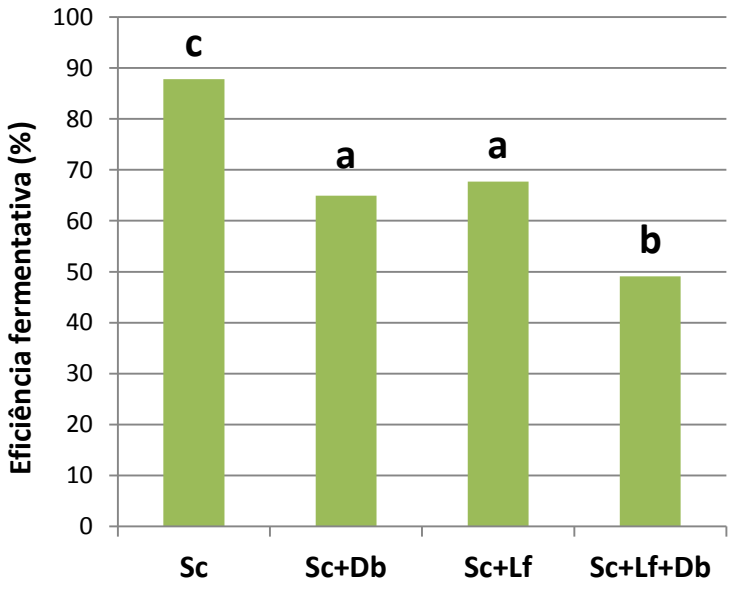

Figura 6 – Valores de eficiência fermentativa média (%) nas fermentações desenvolvidas em caldo de cana, com a cultura pura de *S. cerevisiae* (Sc) e contaminadas com *D. bruxellensis* (Db) e ou *L. fermentum* (Lf), ao longo de seis ciclos fermentativos de 12 horas, a 30 ºC. Letras diferentes indicam diferença significativa a 5% de significância pelo teste de Tukey

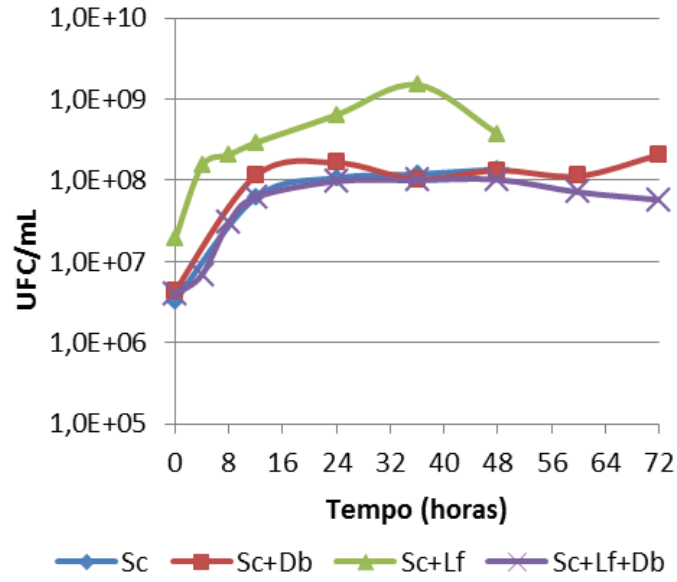

Figura 7 – Crescimento de *S. cerevisiae* (Sc) em meio de melaço, a 30 ºC, 160 rpm, em cultura pura e em co-cultura com *D. bruxellensis* (Db) e ou *L. fermentum* (Lf)

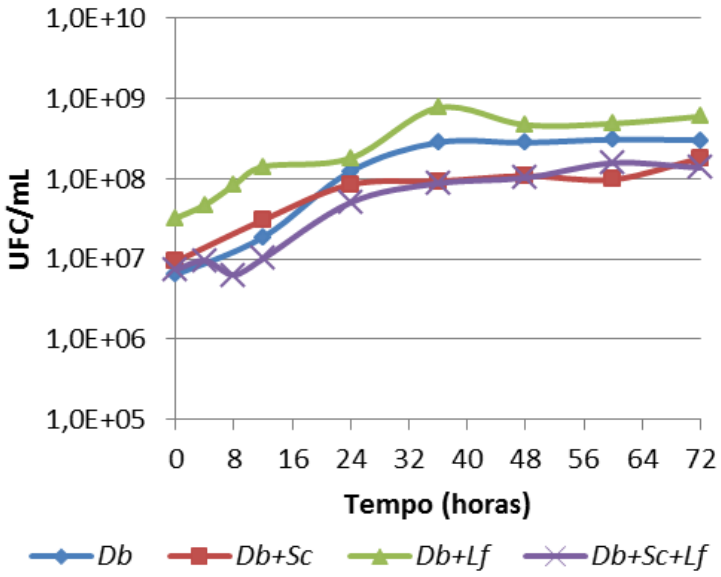

Figura 8 – Cescimento de *D. bruxellensis* (Db) em meio de melaço, a 30 ºC, 160 rpm, em cultura pura e em co-cultura com *S. cerevisiae* (Sc) e ou *L. fermentum* (Lf)

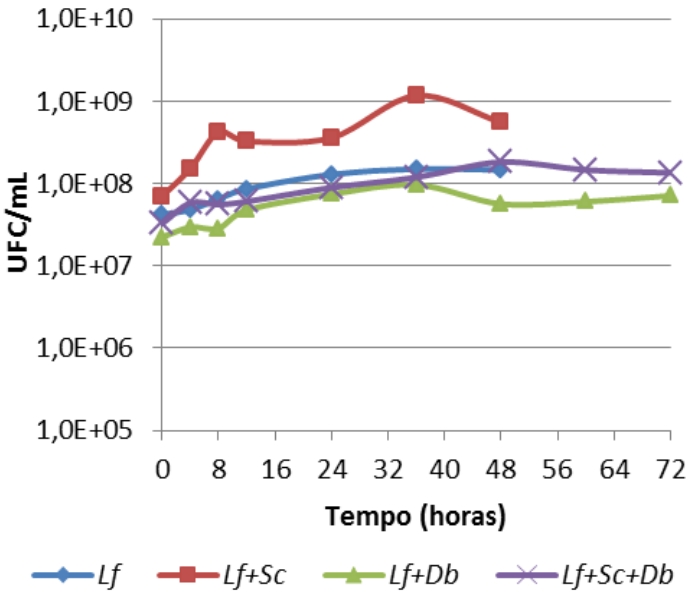

Figura 9 – Crescimento de *L. fermentum* (Lf) em meio de melaço, a 30 ºC, 160 rpm, em cultura pura e em co-cultura com *S. cerevisiae* (Sc) e ou *D. bruxellensis* (Db)

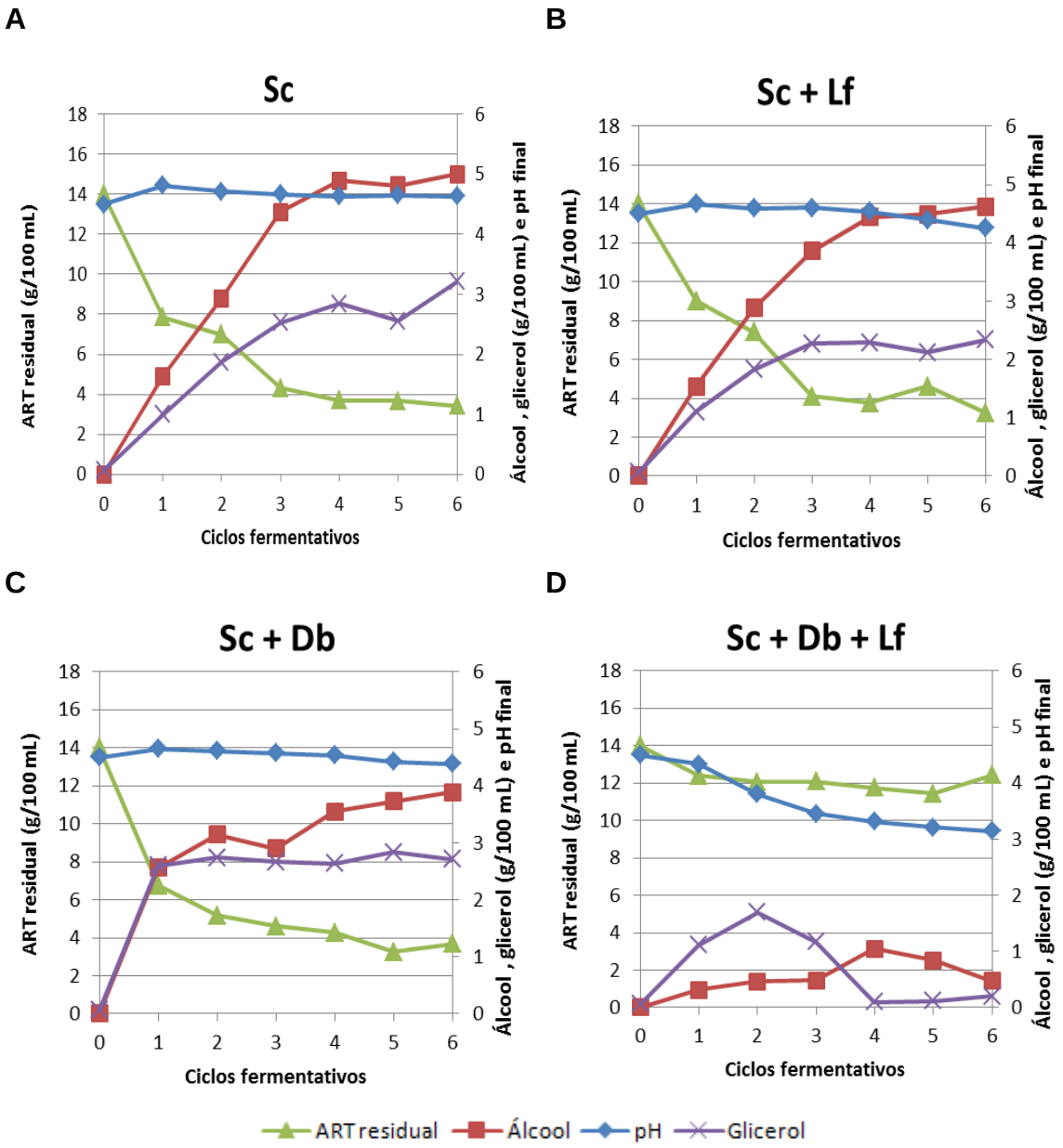

Figura 10 – pH final, concentrações de açúcar redutor total (ART) residual, glicerol e álcool nas fermentações desenvolvidas em melaço, com a cultura pura de *S. cerevisiae* (Sc) e contaminadas com *D. bruxellensis* (Db) e ou *L. fermentum* (Lf), ao longo de seis ciclos fermentativos de 12 horas, a 30 ºC. Ciclo 0 refere-se aos resultados no início do 1º. ciclo

