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# *Evaluation of behavior of Lachancea thermotolerans biocontrol agents on grape fermentations*

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**Short running head:** Biocontrol *Lachancea* behavior

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## SIGNIFICANCE AND IMPACT OF THE STUDY

Generally it is not evaluated if the biofungicide yeasts sprayed on vegetables alter the quality of the fermented products. This work focused in the importance of assessing the possible effects of fungicides based on yeasts used in vineyards on grape fermentative, especially on *S. cerevisiae* growth. In this context, the competition between biofungicide yeasts and *S. cerevisiae* under winemaking conditions will be investigated.

## ABSTRACT

In previous researches, *L. thermotolerans* RCKT4 and RCKT5 were showed that inhibited *Aspergillus* growth. However, currently there are no data about their nutritional preferences, as a possible substrate competitor against *S. cerevisiae*, and their effects on fermentative process. In the present work we observed that the biocontrol yeasts and *S. cerevisiae* BSc203, based in the utilization of 16 carbonate sources, reveled significantly differences in the nutritional profile (biocontrol yeasts NS:0.25, BSc203 NS:0.56). *L. thermotolerans* strains did not occupy the same niche that BSc203 (NOI:0.44). The biocontrol agents and BSc203 presented similar competitive attitude in terms of the sugar, ethanol, and sulphite tolerances. In fermentative conditions, the biocontrol yeasts found to tolerate until 12%v/v ethanol,

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250mg $ml^{-1}$ of total $S0_2$ and 30°Brix sugar. In mixed cultures, *L. thermotolerans* strains did not negatively affect BSc203 growth and the wine quality, except when RCKT4 was initially inoculated at a high proportion in the mixed culture 1MSK4 (1%BSc203/99%RCKT4), resulting in a lower production of $CO_2$ and ethanol, in comparison with BSc203 pure. RCKT5, at a high proportion in 1MSK5 (1%BSc203/99%RCKT5), presented promising oenological properties. This fermentation showed lower acetic acid contents and higher total acidity than pure BSc203.

**Keywords:** *L. thermotolerans*, biocontrol, *S. cerevisiae*, substrate competition, behavior, fermentations.

## INTRODUCTION

Grapes are susceptible to fungal diseases, especially grey rot, downy mildew and black rot (Covarelli *et al*., 2012). Conventional approaches to fungal control have focused on chemical applications. However, sole reliance on this approach is not sustainable because of the emergence of fungicide resistance in vineyards (Leroch *et al*., 2011) and the adverse effects of chemical pesticides on the environment and human health (Komárek *et al*., 2010). A biological approach is highly desirable to control fungal growth on grapes, as this would help to reduce the amount of agrochemical residues on grape, wine and related products (Cabras and Angioni, 2000). Among the various potential antagonists, yeasts have been studied as fungal biocontrol agents on grapes (Nally *et al*., 2012; 2013; Calvo- Garrido *et al*., 2013; Ponsone *et al*., 2011; 2016). The major mode of action of these yeasts is the competition for nutrient and space (Nally *et al*., 2015). Few data have been published about the influence of yeast-based biofungicide used in vineyards on grape fermentative process (Calvo-Garrido *et al.*, 2013; Guzzon *et al*., 2014). Generally it is not evaluated if the antifungal yeasts sprayed on vegetables alter the quality of the fermented products, and if these microorganisms

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continue competing for nutrients and space, especially with *S. cerevisiae* in fermentative process.

Some investigators have been reported that strains belonging to *L. thermotolerans* specie have been increased the acidity (Kapsopololus *et al.*, 2007; Balikci et al., 2016), the aroma complexity (Escribano et al., 2018) or secondary ones as biogenic amines reduction (Benito *et al.* 2015), aroma complexity (Escribano *et al.*, 2018) or reduction in anthocyanin loses during fermentation (Benito *et al.* 2018).