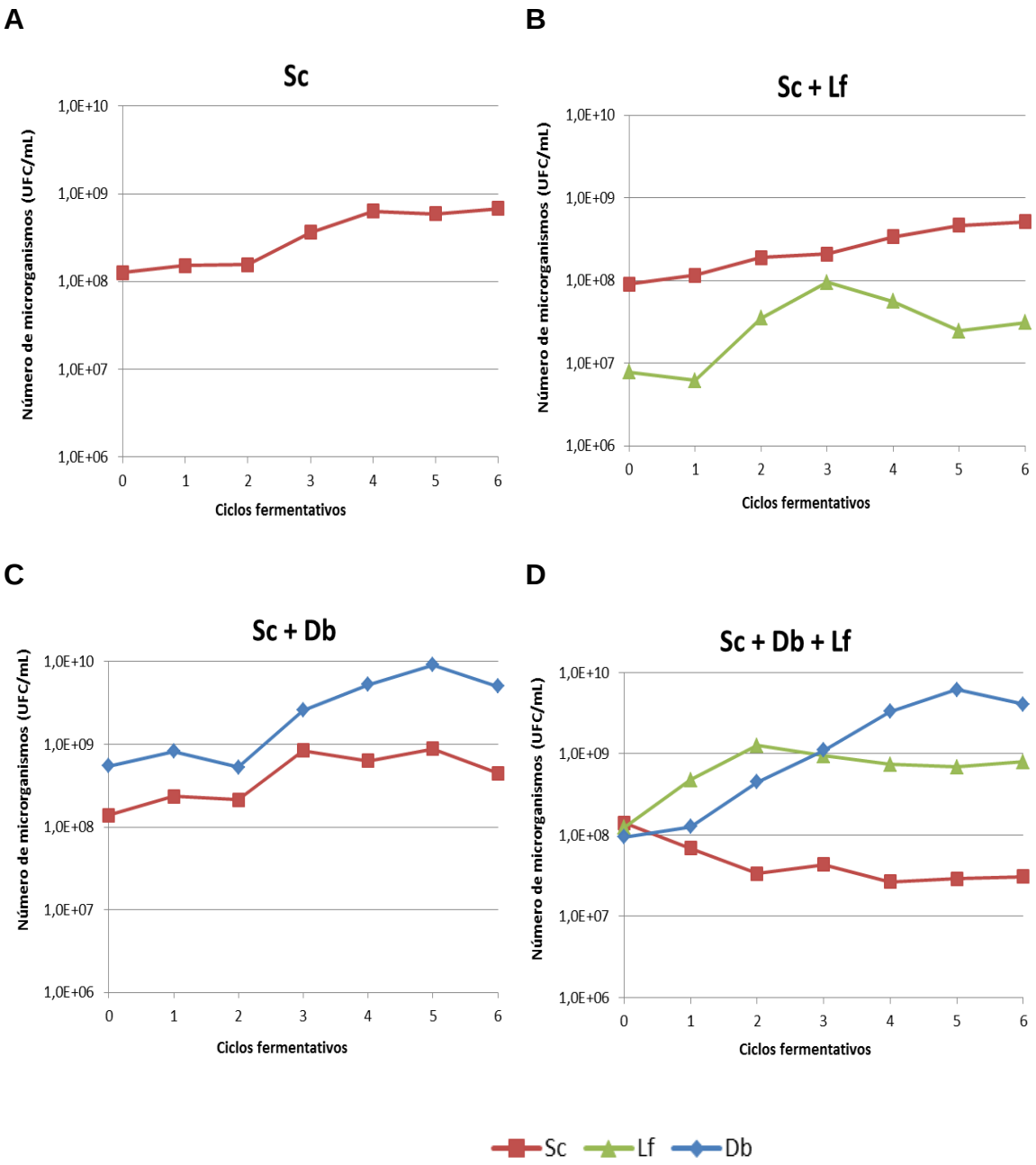

Figura 11 – Número de microrganismos nas fermentações desenvolvidas em melaço, com as culturas pura de *S. cerevisiae* (Sc) e contaminadas com *D. bruxellensis* (Db) e ou *L. fermentum* (Lf), ao longo de seis ciclos fermentativos de 12 horas, a 30 ºC

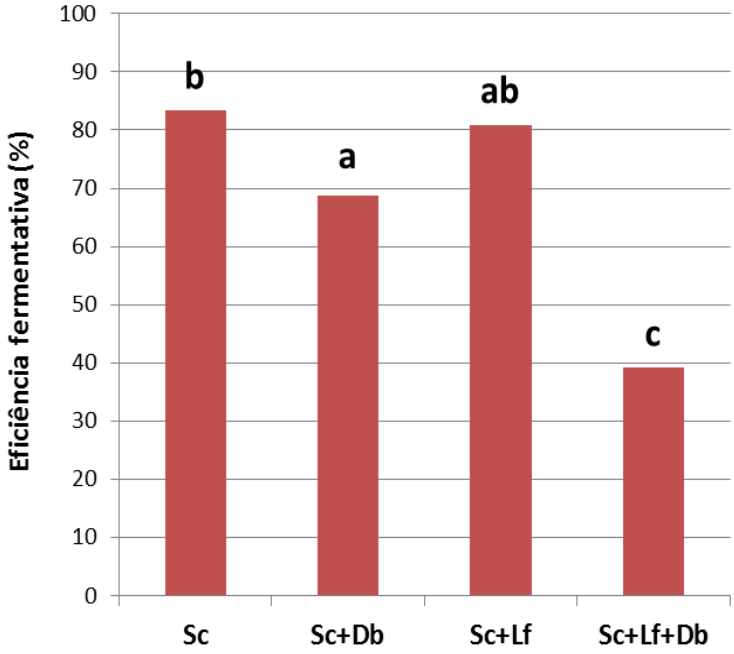

Figura 12 – Valores de eficiência fermentativa média (%) nas fermentações desenvolvidas em melaço, com a cultura pura de *S. cerevisiae* (Sc) e contaminadas com *D. bruxellensis* (Db) e ou *L. fermentum* (Lf), ao longo de seis ciclos fermentativos de 12 horas, a 30 ºC. Letras diferentes indicam diferença significativa a 5% de significância pelo teste de Tukey

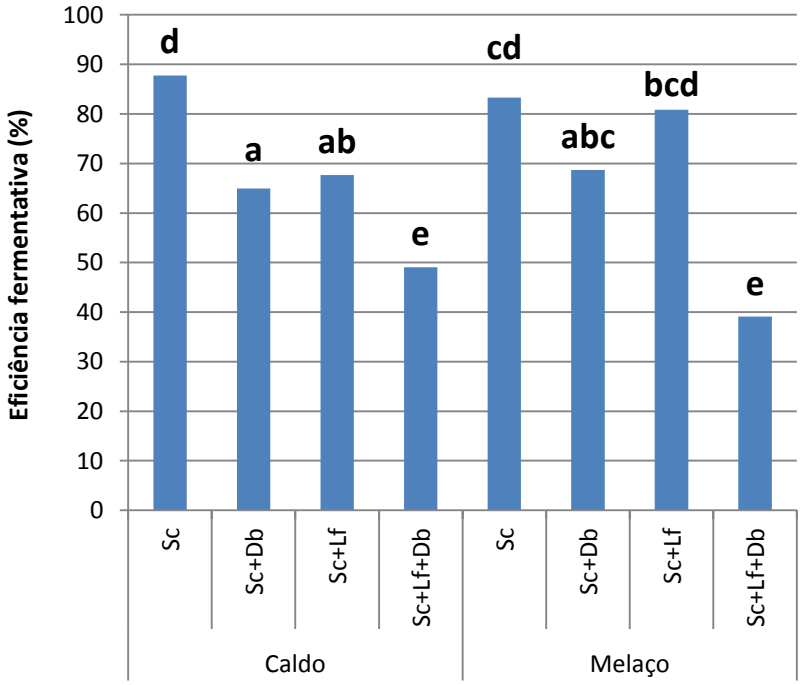

Figura 13 – Valores de eficiência fermentativa média (%) nas fermentações desenvolvidas em caldo e melaço, com a cultura pura de *S. cerevisiae* (Sc) e contaminadas com *D. bruxellensis* (Db) e ou *L. fermentum* (Lf), ao longo de seis ciclos fermentativos de 12 horas, a 30 ºC. Letras diferentes indicam diferença significativa a 5% de significância pelo teste de Tukey

Figuras referentes ao Capítulo 2

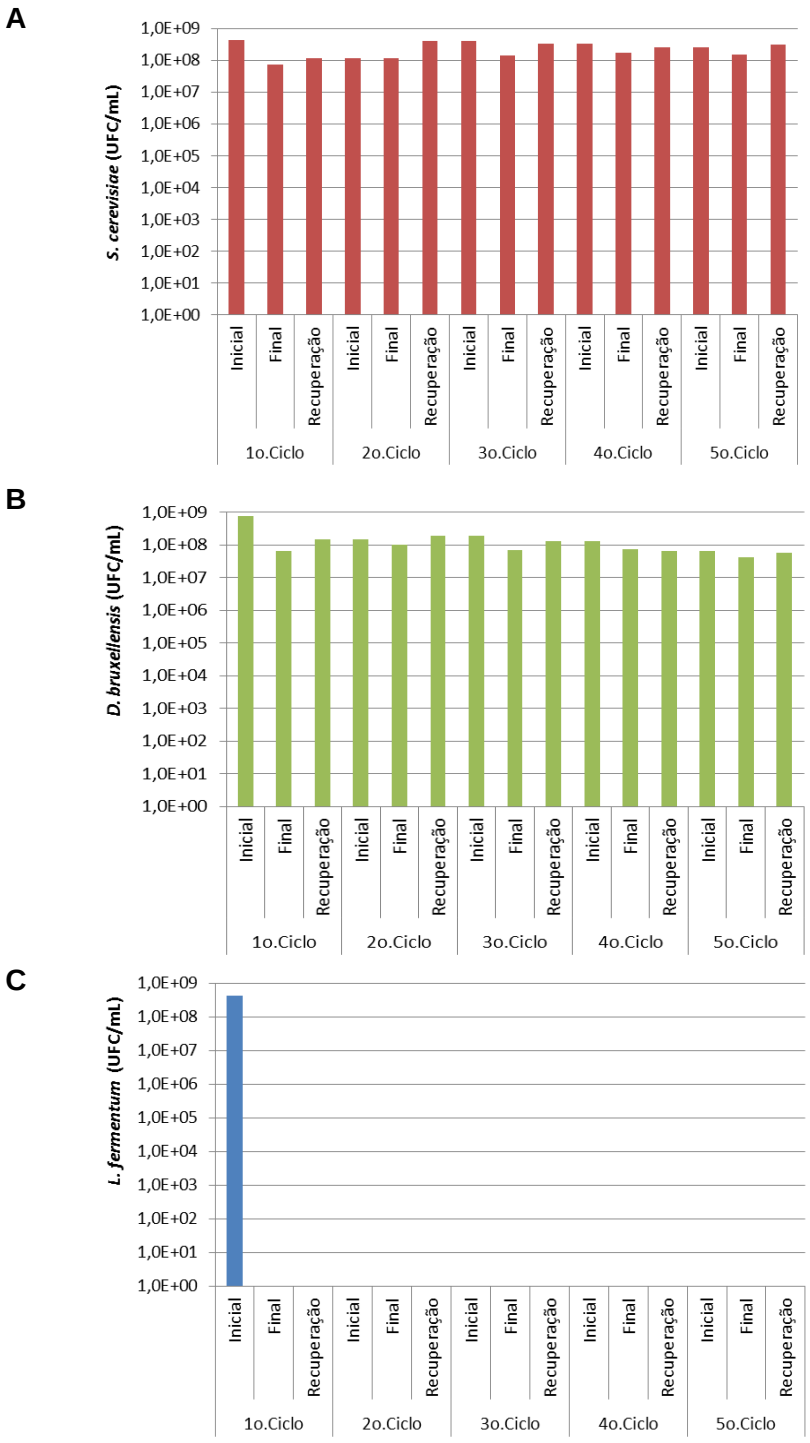

Figura 1 – Número de UFC/mL durante os ciclos sucessivos de tratamento com solução de ácido sulfúrico (pH 2,0) e etanol (13% v/v), em cultura mista de *S. cerevisiae* PE-2 + *D. bruxellensis* (CCA155) + *L. fermentum*. Legenda: **Inicial:** contagem de colônias antes do tratamento; **Final:** contagem de colônias após o tratamento; **Recuperação:** contagem de colônias após 8 horas de incubação das células tratadas em meio caldo de cana 4 ºBrix, incubação a 30 ºC, 160 rpm

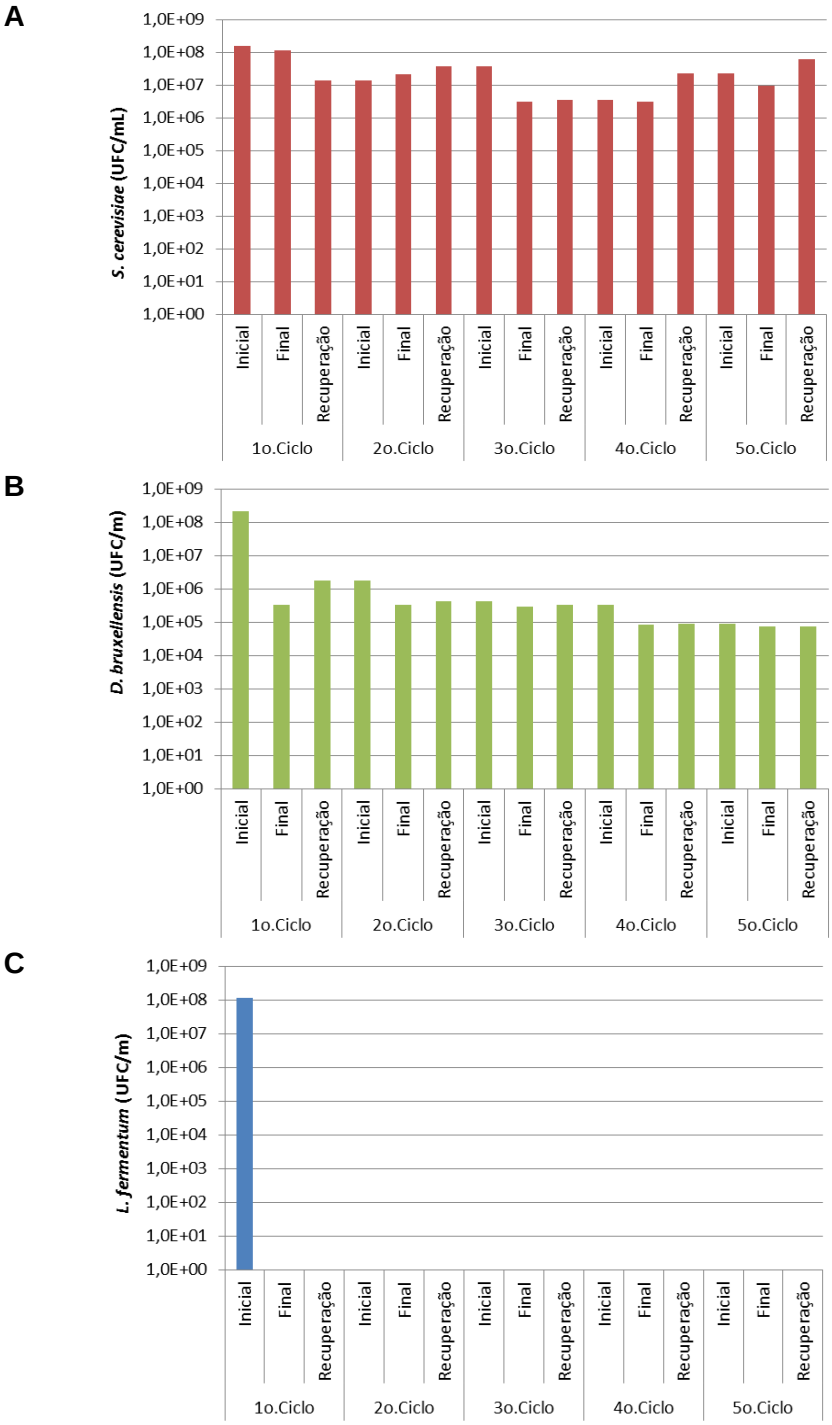

Figura 2 – Número de UFC/mL durante os ciclos sucessivos de tratamento com solução de ácido sulfúrico (pH 2,0) e etanol (13% v/v), em cultura mista de *S. cerevisiae* PE-2 + *D. bruxellensis* (CCA059) + *L. fermentum*. Legenda: **Inicial:** contagem de colônias antes do tratamento; **Final:** contagem de colônias após o tratamento; **Recuperação:** contagem de colônias após 8 horas de incubação das células tratadas em meio caldo de cana 4 ºBrix, incubação a 30 ºC, 160 rpm

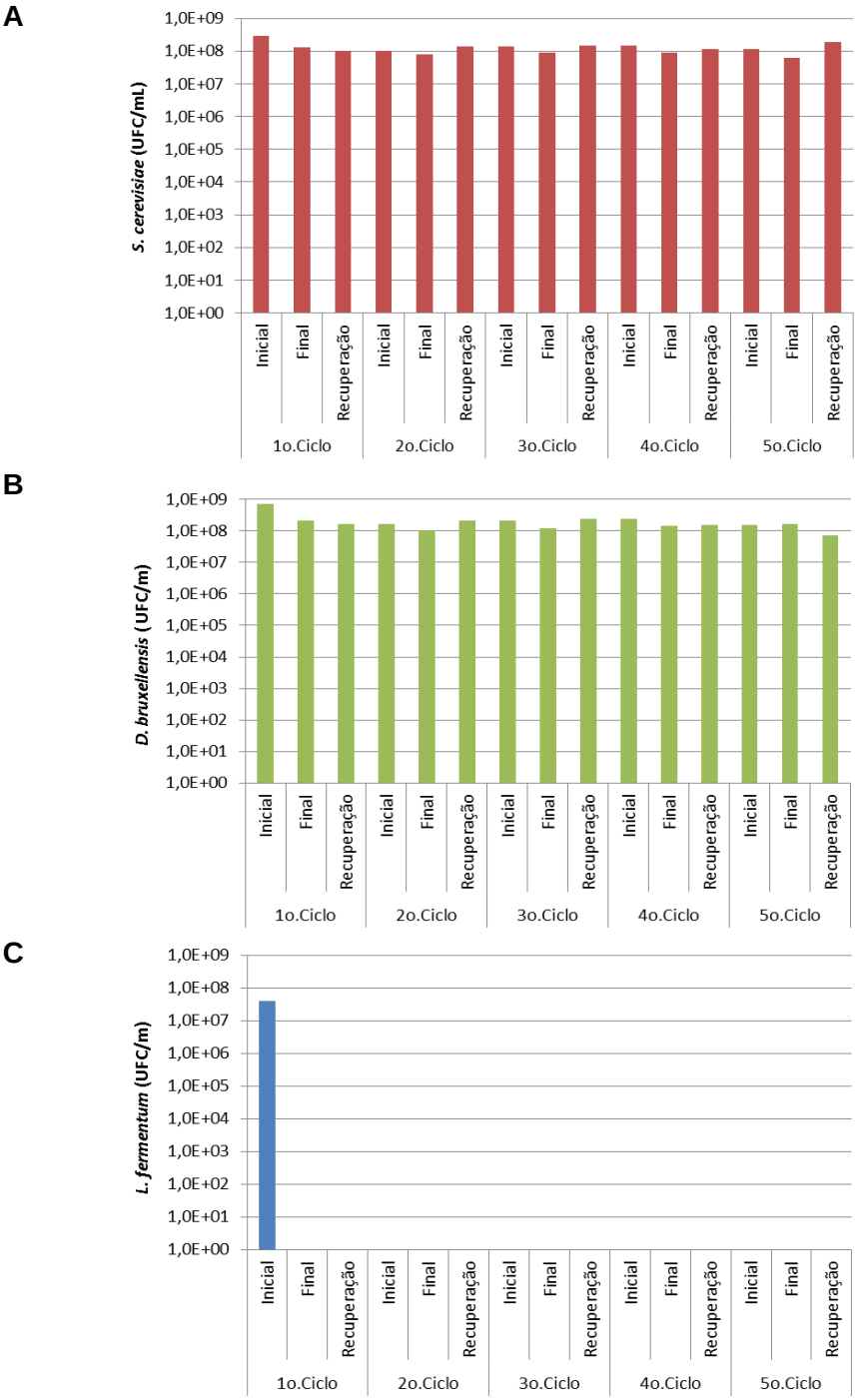

Figura 3 – Número de UFC/mL durante os ciclos sucessivos de tratamento com solução de ácido sulfúrico (pH 2,0) e etanol (13% v/v), em cultura mista de *S. cerevisiae* PE-2 + *D. bruxellensis* (CCA077) + *L. fermentum*. Legenda: **Inicial:** contagem de colônias antes do tratamento; **Final:** contagem de colônias após o tratamento; **Recuperação:** contagem de colônias após 8 horas de incubação das células tratadas em meio caldo de cana 4 ºBrix, incubação a 30 ºC, 160 rpm

Artigo referente ao Capítulo 3

Letters in Applied Microbiology

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ORIGINAL ARTICLE

Potassium metabisulphite as a potential biocide against *Dekkera bruxellensis* in fuel ethanol fermentations

A.P.G. Bassi, A.L. Paraluppi, V.R. Reis and S.R. Ceccato-Antonini

Laboratory of Molecular and Agricultural Microbiology, Department of Tecnologia Agroindustrial e Socio-Economia Rural, Universidade Federal de São Carlos – Centro de Ciencias Agrarias, Araras, SP, Brazil

Significance and Impact of the Study: This study is the first to evaluate the action of potassium metabisulphite to control the growth of *Dekkera bruxellensis* in the fermentation process for fuel alcohol production. As near as possible of industrial conditions, the study simulates the addition of that substance in different points in the fermentation process, verifying in which situation the effects over the starter yeast and alcohol yield are minimal and over *D. bruxellensis* are maximal. Co-culture fermentations were carried out in cell-recycled batch system. The feasibility of using this substance for this specific fermentation is discussed in light of the possible biological and chemical interactions.

Keywords
biocide, ethanol, fermentation, sulphite, yeast contaminants.

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Abstract

Dekkera bruxellensis is an important contaminant yeast of fuel ethanol fermentations in Brazil, whose system applies cell repitching between the fermentative cycles. This work evaluated the addition of potassium metabisulphite (PMB) on yeast growth and fermentative yields in pure and co-cultures of *Saccharomyces cerevisiae* and *D. bruxellensis* in two situations: addition to the acidic solution in which the cells are treated between the fermentative cycles or to the fermentation medium. In the range of 200–400 mg l^{-1}, PMB was effective to control the growth of *D. bruxellensis* depending on the culture medium and strain. When added to the acidic solution (250 mg l^{-1}), a significant effect was observed in mixed cultures, because the inactivation of SO_2 by *S. cerevisiae* most likely protected *D. bruxellensis* from being damaged by PMB. The physiological response of *S. cerevisiae* to the presence of PMB may explain the significant decrease in alcohol production. When added to the fermentation medium, PMB resulted in the control but not the death of *D. bruxellensis*, with less intensive effect on the fermentative efficiency. In co-culture with the addition of PMB, the fermentative efficiency was significantly lower than in the absence of PMB.

Introduction

Dekkera bruxellensis is a two-faced yeast to the fermentation industry. Due to its more efficient energy metabolism compared to *Saccharomyces cerevisiae*, ability to use nitrate as its sole nitrogen source, ability to produce ethanol under aerobic conditions and grow without oxygen, high tolerance to ethanol and acids and capacity to utilize a wide range of carbon sources, this yeast is considered a relevant ethanol-producing organism (Passoth *et al.* 2007; Galafassi *et al.* 2011, 2013; Blomqvist *et al.* 2012; Souza *et al.* 2012). However, that yeast is involved in the production of volatile phenols in wines, with the formation of unpleasant odours that cause important economic losses in the wine industry (Oelofse *et al.* 2008). *Dekkera bruxellensis* has also been reported as a contaminant in ethanol-production plants due to its ability to outcompete *S. cerevisiae* (Liberal *et al.* 2007). It is a low-rate ethanol producer that exhibits expressive growth in oxygen-limited conditions and leaves substantial residual amounts of sugars at the end of the fermentation period. The exhaustion of reducing sugars would extend the fermentation time to an impractical level (Meneghin *et al.* 2013).

The effect of sulphur dioxide (SO_2) on *D. bruxellensis* has been studied extensively in the winemaking industry (Barata *et al.* 2008; Mendoza and Farias 2010; Divol *et al.*

2012) to achieve high-quality wines. Despite controversy about the dose required to cause cell inactivation, results have demonstrated that control of *D. bruxellensis* populations growing in red wine can only be achieved in the presence of relatively high doses of molecular SO_2 (Barata *et al.* 2008). Moreover, the chemistry of SO_2 in wine is fairly complex because of its dissociation into different species, such as bisulphite and sulphite, depending on the pH and its binding to compounds present in the fermentation medium, such as sugars, pigments and other fermentation by-products (Divol *et al.* 2012). SO_2 is added directly or indirectly by means of sulphite salts, such as sodium sulphite, sodium bisulphite, potassium bisulphite, sodium metabisulphite and potassium metabisulphite (PMB, Favero *et al.* 2011). Potassium metabisulphite ($K_2S_2O_5$), here designated 'PMB', renders 57·6% of SO_2 in aqueous solution (Rotter 2011). SO_2 inhibits microbial growth by interfering with metabolites from glycolysis and binds to glucose, dihydroxyacetone phosphate, pyruvate, acetaldehyde, oxaloacetic acid and alpha-ketoglutaric acid, preventing them from being used as substrates for metabolic pathways (Divol *et al.* 2012). Tolerance to SO_2 varies with the yeast species and also between strains. *Saccharomyces cerevisiae* is generally considered to be tolerant to SO_2 (Divol *et al.* 2006; Nardi *et al.* 2010).