Ponsone *et al*. (2011, 2016) found that two *L. thermotolerans* strains, RCKT4 and RCKT5, increased the lag phase, diminished the *in vitro* growth rate of *Aspergillus* and also decreased OTA accumulation in wine grapes. The use of biofungicide *Lachancea* yeasts in vineyards produce wine without presence of mycotoxins. However, it is unknown if these microorganisms affect the fermentative process. Previous investigations reported that some non-*Saccharomyces* were capable to persist throughout the fermentation process and compete with *Saccharomyces* for nutrients, causing a fermentative stuck (Fleet and Heard, 1992; Bisson, 1999). Because there is little information about the oenological behavior of biofungicide yeasts during grape fermentations, the aims of this study were: **1**-To evaluate the competition for nutrients between biocontrol yeasts and *S. cerevisiae*: Nutritional size (NS), Niche Overlap index (NOI). **2**-To evaluate behavior of biocontrol yeasts in fermentative conditions: $S0_2$, ethanol, sugar tolerances, effects on BSc203 growth, persistence time and wine quality in mixed cultures Biofungicide/*Lachancea.*

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## RESULTS AND DISCUSSION

### Nutritional and oenological behavior of *L. thermotolerans* RCKT4 and RCKT5.

### Nutritional Size (NS) and Niche overlap index (NOI)

The two biocontrol yeasts assimilated the same carbon sources *in vitro*. From 16 carbon sources tested, 4 were utilized by RCKT4 and RCKT5 (NS:0.25), and 9 were utilized by *S. cerevisiae* BSc203 (NS:0.56) **(Table 1).** Glucose, sucrose, raffinose and arginine were used by all the yeasts strains tested. Proline, asparagine, alanine, fructose and melibiose were used only by BSc203. The biocontrol strains did not occupy the same ecology niche than BSc203 (NOI:0.44), showing a low level of competence between biocontrol yeasts and BSc203 **(Table 1).** These results suggested that biocontrol strains were not able to successfully assimilate a wide variety of nutrients of the wine grape, making them available to BSc203. The NOI between yeast-filamentous fungi (La Penna *et al.,* 2004; Nally *et al.,* 2015), bacteria-bacteria (Jaspers and Overmann, 2004) and bacteria-filamentous fungi (Nesci *et al.,* 2005) have been previously studied. There is only one publication about the NOI between yeasts the same genera (Janisiewicz, 1996). At present, this work provides new data on NOI between yeasts isolates from grape musts which belong to different genera.

### Tolerances to $S0_2$, sugar and ethanol

In the present study it was observed that biocontrol yeasts were able to ferment in media with 25 to 250mg $l^{-1}$ $SO_2$, but they were unable to ferment in media with 300-400mg $l^{-1}$ $SO_2$ **(Table 2).** Comitini *et al.* (2011) showed that *L. thermotolerans* isolates assayed were less resistant to $SO_2$ than RCKT4 and RCKT5. These strains did not ferment grape musts with 20-

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30mg $l^{-1}$ $SO_2$. The discrepancy between results may be explained by differences in the concentration of extracellular acetaldehyde (Nadai *et al.,* 2016; Stanley *et al.,* 1993). RCKT4 and RCKT5 were able to tolerate high ethanol (7-11%v/v) and sugar (21 and 30° Brix) levels **(Table 2).** Levels of ethanol tolerance in the present study exceeded values reported by Kapsopoulou *et al.* (2005), who observed that *L. thermotolerans* strains did not tolerate must with 9%v/v ethanol. Gobbi et al. (2013) mentioned that *L. thermotolerans* presented a high fermentation power (10.46%) too. The discrepancy between results on tolerances may be explained by differences in the plasma membrane fluidity, integrity of strains assayed (Henderson and Block, 2014) or by the strain variability in the fermentations power (Comitini *et al*., 2011).

In general, the biocontrol agents and BSc203 presented similar competitive attitude in terms of the sugar, ethanol, and sulphite tolerances. The current study is the first that provides data on tolerance of *L. thermotolerans* biocontrol agents to ethanol, sugar and $SO_2$ concentrations under winemaking conditions.

**Impact of *L. thermotolerans* strains on *S. cerevisiae* BSc203 and on the wine quality**

In pure cultures, viable cells of RCKT4, RCKT5 and BSc203 were present until the end of the fermentations (22d) **(Figure 1 A, B).** In biocontrol/BSc203 co-cultures, the survival time of RCKT4 and RCKT5 depended on the biocontrol strain used, and the initial ratio of the yeasts assayed. In general, RCKT4 persisted more time than RCKT5. In the RCKT4/BSc203 co-cultures, RCKT4 was detected until day 5 (3MSK4), 11 (2MSK4) and 12 (1MSK4) **(Figure 1A).** RCKT5 was detected until day 2 (3MSK5), 8 (2MSK5) and 10 (1MSK5) **(Figure 1B).** Analyzing investigations, cell viability of *L. thermotolerans* (indicated as *L.t.*) in mixed cultures with *S. cerevisiae* (indicated as *S.c*.) was different. In 50%*L.t.*-50%*S.c.* mixed culture, *L.t.* disappeared in day 7 (Kapsopoulou *et al.,* 2007), 15 (Comitini *et al.,*

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2011), and 17 (Ciani *et al*., 2006). In mixed 90%*L.t.*-10%*S.c.*, *L.t.* was present until day 22 (Gobbi *et al.,* 2013); and in 99%*L.t.*-1%*S.c*. co-culture; *L.t*. was present during 22 days (Comitini *et al.*, 2011). The discrepancy about survival time of *L. thermotolerans* in mixed cultures with *S. cerevisiae* may be to the different initial sugar concentration of the medium used to perform the fermentative assays (between 16 and 27% of sugar). This showed an important notion of how media and the characteristics of the yeast strains may pre-determined the selection of them.

In RCKT4/BSc203 mixed cultures, BSc203 reached its maximum cell population on day 4 in 3MSK4 (7.65$Log_{10}$CFU $ml^{-1}$) and 2MSK4 (7.59$Log_{10}$CFU $ml^{-1}$), and on day 6 in 1MSK4 (6.44$Log_{10}$CFU $ml^{-1}$) **(Figure 2A).** These values were similar to a culture pure BSc203 (7.69$Log_{10}$CFU $ml^{-1}$ on day 3), except for 1MSK4. In BSc203/RCKT5 mixed fermentations, BSc203 reached a maximum cell density: on day 4 in 3MSK5 (7.68$Log_{10}$CFU $ml^{-1}$) and 2MSK5 (7.76$Log_{10}$CFU $ml^{-1}$), and on day 6 in 1MSK5 (7.65$Log_{10}$CFU $ml^{-1}$) **(Figure 2B).** These values were not significantly different to pure BSc203 ($p \leq 0.068$). On day 22, the BSc203 cell concentration in all mixed fermentations assayed was not significantly different to pure BSc203, except for co-culture 1MSK4 **(Figure 2A).** The BSc203 cell concentration in 1MSK4 was 1.38 $Log_{10}$ cycle lower than in a pure BSc203 culture (negative interference). In the present study, a correlation between the decrease in BSc203cell growth in 1MKT4 and the high cell concentration of RCKT4 (6-7$Log_{10}$ CFU $ml^{-1}$ during 12 days) was observed **(Figure 2A).** Several researchers have reported that other non-*Saccharomyces* yeast strains such as *Pichia anomala* and *Hanseniaspora guilliermondii* (Rojas *et al*., 2003) also decreased the final cell density of *S. cerevisiae* in mixed cultures, which is in agreement with our results.