Because of *D. bruxellensis* role in the industrial fermentation of fuel alcohol in Brazil and the fact that SO_2 has not yet been reported as an antimicrobial for this particular fermentation, this study aimed to evaluate the effect of adding SO_2 in the form of PMB, under both growing and fermenting conditions with *S. cerevisiae* and *D. bruxellensis* in pure and mixed cultures, on the growth of the yeasts and the fermentative yield. The addition of PMB was evaluated at two different steps during the process: it was added to the acidic solution used to treat the cells between industrial fermentative cycles (Amorim *et al.* 2011) and to the fermentation medium (sugar cane juice) in multiple-recycled fermentations with cell repitching.

Results and discussion

The effect of PMB concentrations ranging from 100 to 400 mg l^{-1} on the growth of *S. cerevisiae* and *D. bruxellensis* in different culture media is depicted in Figs S1–S3. In YPD and sugar cane juice medium, the growth of *S. cerevisiae* was not affected in any concentration of PMB, but for *D. bruxellensis*, a slower growth was observed with 100 and 200 mg l^{-1} of PMB and almost complete inhibition in 400 mg l^{-1}, but for strain CCA155. In molasses medium, there was a slower growth in any concentration of PMB for all yeasts and 400 mg l^{-1} was not inhibitory to *D. bruxellensis*. Comparative analysis of PMB concentrations in 72 h of cultivation in different media is displayed in Fig. 1a–d. In addition to sugars, molasses also present colouring compounds, such as tannins, which can bind to sulphite and inactivate it. The resulting substances, hydroxysulphonates, lack antimicrobial activity (Rotter 2011). This may be the reason why PMB was less inhibitory to the yeasts in molasses than in sugar cane juice or YPD.

Individual *D. bruxellensis* strains were affected differently by PMB as already verified by Barata *et al.* (2008) and Curtin *et al.* (2012). There is some controversy about the level of SO_2 required to cause inactivation or death of *D. bruxellensis* yeast cells. Growth of this yeast was prevented by the maintenance of 25–35 mg l^{-1} of free SO_2 (*c.* 45–60 mg l^{-1} of PMB) in bulk wines (du Toit *et al.* 2005), 30 mg l^{-1} at pH 3·4–3·5 (Ribéreau-Gayon *et al.* 2006) or 50 mg l^{-1} for non-*Saccharomyces* yeasts in general (Mendoza and Farias 2010). The most tolerant yeast was *S. cerevisiae*, growing under 200 mg l^{-1} of PMB, while *D. bruxellensis* grew in the range of 60–90 mg l^{-1} in synthetic culture media at an initial pH of 3·5 (Barata *et al.* 2008). For further experiments simulating industrial fermentations, the strain CCA155 of *D. bruxellensis* was chosen because of the higher resistance to PMB in sugar cane juice and YPD. The minimum inhibitory concentration of PMB was determined, and at 250 mg l^{-1}, its growth was greatly reduced with no substantial difference in relation to the higher concentrations tested (Fig. 1e).

Tests to evaluate the effects of PMB on fermentation were carried out by adding PMB at the acid treatment step used industrially to minimize bacterial contamination (Amorim *et al.* 2011). In this situation, a significant effect ($P < 0·05$) was observed for *S. cerevisiae* after treatment; however, the number of CFU was re-established after 12 h of fermentation, which did not occur with *D. bruxellensis*. Even with the cell population recovery, there was a significant effect ($P < 0·05$) of PMB addition on the alcohol production by *S. cerevisiae* (Fig. 2a,b). Two points must be explored to explain these results. When PMB is added to an aqueous solution, it dissociates into three molecular species: molecular SO_2, the most active antimicrobial species; bisulphite (HSO_3^-); and sulphite (SO_3^{2-}). The chemical equilibrium between these species is dependent on the pH. Molecular SO_2 is prevalent from pH 0 to 2, bisulphite from pH 2 to 7 and sulphite from pH 7 to 10 (Divol *et al.* 2012). In the acidic solution (pH 2·0), in which the yeast cells were treated for 90 min at 30°C, there was almost 40% SO_2, corresponding to *c.* 58 mg l^{-1} of SO_2. At pH 4·5, which was the pH value of the growth medium, no SO_2 was available, with bisulphite predominating. The second point concerns the mechanism by which SO_2 inhibits growth. SO_2 is a highly reactive molecule that can bind to many metabolites and enzymes in the cell, such as glyceraldehyde-3-phosphate dehydrogenase

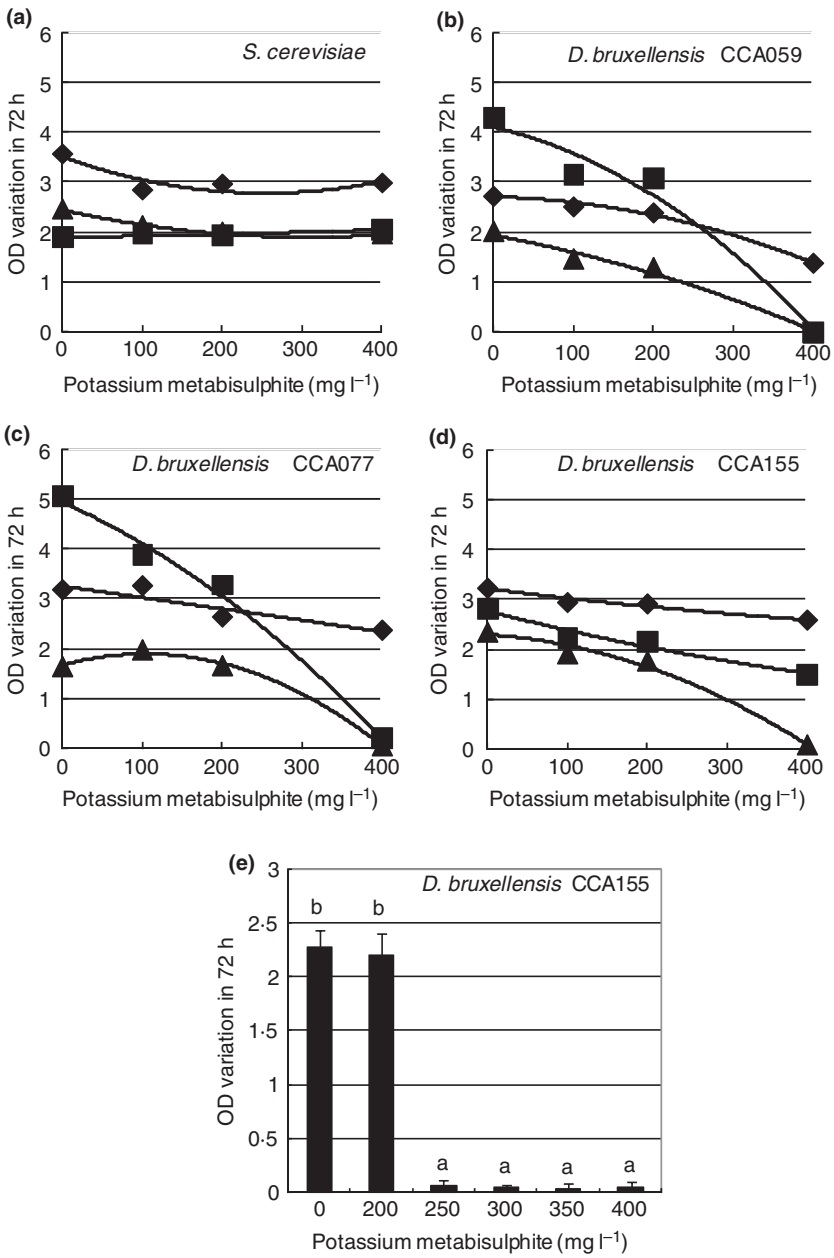
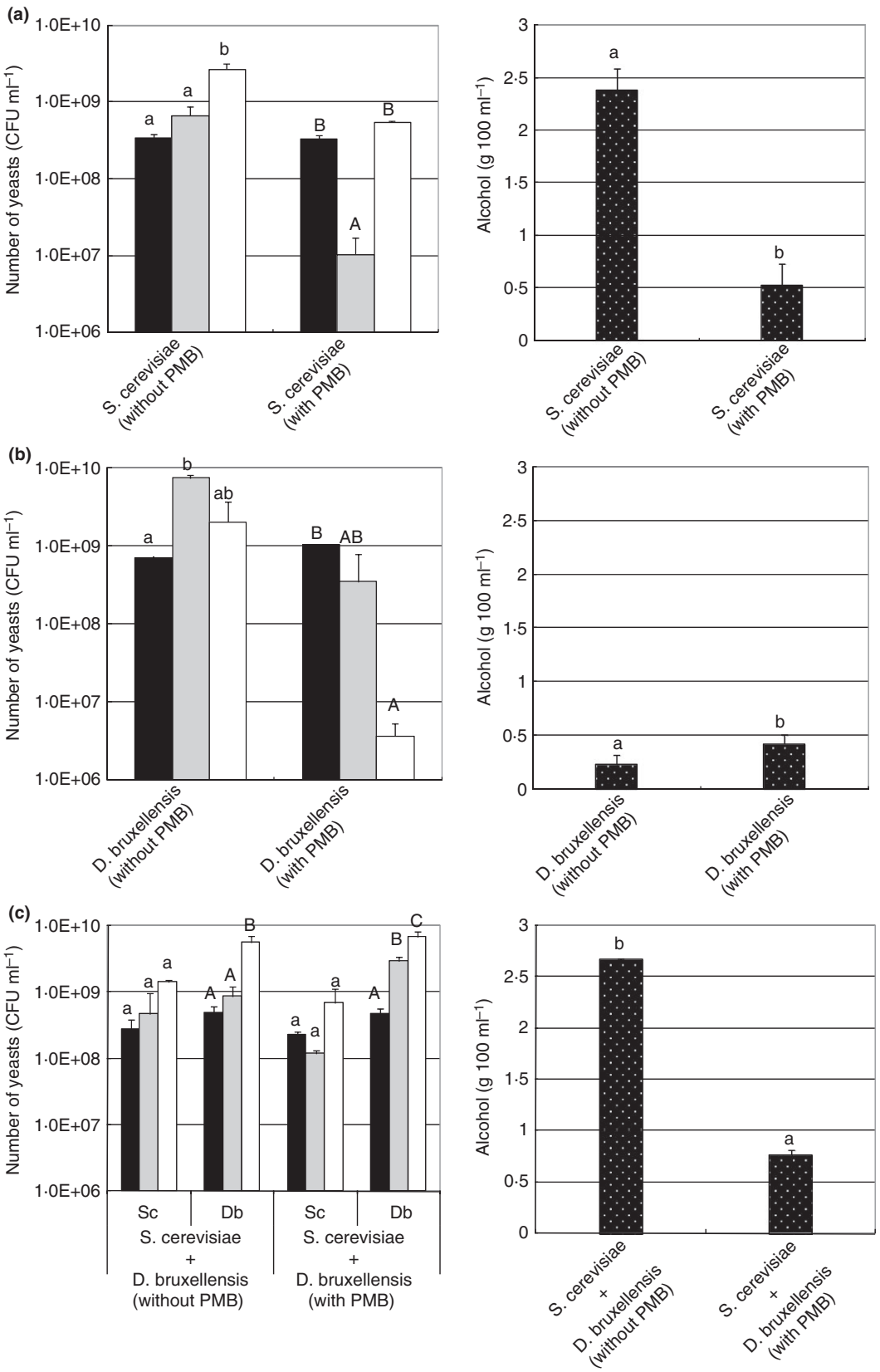


Figure 1 Growth of *Saccharomyces cerevisiae* (in a) and *Dekkera bruxellensis* strains (in b–d for the strains CCA059, 077 and 155, respectively) in YPD (■), sugar cane juice (▲) and molasses (♦), pH 4·5, with potassium metabisulphite (PMB) added in the concentrations of 0, 100, 200 and 400 mg l^{-1}. In (e), the minimum inhibitory concentration of PMB for *D. bruxellensis* CCA155 in sugar cane juice medium. Cultures were maintained at 30°C, under 160 rpm, for 72 h. OD (optical density at 600 nm) variation was calculated by the difference in the values obtained at the end of 72 and 0 h for each PMB concentration. Different letters over the bars in (e) indicate significant differences ($P < 0{\cdot}05$).