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On day 22, in pure cultures, RCKT4 and RCKT5 produced significantly lower ethanol concentrations (7.91 and 6.05%v/v, respectively) than BSc203 (12.81%v/v) **(Table 3).** RCKT4 and RCKT5 presented a residual sugar concentration of 22.59g $l^{-1}$ and 29.97g $l^{-1}$, respectively, whereas BSc203 completed the fermentation (residual sugar 1.91g $l^{-1}$). The sugar consumption rate of BSc203 during the first three days (16g $l^{-1}$ of sugar consumed) was significantly higher than that of RCKT4 and RCKT5 (6.5 and 6g $l^{-1}$, respectively) ($p \leq 0.05$). As expected, all multistarter cultures with RCKT4 and RCKT5 **(Table 3)** showed ethanol values that were not significantly different to those produced by pure BSc203, ranging from 12.81 to 12.92%v/v, except for 1MSK4. In the latter fermentation, the amounts of ethanol and $CO_2$ produced were significantly lower than in pure BSc203 and the other mixed cultures assayed ($p \leq 0.05$). Similarly, Gobbi *et al.* (2013) reported that ethanol production in a mixed fermentation 90%*L.t*:10%*S.c* was significantly lower than in *S. cerevisiae* used as control. Fermentations in all mixed cultures assayed were completed (residual sugar≤1.96g $l^{-1}$) except for 1MSK4 that presented 8.94g $l^{-1}$ of residual sugar.

Mixed cultures with *L. thermotolerans* presented values for total acidity, volatile acidity and pH that were not significantly different to those in a pure culture of BSc203 ($p \leq 0.05$), except for 1MSK5. In the latter culture, total acidity in wines increased 27.65% and acetic acid reduced 28.57% compared with pure BSc203 culture **(Table 3).** In agreement with these results, other studies showed that *L.t*/*S.c* associations significantly affected positively the final wine composition by enhancing total acidity and reducing the pH (Kapsopoulou *et al.,* 2007) and volatile acidity (Comitini *et al.,* 2011). The oenological industry shows great interest in correcting insufficient acidity (Kapsopoulou *et al.,* 2007) and high volatile acidity (Schutz and Gafner, 1993) of some grape musts from warm regions as San Juan and Mendoza (Argentina).

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These data suggests that at high initial concentrations, RCKT5 is a good candidate for used as biofungicide in wine grapes, because this strain did not affect the *S. cerevisiae* growth and the wine quality. With respect to RCKT4 interfered negatively on fermentative process, especially on *S. cerevisiae* growth and on the ethanol production.

## MATERIALS AND METHODS

### Yeast strains

Biocontrol yeasts: *L. thermotolerans* RCKT4 and RCKT5 were isolated from the grape surfaces from vineyards in Mendoza province, Argentina (Ponsone *et al.*, 2011; 2016).

Oenological yeast: *S. cerevisiae* BSc203 was isolated from fermentation grape must in San Juan province, Argentina. This yeast has proven good fermentative characteristics (Vazquez *et al.*, 2014).

Both the biocontrol strains and BSc203 were identified by restriction fragment length polymorphism (RFLP) (Ponsone *et al.*, 2011; Nally *et al.*, 2012).

### Media

**YEPD-agar:** 10g $l^{-1}$ Yeast Extract, 20g $l^{-1}$ Peptone, 20g $l^{-1}$ Dextrose, 20g $l^{-1}$ agar.

**YEPD-MB-Phosphate Citrate Buffer-agar:** 10g $l^{-1}$ Yeast Extract, 20g $l^{-1}$ Peptone, 20g $l^{-1}$ Dextrose, 0.01% Methylene Blue, 0.1M Phosphate Citrate Buffer, 20g $l^{-1}$ agar.

**CAS-HDTMA-PIPES-YNB-Glucose-agar:** 60.5mg $l^{-1}$ CAS (Chrome Azurol S), 72.9mg $l^{-1}$ HDTMA (Hexadecyltrimethylammonium Bromide), 30.24g $l^{-1}$ PIPES (Piperazine-1,4-bis(2-ethanesulfonic acid)), 6.7g $l^{-1}$ YNB, 1mmol $l^{-1}$ $FeCl_3 \cdot 6H_2O$ in 10mmol $l^{-1}$ HCl, 20g $l^{-1}$ glucose, 20g $l^{-1}$ agar.