(GAPDH), a critical enzyme in the glycolytic pathway that can lose all activity after incubation of *S. cerevisiae* with 2 mmol l^{-1} of sulphite for 45 min (Hinze and Holzer 1986; Casalone *et al.* 1992). Low levels of sulphite inhibited the action of alcohol dehydrogenase, affecting the conversion of acetaldehyde to ethanol (Maier *et al.* 1986). Moreover, acetaldehyde has a strong affinity for unbound SO_2, reducing the effectiveness of sulphite

Figure 2 Number of yeasts (CFU ml^{-1}) and alcohol production (g 100 ml^{-1}) in pure cultures of *Saccharomyces cerevisiae* (in a), *Dekkera bruxellensis* (in b) and mixed culture of both (in c) inoculated in sugar cane juice medium, 16 g 100 ml^{-1} of reducing sugars, pH 4·5, at 30°C, for 12 h. Potassium metabisulphite (PMB) (250 mg l^{-1}) was added to the acidic solution (pH 2·0) in which the cells were treated before inoculation in the fermentation medium, at 30°C, for 90 min, under 160 rpm. Samples were taken before the acid treatment (■), after the acid treatment (▣) and after 12 h fermentation (□). Different letters over the bars indicate significant differences ($P < 0{\cdot}05$). For CFU countings, mean values were compared statistically among the treatments and not among the yeasts.


(a)
Number of yeasts (CFU ml−1)
1·0E+10
1·0E+09
1·0E+08
1·0E+07
1·0E+06
a
a
b
B
A
B
S. cerevisiae (without PMB)
S. cerevisiae (with PMB)
Alcohol (g 100 ml−1)
3
2·5
2
1·5
1
0·5
0
a
b
S. cerevisiae (without PMB)
S. cerevisiae (with PMB)
(b)
Number of yeasts (CFU ml−1)
a
b
ab
B
AB
A
D. bruxellensis (without PMB)
D. bruxellensis (with PMB)
Alcohol (g 100 ml−1)
a
b
D. bruxellensis (without PMB)
D. bruxellensis (with PMB)
(c)
Number of yeasts (CFU ml−1)
a
a
a
A
A
B
a
a
a
A
B
C
Sc
Db
Sc
Db
S. cerevisiae + D. bruxellensis (without PMB)
S. cerevisiae + D. bruxellensis (with PMB)
Alcohol (g 100 ml−1)
b
a
S. cerevisiae + D. bruxellensis (without PMB)
S. cerevisiae + D. bruxellensis (with PMB)

against bacteria and yeast (Divol *et al.* 2012). These events would clearly impair fermentation by yeasts.

Moreover, the combination of acetaldehyde with the bisulphite ion can also result in substantial overproduction of glycerol by *S. cerevisiae* (Wang *et al.* 2001). The diverted route to glycerol may also impact the ethanol yield.

The ability to synthesize acetaldehyde may explain sulphur dioxide tolerance in *S. cerevisiae*. Acetaldehyde concentration rapidly increased in *S. cerevisiae* strains after the addition of 10 and 50 mg l^{-1} of sulphur dioxide (Divol *et al.* 2006). Acetaldehyde bound to SO_2 results in hydroxysulphonate, which is not active against microorganisms (Rotter 2011). Whether this investment in acetaldehyde production occurs at the expense of energy devoted to fermentation or growth is not clearly understood. In nonproliferative conditions, when PMB was added to the acidic solution, *S. cerevisiae* cells may have produced acetaldehyde to inactivate SO_2, which occurred at a high concentration; thus, alcohol production was impaired substantially. This finding explains the result obtained for the mixed *S. cerevisiae* and *D. bruxellensis* culture. In this situation, *D. bruxellensis* was not affected by the addition of PMB, most likely because the inactivation of SO_2 by *S. cerevisiae* 'protected' the *Dekkera* cells from damage by PMB. The effect on alcohol production was also significant ($P < 0{\cdot}05$), indicating that the major effect of PMB was on *S. cerevisiae* and not *D. bruxellensis* (Fig 2c). In summary, the addition of PMB to the acidic solution in which the yeast cells are commonly treated in the fermentation industry specifically overloaded *S. cerevisiae*, impairing alcohol production and not affecting the contaminant *D. bruxellensis* yeast, despite the sensitivity displayed by the latter to sulphite in pure cultures in both growing and fermenting conditions. Comparing the experiments by Bassi *et al.* (2013), in which a decrease of 14% in alcohol production and 10-fold in the number of *D. bruxellensis* were verified when 13% ethanol was added to the acidic solution, with those of the present study carried out in the same conditions, it is better to add ethanol to the acidic solution than PMB.

What would be the effect on alcohol production if PMB was added directly to the fermentation medium? Recycled-cell fermentations were carried out with mixed cultures of *S. cerevisiae* and *D. bruxellensis* with and without PMB and compared to pure cultures of *S. cerevisiae* without PMB to evaluate whether the addition of PMB is more harmful to fermentation than contamination by *D. bruxellensis* itself (Figs 3–5). Without the addition of PMB, the population of *D. bruxellensis* increased significantly 100-fold in the last two 12-h fermentation cycles (Fig 3a), while it remained constant when PMB was added at 250 mg l^{-1} (Fig 4a). The *S. cerevisiae* population presented slight fluctuations during the fermentation cycles regardless of PMB addition, with a significant increase in the number in the last cycle with PMB (Fig 4a). This is an interesting point achieved by adding PMB to each fermentation cycle: *D. bruxellensis* growth is controlled, but cell death does not occur. Bisulphite, which is the molecular species found to predominate in the medium at pH 4·5, is considered fungistatic (Ribéreau-Gayon *et al.* 2006). However, the effects on the physiology of *S. cerevisiae* persisted because the fermentative efficiency was significantly lower ($P < 0{\cdot}05$) when PMB was added than in the absence of PMB. Contamination by *D. bruxellensis* caused also a significant decrease in the fermentative efficiency compared to fermentation without contamination, but the usage of PMB at a concentration of 250 mg l^{-1} in each fermentative cycle diminished the alcohol yield still more (Fig 5d).

However, the effect of PMB on alcohol production was lower when it was added to the fermentation medium instead of the acidic solution. Different molecular species were present in each situation, with molecular SO_2 being much more reactive than bisulphite (Ribéreau-Gayon *et al.* 2006).

Our experiments lasted six fermentation cycles, and whether extension of *D. bruxellensis* growth would take a long time without any type of control is a question to be considered. Would it be better to control *D. bruxellensis* growth using PMB, even at the expense of lower alcohol yield? An ethanol-based cell treatment, as proposed by Bassi *et al.* (2013), despite slowing the fermentation, decreased and controlled the *D. bruxellensis* population while *S. cerevisiae* took over the fermentation during the growth cycles. Both PMB added to the fermentation medium in each cycle (this study) and ethanol added to the acidic solution helped to control *D. bruxellensis* growth but with a lower alcohol yield. In both experiments, *D. bruxellensis* was inoculated into the fermentation system in high cell concentration (around 10^9 CFU ml^{-1}).

The outstanding population growth capacity of *D. bruxellensis* under fermentation conditions was demonstrated by Meneghin *et al.* (2013), who showed that an initial contamination of 10^3 cells ml^{-1} could render slower alcohol production and results in a cellular increase of *c.* 3 log cycles throughout 14 fermentative cycles lasting 12 h each. These results demonstrate that if a few cells enter the fermentation tank, they will be recycled together with the *S. cerevisiae* and may achieve high numbers after some time. The authors concluded that this peculiarity of the Brazilian fermentation systems—cell repitching—favours the development and establishment of *D. bruxellensis* in the system.

Whether PMB should be considered for use in the fermentation industry to control the growth of *D. brux-*

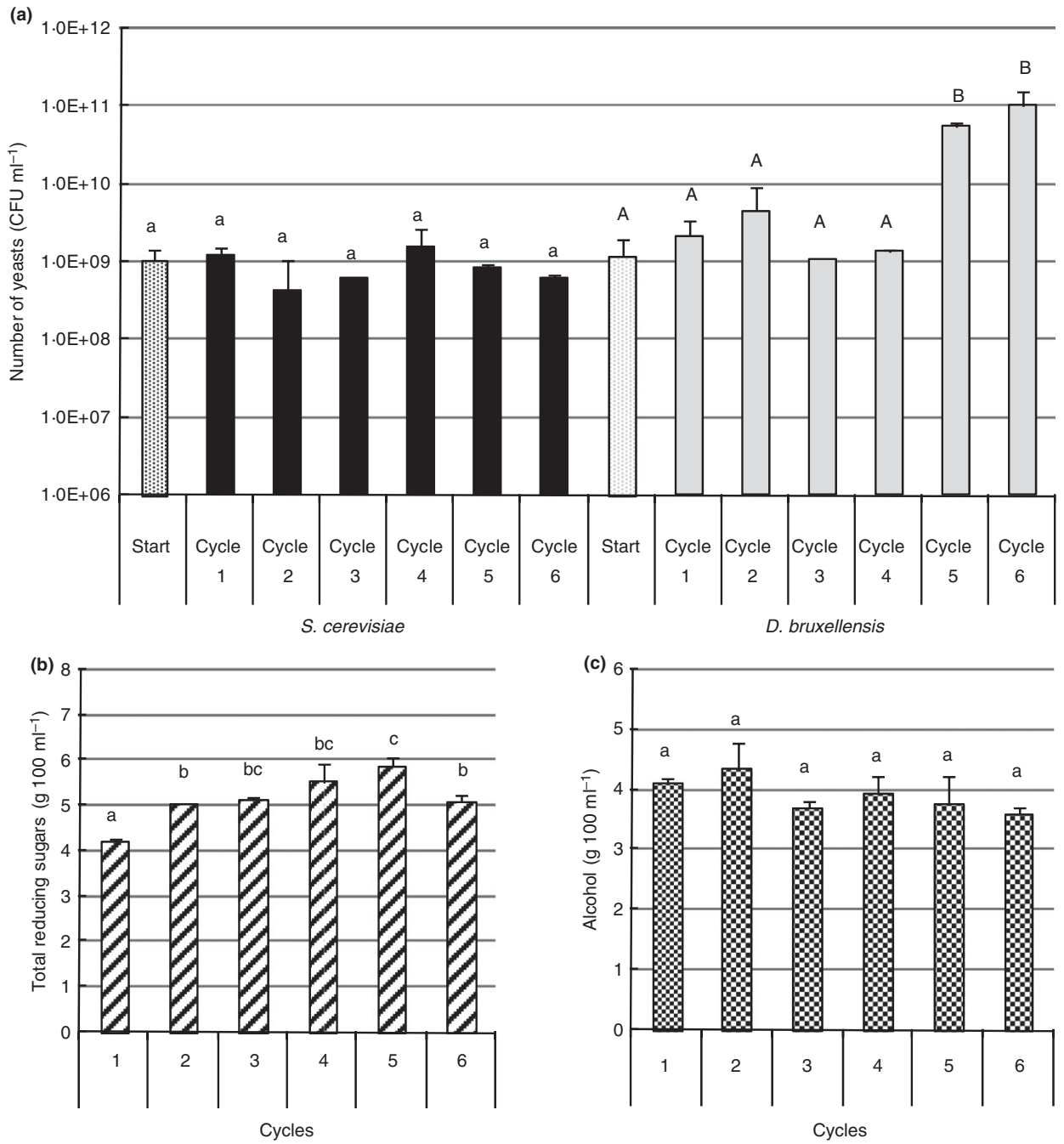


Figure 3 Number of yeasts (CFU ml^{-1}, in a), residual total reducing sugars (g 100 ml^{-1}, in b) and alcohol production (g 100 ml^{-1}, in c) in the mixed culture of *Saccharomyces cerevisiae* and *Dekkera bruxellensis* inoculated in sugar cane juice medium, 16 g 100 ml^{-1} of reducing sugars, pH 4·5, at 30°C, for six fermentation cycles lasting 12 h each. Potassium metabisulphite was not added to the fermentation medium. Cells were recycled from one fermentation cycle to another after centrifugation of the fermented medium. 'Start' means the number of yeasts inoculated at the beginning of the fermentation process. Number of yeasts was quantified after 12 h of fermentation in each fermentative cycle. Different letters over the bars indicate significant differences ($P < 0·05$). For CFU countings, mean values were compared statistically among the cycles and not among the yeasts.

ellensis demands further investigation, which should also include an economic study. The role of acetaldehyde as a defence mechanism against SO_2 and the interaction between ethanol and other sugar derived medium components and SO_2 are questions that arise from our study.

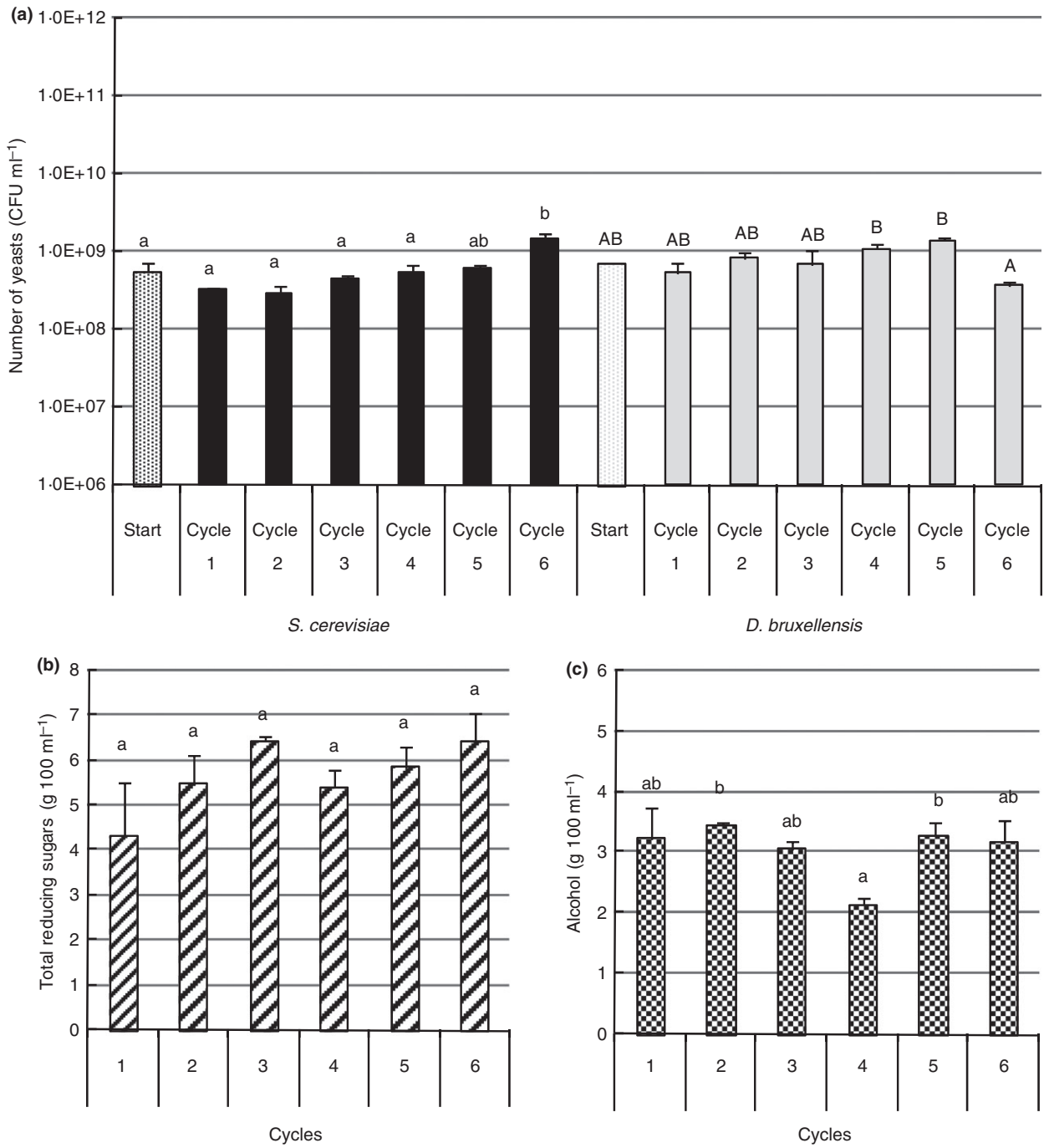


Figure 4 Number of yeasts (CFU ml^{-1}, in a), residual total reducing sugars (g 100 ml^{-1}, in b) and alcohol production (g 100 ml^{-1}, in c) in the mixed culture of *Saccharomyces cerevisiae* and *Dekkera bruxellensis* inoculated in sugar cane juice medium, 16 g 100 ml^{-1} of reducing sugars, pH 4·5, at 30°C, for six fermentation cycles lasting 12 h each. Potassium metabisulphite (PMB) (250 mg l^{-1}) was added to the fermentation medium at the start of each fermentation cycle. Cells were recycled from one fermentation cycle to another after centrifugation of the fermented medium. 'Start' means the number of yeasts inoculated at the beginning of the fermentation process. Number of yeasts was quantified after 12 h of fermentation in each fermentative cycle. Different letters over the bars indicate significant differences ($P < 0·05$). For CFU countings, mean values were compared statistically among the cycles and not among the yeasts.

Material and methods

Yeast strains and culture conditions

Three strains of *D. bruxellensis* (CCA059, CCA077 and CCA155) isolated from the fermentation process for fuel alcohol production were utilized in the experiments after they were identified by DNA sequencing at the D1/D2 26S locus and ITS region of the ribosomal DNA. An industrial strain of *S. cerevisiae* (PE-2) was also used in these experiments (Basso *et al.* 2008). The strains were maintained on YPD (in w/v: 1% yeast extract, 2% glucose, 2% peptone, 2% agar; for broth, agar was not included) slants at 4°C with constant transfers to new medium.

Tolerance to PMB in growing conditions with different culture media

Initially, the tests were carried out in 50-ml Falcon tubes with 15 ml of YPD broth (final volume), in duplicate, with an initial pH of 4·5. A 1 ml aliquot of a concentrated cell suspension was added to each tube for a final optical density (at 600 nm) of *c.* 0·2–0·3. PMB (*Synth*) solutions were prepared in sterile distilled water, and aliquots of 1 ml were added to the media to a final concentration of 0, 100, 200 and 400 mg l^{-1}. The tubes were incubated at 30°C, at 160 rpm, for 72 h. Samples were taken every 24 h to evaluate the optical density at 600 nm in a Bio-Mate® spectrophotometer (Thermo Electron Corporation, Madison, WI, USA). The same procedures were carried out with diluted molasses and sugar cane juice instead of YPD, with an initial pH of 4·5 and a total sugar concentration of 4 g 100 ml^{-1}.

To determine the minimum inhibitory concentration of PMB for the *D. bruxellensis* strain CCA155, we used diluted sugar cane juice (as above) in Erlenmeyer flasks containing 25 ml of medium. Concentrations of 0, 200, 250, 300, 350 and 400 mg l^{-1} of PMB were tested. Growth was evaluated by optical density at 600 nm, as described above.

Effect of the addition of PMB to the acid cell treatment on the fermentative yield

For this set of experiments, pure and mixed cultures of *S. cerevisiae* and *D. bruxellensis* (CCA155) were utilized. Cells were grown in sugar cane juice (4 g 100 ml^{-1} of total sugars, pH 5·5) until a cell concentration of *c.* 5×10^9 cells ml^{-1} was achieved. Daily transfers to new media were performed if necessary to achieve the desired cell concentration. Aliquots of 10 ml of the concentrated suspension (previously centrifuged and resuspended in distilled water) were added to a sterile acidic solution, prepared with sulphuric acid and water, to a final pH of 2·0 and added to PMB to achieve a concentration of 250 mg l^{-1} in 250-ml Erlenmeyer flasks for pure cultures of both yeast strains. Mixed cultures were prepared by adding 5 ml of concentrated suspension to the acidic solution for each yeast strain. The flasks were maintained for 90 min at 30°C under 150 rpm. The flask content was centrifuged, the supernatant was discarded, and the cells were washed with sterile distilled water. The cells were then resuspended in the fermentation medium (100 ml of sugar cane juice with 16 g 100 ml^{-1} of total sugars, pH 5·5), and the 250-ml Erlenmeyer flasks were incubated at 30°C for 12 h without shaking. Samples were taken immediately after the cells were added to the acidic solution, after the cells were treated in the acidic solution with and without PMB and after the 12-h fermentation. Aliquots (1 ml) were serially diluted in saline solution and plated on YPD medium with and without actidione (50 mg l^{-1}) for *D. bruxellensis* and *S. cerevisiae,* respectively. The plates were incubated at 30°C for 5 days and colonies were then counted. The remaining sample was centrifuged, and the supernatant was evaluated for alcohol content (distillation of the sample and density determination of the hydroalcoholic solution using an *Anton Paar* digital densimeter).

Effect of PMB addition to the fermentation medium

For this set of experiments, a pure culture of *S. cerevisiae* and mixed cultures of *S. cerevisiae* and *D. bruxellensis* (CCA155) were utilized. Cells were prepared as above.

Aliquots of 20 ml of the concentrated suspension (previously centrifuged and resuspended in fermentation medium as above) were inoculated in a final volume of 100 ml, in duplicate, in 250-ml Erlenmeyer flasks containing the fermentation medium. PMB was added at a final concentration of 250 mg l^{-1} to the fermentation medium when required. The flasks were incubated for 12 h at 30°C without shaking. Equal concentrations of *S. cerevisiae* and *D. bruxellensis* cells (mixed fermentations) were added to fermentation flasks, as previously described. The fermentation medium was first sampled to estimate the numbers of *S. cerevisiae* and *D. bruxellensis* cells (as described above) and subsequently centrifuged to separate the supernatant from the yeast cell mass, which was inoculated into new fermentation media with PMB, when required. Six 12-h fermentative cycles were performed. In the supernatant, the alcohol content (g 100 ml^{-1}) was evaluated in the hydroalcoholic solution using a *DMA-45 Anton Paar* densitometer after sample distillation, and the total amount of reducing sugar was determined by the 3,5-dinitrosalicylic acid method after hydrolysis of the samples (Miller 1959). The fermentative efficiency (%) was calculated based on the alcohol yield (g alcohol pro-

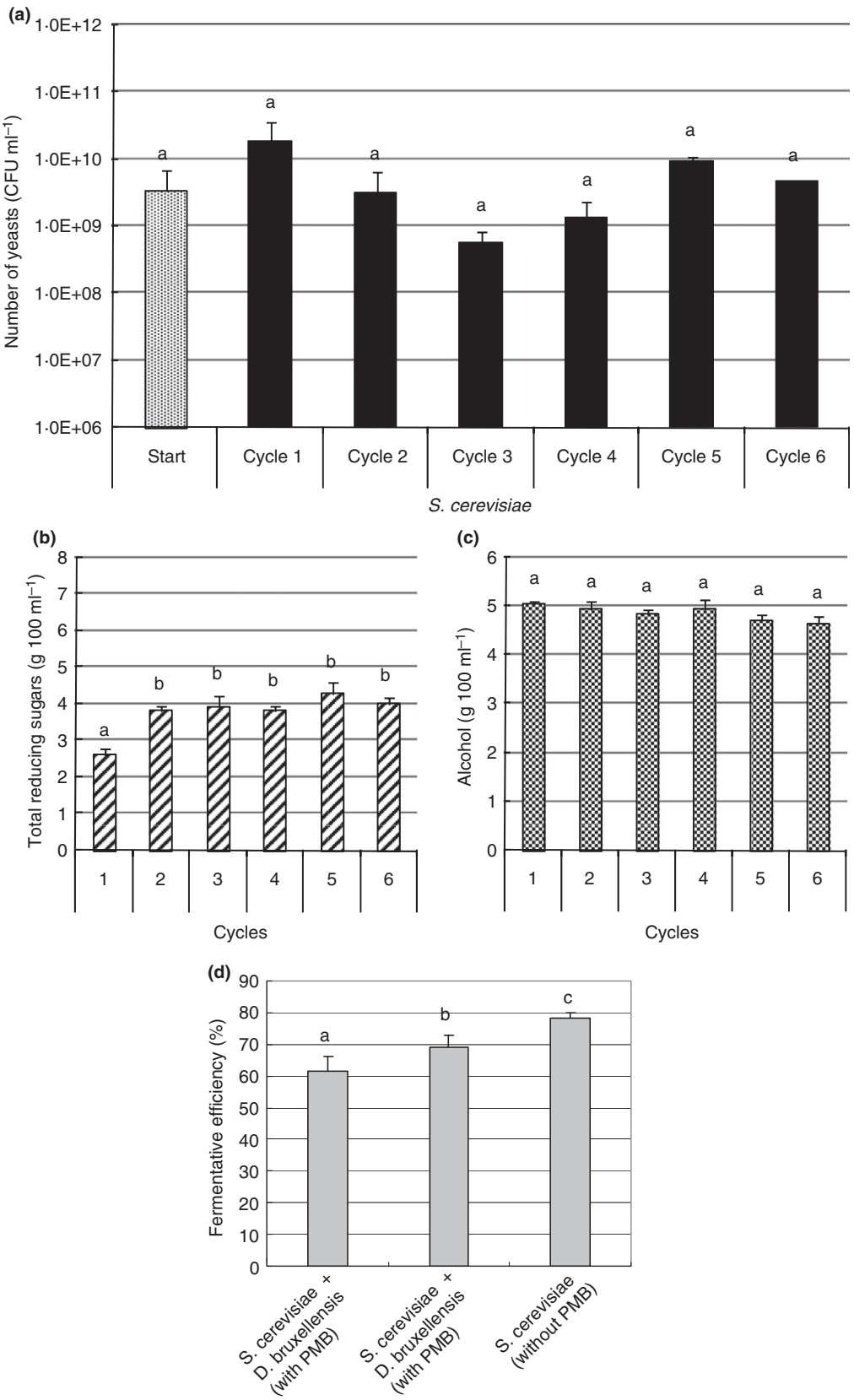

(a)
Number of yeasts (CFU ml−1)
1·0E+12
1·0E+11
1·0E+10
1·0E+09
1·0E+08
1·0E+07
1·0E+06
Start
Cycle 1
Cycle 2
Cycle 3
Cycle 4
Cycle 5
Cycle 6
S. cerevisiae
(b)
Total reducing sugars (g 100 ml−1)
Cycles
(c)
Alcohol (g 100 ml−1)
Cycles
(d)
Fermentative efficiency (%)
S. cerevisiae + D. bruxellensis (with PMB)
S. cerevisiae + D. bruxellensis (with PMB)
S. cerevisiae (without PMB)