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## Characterization of *L. thermotolerans*-based biocontrol agents

### Nutritional profiles: NOI and NS

Biocontrol yeasts and BSc203 aliquots (20µL, $10^6$cells $ml^{-1}$) were inoculated on plates. Each plate contained one carbonate source (10mM), YNB with 20g $l^{-1}$ agar, pH5.5. The carbonate sources assayed are present in wine grapes and represent the niche size: proline, asparagine, alanine, glutamic acid, tyrosine, arginine, lysine, methionine, glycine, malic acid, tartaric acid, fructose, melibiose, raffinose, rhamnose, sucrose and glucose. Plates were incubated at 25°C for 14 days. NOI were evaluated as the ratio between the number of carbonate sources used in common (biocontrol agent and BSc203) and the total number of carbonate sources utilized only by BSc203. NOI values of > 0.9 represent competence between yeasts while scores of<0.9 represent occupation of separate niches. NS values were evaluated as the ratio between number of compounds used by each of the yeasts and number of compounds assayed in total (Collazo *et al.*, 2017).

### Oenological behavior of the biocontrol yeasts. Tolerance to $S0_2$, ethanol and sugar concentrations

Yeast tolerance towards $SO_2$, ethanol and sugar was assayed according to slightly modified methods described by Parish and Carrol (1987). $SO_2$ concentrations evaluated in the present study were 0, 25, 50, 75, 100, 150, 200, 250, 300, 400mg $l^{-1}$ and added to YNB plus 10g $l^{-1}$ of glucose medium (pH3.5). The ability to start fermentations at 7, 8, 9, 10, 11 and 12%v/v of ethanol was determined similarly. Tubes only containing YNB medium without glucose were used as negative controls.

Strain resistance to osmotic-stress was examined by winemaking tests using commercial concentrated grape must from *V. vinifera* L. adjusted to 21°Brix and 30°Brix. The grape juice

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obtained was pasteurized for 30min at 80°C. This process did not produce caramelization of the grape juice following the Maillard reaction (Bozkurt *et al*., 1999).

All assays were carried out in 20ml tubes with 5ml of medium, and tubes were inoculated with $10^6$cells $ml^{-1}$. All microfermentations were checked for $CO_2$ production and considered positive when, after a 3d incubation period at 25°C, Dürham bells located in the tubes were filled up for at least one-third of their capacity (Ubeda *et al.,* 1995). The results are expressed as + (ability to ferment) and - (not ability to ferment). *S. cerevisiae* BSc203 was used as positive control.

**Influence of *L. thermotolerans* strains on *S. cerevisiae* growth during fermentative process**

Commercial must from *V. vinifera* L. was pasteurized as above mentioned. The initial grape must composition was 22°Brix and the pH 3.5. Biocontrol strains and BSc203 were pre-adapted in the same must at 13°Brix and pH3.5, during 12h at 22°C. Microvinifications were carried out in 5l glass flasks with 3l of pasteurized commercial must, and topped with Müller valves (Ciani and Rossini, 1987). The following mixed cultures were assayed: **1MSK4**:1%BSc203/99% RCKT4; **1MSK5**:1%BSc203/99%RCKT5; **2MSK4**:50%BSc203/50%RCKT4; **2MSK5**:50%BSc203/50%RCKT5; **3MSK4**:99%BSc203/1%RCKT4; **3MSK5**:99%BSc203/1%*L.* RCKT5. Pure and mixed cultures were inoculated at an initial concentration of $10^6$cells $ml^{-1}$ and were incubated at 18°C. Pasteurized non-inoculated must was used as negative control under the same assay conditions. Fermentations under static conditions were monitored for $CO_2$ release measuring weight loss every 24h until the end of the fermentation (constant weight). The sugar consumption rate was calculated as the amount of sugar consumed (g $l^{-1}$) in 72h.

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Fermentation samples were withdrawn every 24h and spread on Wallerstein Laboratory Nutrient (WLN). This medium allows putative identification of yeasts according to color of the colonies. On WLN, BSc203 present creamy colonies, whereas RCKT4 and RCKT5 light-green colonies (Vazquez *et al.*, 2014). At the end of the assay, fermented products were centrifuged at 11,000x*g* (10min, 4°C), filtered and stored at 4°C until further analysis. The most important wine quality parameters (ethanol, volatile acidity, total acidity, pH, residual sugar) were analyzed according to the official methods of the OIV (2013) and INV (2015).