Figure 5 Number of yeasts (CFU ml^{-1}, in a), residual total reducing sugars (g 100 ml^{-1}, in b), alcohol production (g 100 ml^{-1}, in c) and fermentative efficiency (%, in d) of the fermentation trials with the pure culture of *Saccharomyces cerevisiae* inoculated in sugar cane juice medium, 16 g 100 ml^{-1} of reducing sugars, pH 4·5, at 30°C, for six fermentation cycles lasting 12 h each. Potassium metabisulphite (PMB) was not added to the fermentation medium. Cells were recycled from one fermentation cycle to another after centrifugation of the fermented medium. 'Start' means the number of yeasts inoculated at the beginning of the fermentation process. Number of yeasts was quantified after 12 h of fermentation in each fermentative cycle. Number of yeasts was quantified after 12 h of fermentation in each fermentative cycle. Different letters over the bars indicate significant differences ($P < 0{\cdot}05$).

duced/g sugar consumed) divided by 0·51 (theoretical value) and multiplied by 100.

Statistical analysis

Yeast counts were transformed into $\log_{10}$ CFU ml^{-1}. The experiments were replicated at least twice using two samples per treatment. Mean values and standard deviations were determined with Microsoft Excel (Microsoft Windows XP). All statistical analyses (ANOVA and Tukey's test) were performed using Statistica® version 6.0 (StatSoft, Tulsa, OK, USA), and data were considered to be significant different when $P < 0{\cdot}05$.

Acknowledgements

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Conflict of Interest

No conflict of interest declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Growth of *S. cerevisiae* (strain PE-2) and *D. bruxellensis* (strains CCA059, 077 and 155) in YPD medium, pH 4·5, with potassium metabisulphite added in the concentrations of 0 (♦), 100 (■), 200 (▲) and 400 (●) mg l^{-1}.

Figure S2. Growth of *S. cerevisiae* (strain PE-2) and *D. bruxellensis* (strains CCA059, 077 and 155) in sugar cane medium, pH 4·5, with potassium metabisulphite added in the concentrations of 0 (♦), 100 (■), 200 (▲) and 400 (●) mg l^{-1}.

Figure S3. Growth of *S. cerevisiae* (strain PE-2) and *D. bruxellensis* (strains CCA059, 077 and 155) in molasses medium, pH 4·5, with potassium metabisulphite added in the concentrations of 0 (♦), 100 (■), 200 (▲) and 400 (●) mg l^{-1}.

Figuras referentes ao Capítulo 4

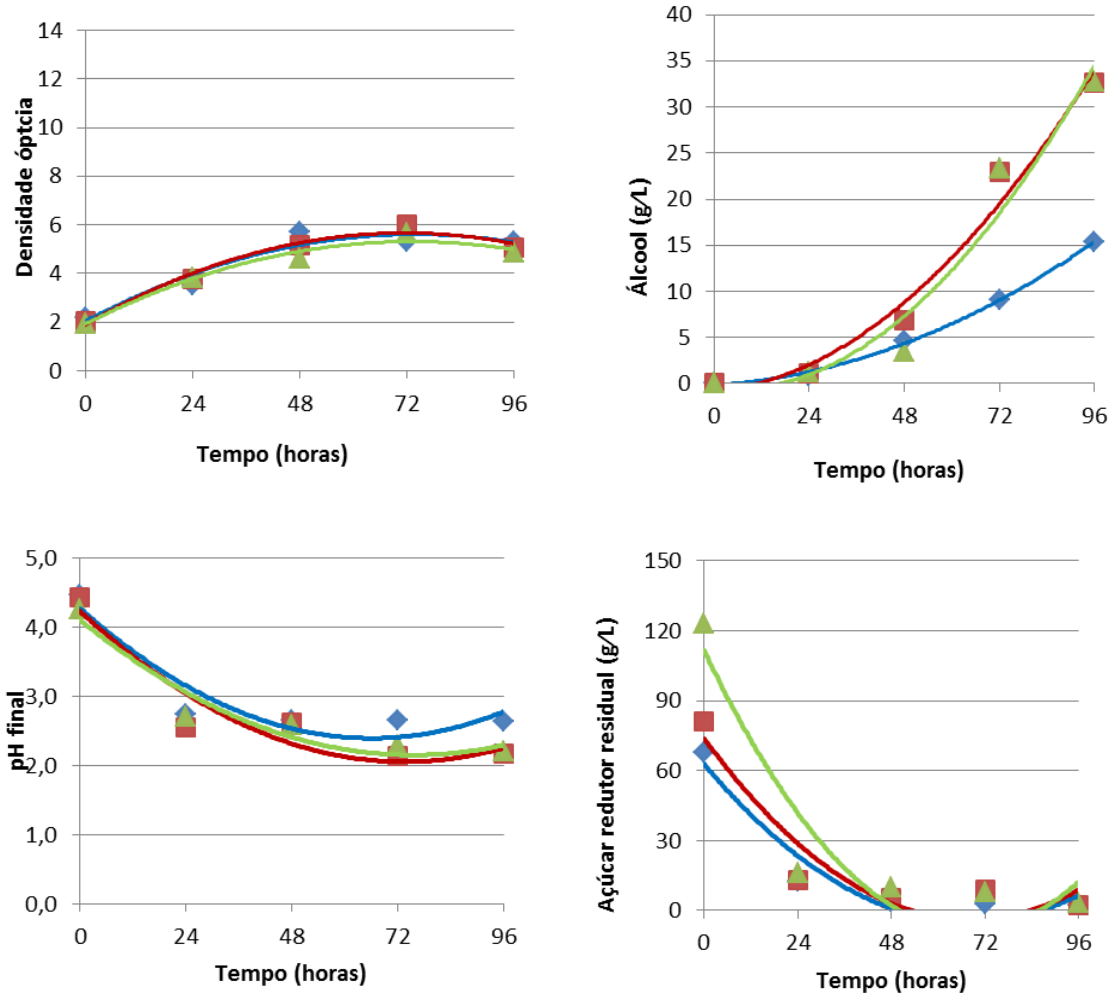

Figura 1 - Densidade óptica (a 600 nm), produção de álcool (g/L), pH final e açúcar redutor total residual (g/L) do cultivo da levedura *Dekkera bruxellensis* (155), em meio contendo glicose como única fonte de carbono, em concentrações de 50 g/L (◆), 100 g/L (■) e 150 g/L (▲), a 0 rpm, 30ºC, por 96 horas.

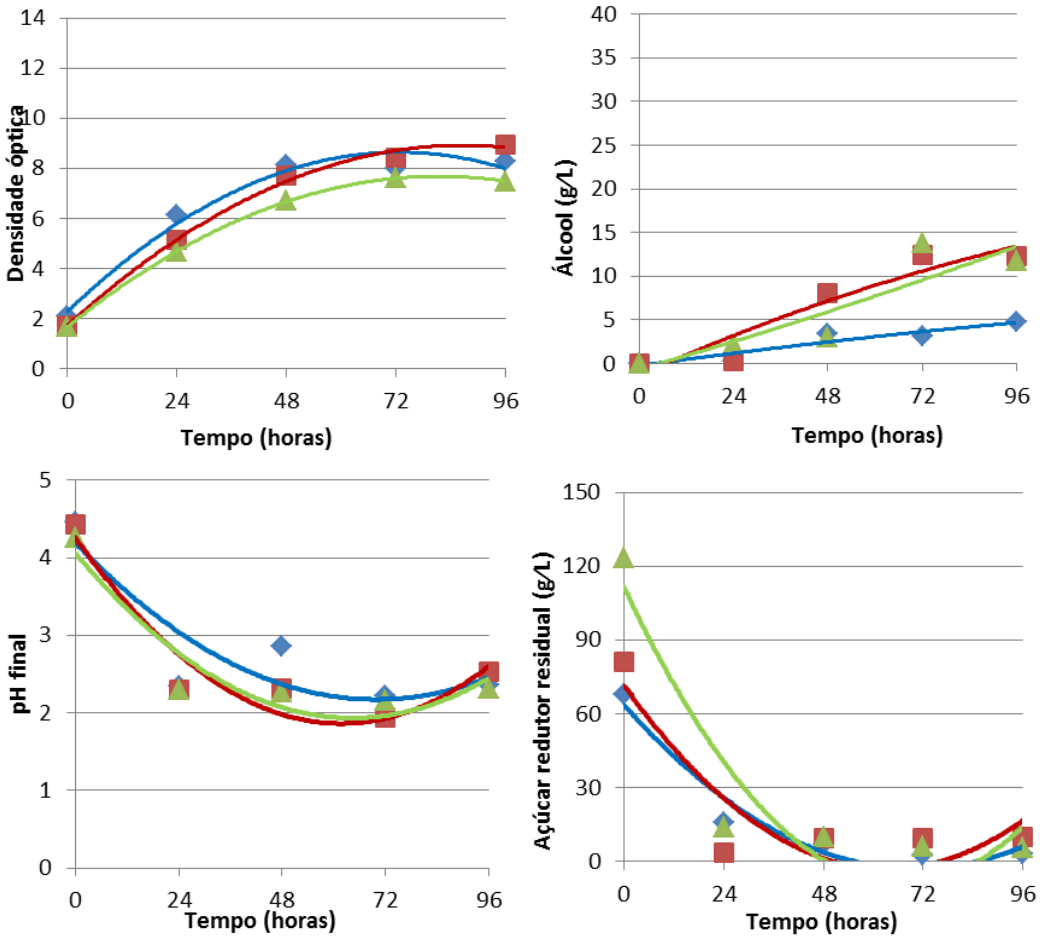

Figura 2 - Densidade óptica (a 600 nm), produção de álcool (g/L), pH final e açúcar redutor total residual (g/L) do cultivo da levedura *Dekkera bruxellensis* (155), em meio contendo glicose como única fonte de carbono, em concentrações de 50 g/L (◆), 100 g/L (■) e 150 g/L (▲), a 150 rpm, 30ºC, por 96 horas.