**Statistical analysis**

In all the assays, three replicates per treatment were performed and the experiment was repeated twice.

To evaluate the effects of *L. thermotolerans* strains on BSc203 growth and on the wine quality, single-factor variance analysis (ANOVA) was carried out after verification of variance homogeneity (Levene test, $p \leq 0.05$). Significant differences were determined using Tukey's Test. SPSS version 21.0 (Chicago I. L.) was used.

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**CONFLICT OF INTERESTS**

The authors declare no conflicts of interest.

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**Table 1.** Nutritional profile analysis of *L. thermotolerans* yeasts and BSc203.

| Nutritional sources | | *L. thermotolerans* | | BSc203 |
|---|---|---|---|---|
| | | RCKT4 | RCKT5 | |
| Amino acids | Proline | - | - | + |
| | Lysine | - | - | - |
| | Arginine | + | + | + |
| | Asparagine | - | - | + |
| | Alanine | - | - | + |
| | Glycine | - | - | - |
| | Methionine | - | - | - |
| | Tyrosine | - | - | - |
| Organic acids | Glutamic acid | - | - | - |
| | Malic acid | - | - | - |
| | Tartaric acid | - | - | - |
| Carbohydrates | Fructose | - | - | + |
| | Glucose | + | + | + |
| | Sucrose | + | + | + |
| | Raffinose | + | + | + |
| | Rhamnose | - | - | - |
| | Melibiose | - | - | + |
| **Nutritional Ecology** | ***NS*** | 4/16=0.25 | 4/16=0.25 | 9/16=0.56 |
| | ***NOI*** | 4/9=0.44 | 4/9=0.44 | |

**REFERENCES:**

**–** = Not assimilate carbonate source, + = Assimilate carbonate source.

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**Table 2.** Tolerance of biocontrol yeasts to different concentrations of the ethanol (7-12% v/v), sulfur dioxide (25-400 mg $l^{-1}$) and sugar (21 and 30 °Brix).

| | Treatments | Yeast strains | | |
|---|---|---|---|---|
| | | **RCKT4** | **RCKT5** | **BSc203** |
| $SO_2$ | 25 mg $l^{-1}$* | + | + | + |
| | 50 mg $l^{-1}$* | + | + | + |
| | 75 mg $l^{-1}$* | + | + | + |
| | 100 mg $l^{-1}$* | + | + | + |
| | 150 mg $l^{-1}$* | + | + | + |
| | 200 mg $l^{-1}$* | + | + | + |
| | 250 mg $l^{-1}$* | + | + | + |
| | 300 mg $l^{-1}$* | - | - | + |
| | 400 mg $l^{-1}$* | - | - | + |
| Ethanol | 7% v/v* | + | + | + |
| | 8% v/v* | + | + | + |
| | 9% v/v* | + | + | + |
| | 10% v/v* | + | + | + |
| | 11% v/v* | + | + | + |
| | 12% v/v* | - | - | + |
| Grape must | 21°Brix | + | + | + |
| | 30°Brix | + | + | + |

**REFERENCES:**

**+:** ability to ferment, -: not ability to ferment

* in YNB+glucose

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**Table 3**. Influence of *L. thermotolerans* strains on the analytical profile of wines in pure and mixed cultures with BSc203. Values followed by the same letter in the same column were not significantly different at $p \leq 0.05$.

| Cultures | Sugar consumption rate in 72h ($g\ l^{-1}$) | Ethanol ($g\ l^{-1}$) | Total acidity ($g\ l^{-1}$) | Volatile acidity ($g\ l^{-1}$) | Residual sugar ($g\ l^{-1}$) | pH |
|---|---|---|---|---|---|---|
| **Pure BSc203** | 16±0.01$^{a}$ | 12.81±0.21$^{a}$ | 5.17±0.12$^{a}$ | 0.56±0.02$^{a}$ | 1.91±0.11$^{a}$ | 3.39±0.01$^{a}$ |
| **3MSK4** | 15.2±0.4$^{ab}$ | 12.75±0.16$^{a}$ | 5.19±0.13$^{a}$ | 0.58±0.03$^{a}$ | 1.9±0.11$^{a}$ | 3.38±0.01$^{a}$ |
| **2MSK4** | 15.3±0.034$^{ab}$ | 12.83±0.05$^{a}$ | 5.21±0.21$^{a}$ | 0.53±0.06$^{a}$ | 1.88±0.03$^{a}$ | 3.38±0.02$^{a}$ |
| **1MSK4** | 14.7±0.05$^{ab}$ | 11.01±0.03$^{b}$ | 5.28±0.3$^{a}$ | 0.5±0.09$^{a}$ | 8.94±0.11$^{b}$ | 3.37±0.02$^{a}$ |
| **3MSK5** | 15.8±0.031$^{a}$ | 12.83±0.08$^{a}$ | 5.36±0.11$^{a}$ | 0.55±0.02$^{a}$ | 1.61±0.14$^{a}$ | 3.39±0.02$^{a}$ |
| **2MSK5** | 14.5±0.02$^{ab}$ | 12.71±0.06$^{a}$ | 5.45±0.06$^{a}$ | 0.5±0.02$^{a}$ | 1.82±0.18$^{a}$ | 3.37±0.03$^{a}$ |
| **1MSK5** | 15.1±0.03$^{a}$ | 12.92±0.04$^{a}$ | 6.6±0.13$^{b}$ | 0.40±0.03$^{b}$ | 1.96±0.02$^{a}$ | 3.35±0.01$^{b}$ |
| **Pure RCKT4** | 6.5±0.04$^{b}$ | 7.91±0.41$^{c}$ | 6.66±0.15$^{b}$ | 0.39±0.01$^{c}$ | 22.59±2.01$^{c}$ | 3.34±0.02$^{b}$ |
| **Pure RCKT5** | 6±0.07$^{c}$ | 6.05±0.09$^{d}$ | 9.43±0.4$^{c}$ | 0.26±0.05$^{d}$ | 29.27±2.18$^{d}$ | 3.32±0.02$^{b}$ |

**REFERENCES:**

**1MSK4:**1%*S.c*/99%RCKT4; **2MSK4:**50%*S.c*/50%RCKT4; **3MSK4:**99%*S.c*/1%RCKT4.

**1MSK5:**1%*S.c*/99%RCKT5; **2MSK5:**50%*S.c*/50%RCKT5; **3MSK5:**99%*S.c*/1%RCKT5.

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**Figure 1**. Population dynamics of **RCKT4** and **RCKT5** in pure and mixed cultures with BSc203 (3MSK4, 2MSK4, 1MSK4 **[A]** and 3MSK5, 2MSK5, 1MSK5 **[B]).** The data are presented as the average of three independent experiments. References: **A**: 100% RCKT4, RCKT4 in 2MSK4 (50%Sc-50%RCT4), RCKT4 in 3MSK4 (99%Sc-1%RCKT4), RCKT4 in 1MSK4 (1%Sc-99%RCT4). **B**: 100% RCKT5, RCKT5 in 2MSK5 (50%Sc-50%RCKT5), RCKT5 in 3MSK5 (99%Sc-1%RCKT5), RCKT5 in 1MSK5 (1%Sc-99%RCT5).

**Figure 2.** Population dynamics of **BSc203** in pure and mixed cultures with RCKT4 (3MSK4, 2MSK4, 1MSK4) **[A]** and with RCKT5 (3MSK5, 2MSK5, 1MSK5) **[B].** The data are presented as the average of three independent experiments. References: **A** 100% BSc203, BSc203 in 2MSK4 (50%Sc-50%RCT4), BSc203 in 3MSK4 (99%Sc-1%RCKT4), BSc203 in 1MSK4 (1%Sc-99%RCT4).