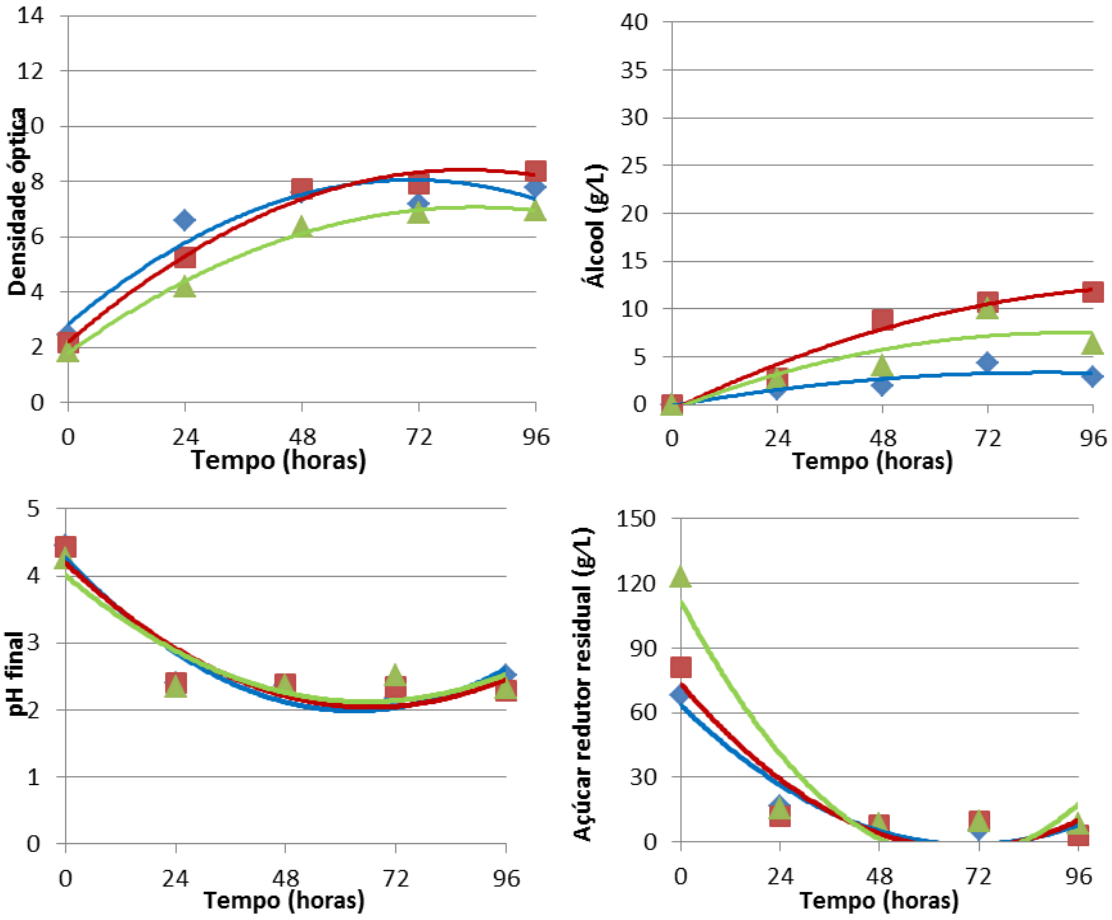

Figura 3 - Densidade óptica (a 600 nm), produção de álcool (g/L), pH final e açúcar redutor total residual (g/L) do cultivo da levedura *Dekkera bruxellensis* (155), em meio contendo glicose como única fonte de carbono, em concentrações de 50 g/L (◆), 100 g/L (■) e 150 g/L (▲), a250 rpm, 30ºC, por 96 horas.

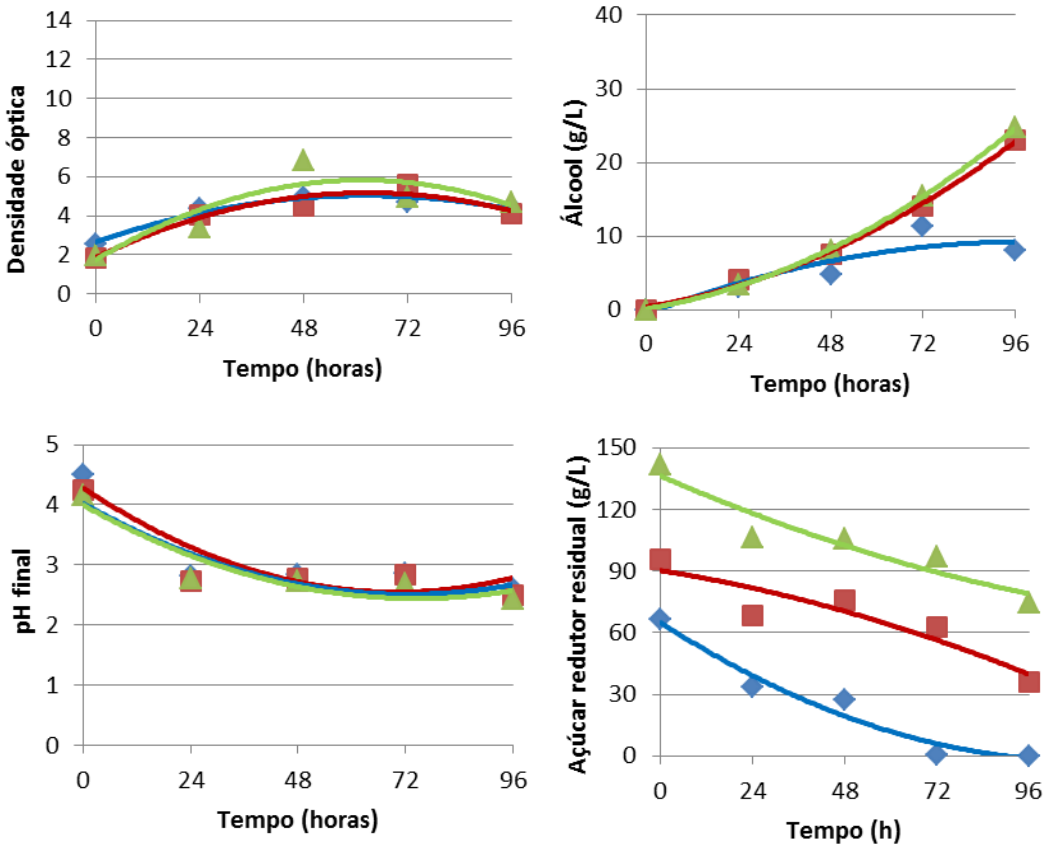

Figura 4 - Densidade óptica (a 600 nm), produção de álcool (g/L), pH final e açúcar redutor total residual (g/L) do cultivo da levedura *Dekkera bruxellensis* (155), em meio contendo sacarose como única fonte de carbono, em concentrações de 50 g/L (◆), 100 g/L (■) e 150 g/L (▲), a 0 rpm, 30ºC, por 96 horas.

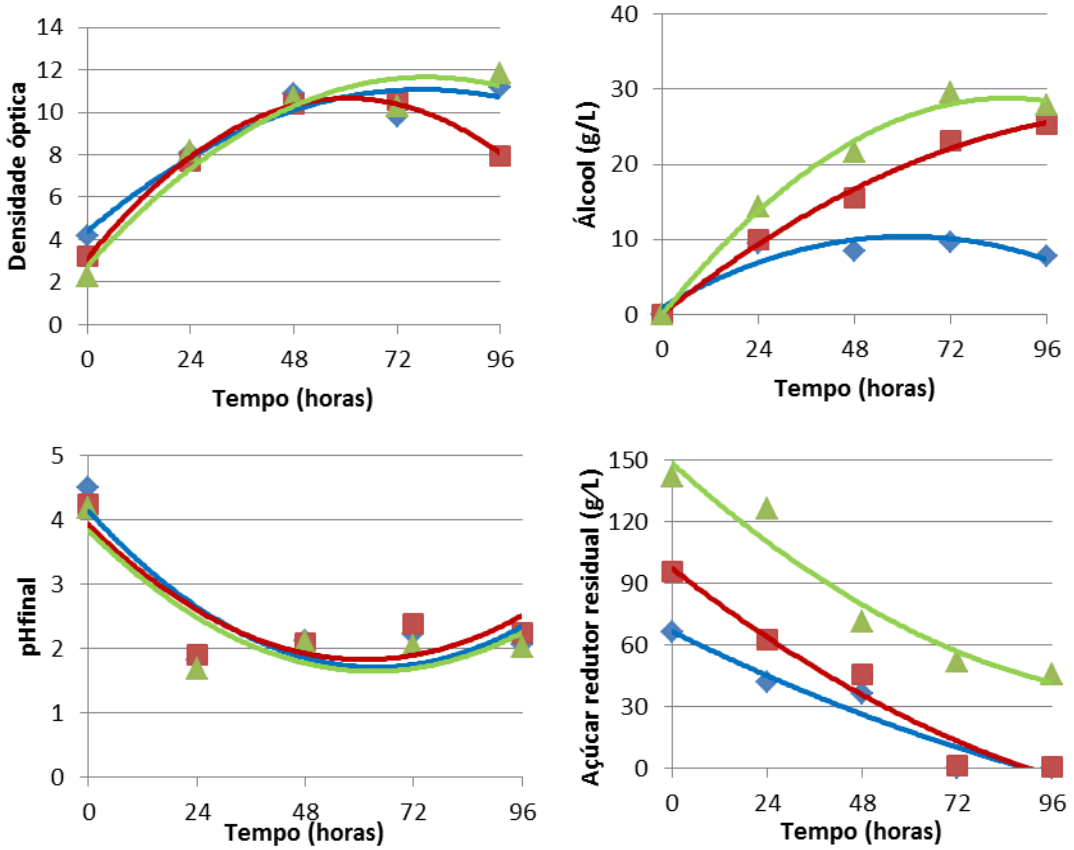

Figura 5 - Densidade óptica (a 600 nm), produção de álcool (g/L), pH final e açúcar redutor total residual (g/L) do cultivo da levedura *Dekkera bruxellensis* (155), em meio contendo sacarose como única fonte de carbono, em concentrações de 50 g/L (◆), 100 g/L (■) e 150 g/L(▲), a150 rpm, 30ºC, por 96 horas.

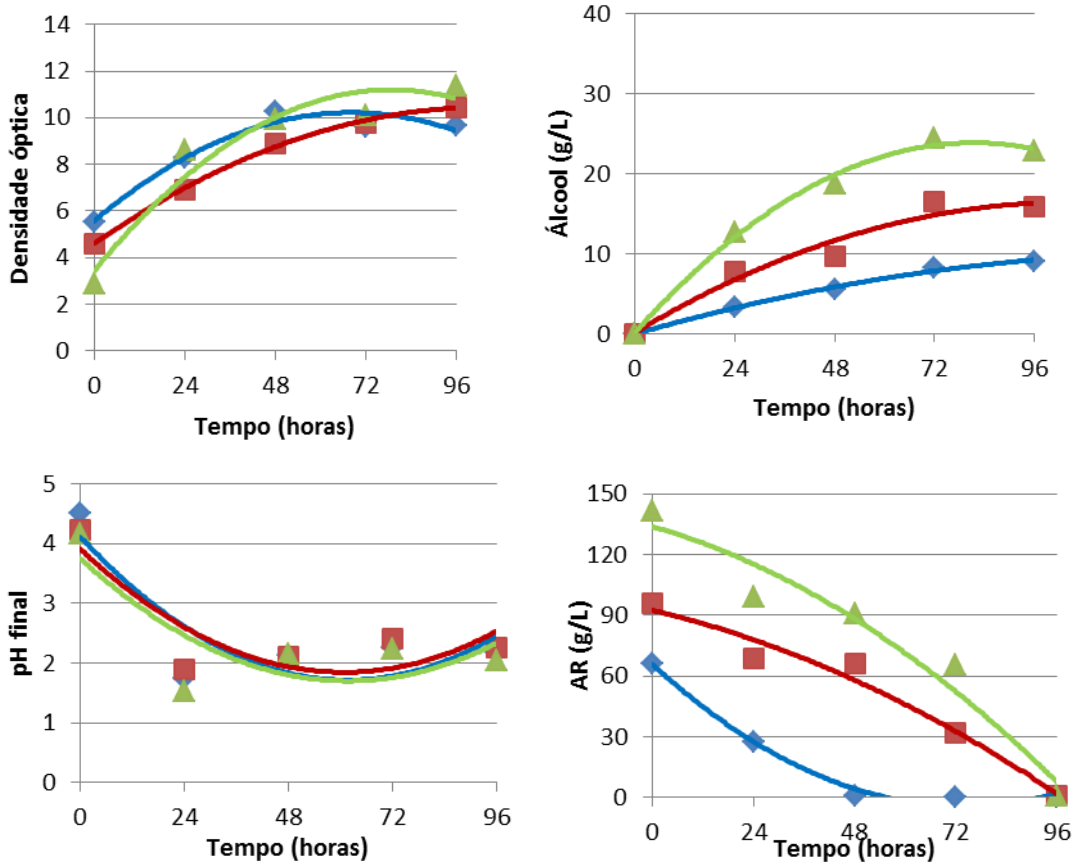

Figura 6 - Densidade óptica (a 600 nm), produção de álcool (g/L), pH final e açúcar redutor residual (g/L) do cultivo da levedura *Dekkera bruxellensis* (155), em meio contendo sacarose como única fonte de carbono, em concentrações de 50 g/L (◆), 100 g/L (■) e 150 g/L (▲), a250 rpm, 30ºC, por 96 horas.