**B**: 100% RCKT5, RCKT5 in 2MSK5 (50%Sc-50%RCKT5), RCKT5 in 3MSK5 (99%Sc-1%RCKT5), RCKT5 in 1MSK5 (1%Sc-99%RCT5).

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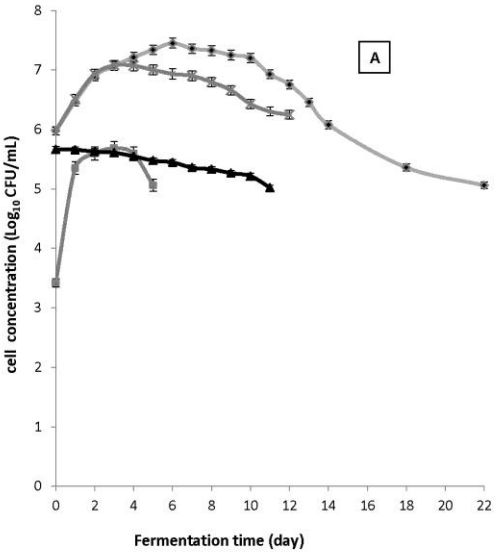


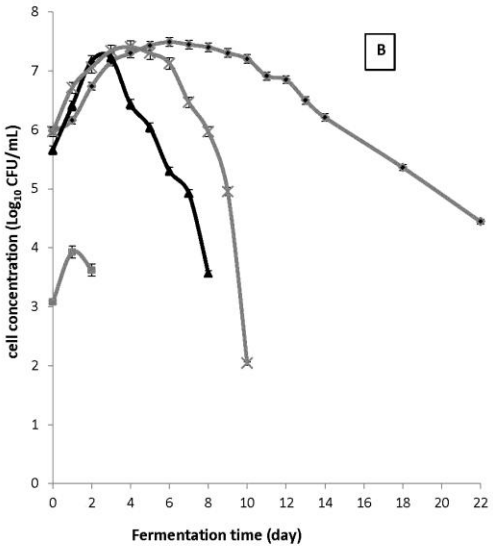


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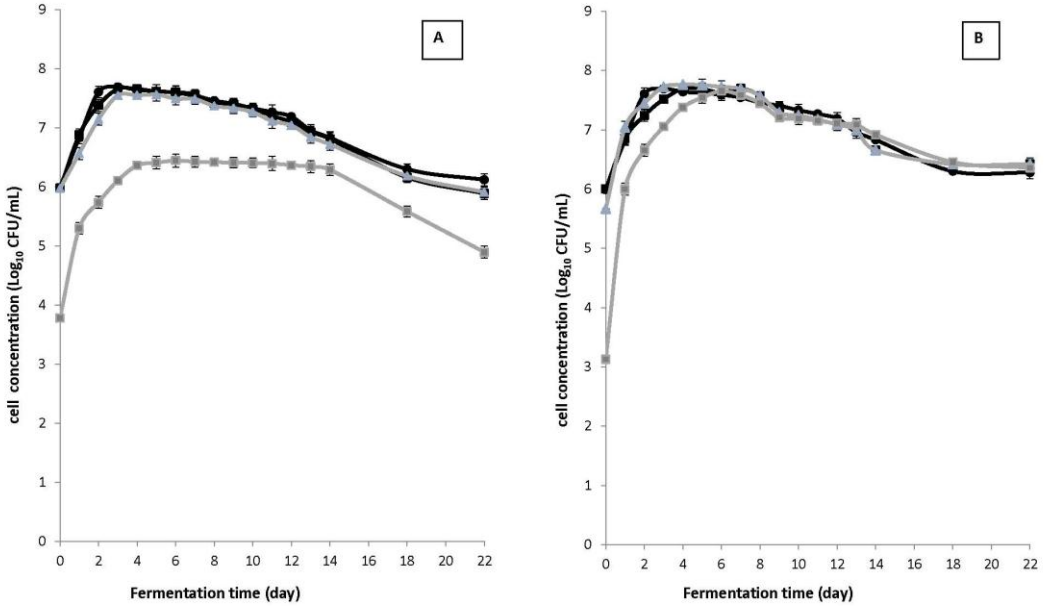


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