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1 ***Saccharomyces kudriavzevii* and *Saccharomyces uvarum* differ from *Saccharomyces***
2 ***cerevisiae* during the production of aroma-active higher alcohols and acetate esters using**
3 **their amino acidic precursors**
4
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12 **Abstract**

13 Higher alcohols and acetate esters are important flavour and aroma components in the food
14 industry. In alcoholic beverages these compounds are produced by yeast during fermentation.
15 Although *S. cerevisiae* is one of the most extensively used species, other species of the
16 *Saccharomyces* genus have become common in fermentation processes. This study analyses
17 and compares the production of higher alcohols and acetate esters from their amino acidic
18 precursors in three *Saccharomyces* species: *S. kudriavzevii*, *S. uvarum* and *S. cerevisiae*.
19 The global volatile compound analysis revealed that *S. kudriavzevii* produced large amounts
20 of higher alcohols, whereas *S. uvarum* excelled in the production of acetate esters. Particularly
21 from phenylalanine, *S. uvarum* produced the largest amounts of 2-phenylethyl acetate, while
22 *S. kudriavzevii* obtained the greatest 2-phenylethanol formation from this precursor.
23 The present data indicate differences in the amino acid metabolism and subsequent production
24 of flavour-active higher alcohols and acetate esters among the closely related *Saccharomyces*
25 species. This knowledge will prove useful for developing new enhanced processes in
26 fragrance, flavour, and food industries.

27

28 *Keywords:* Higher alcohols, acetate esters, non-conventional yeasts, flavour compounds

29

30 ## 1. Introduction

31 It is widely accepted that the acetate esters and higher alcohols produced during
32 fermentation by yeast are particularly important for the food industry. Given their desired
33 fruity and floral aroma, acetate esters significantly contribute to the aroma character of
34 alcoholic beverages (Lambrechts and Pretorius, 2000; Swiegers et al., 2005). The most
35 significant esters are ethyl acetate (solvent-like aroma), isobutyl acetate (fruity), isoamyl
36 acetate (banana), and 2-phenylethyl acetate (flowery, rose-like) (Lambrechts et al., 2000;
37 Styger et al., 2011). These esters are synthesised by alcohol acetyltransferases from acetyl-
38 CoA and ethanol (ethyl acetate) or aliphatic or aromatic higher alcohols. Aliphatic (also
39 branched-chain) higher alcohols, which include isobutyl alcohol, active amyl alcohol, and
40 isoamyl alcohol, are formed as part of the degradation of branched-chain amino acids valine,
41 isoleucine and leucine, respectively. The major component of aromatic higher alcohols is 2-
42 phenylethanol, which is produced from aromatic amino acid phenylalanine (Dickinson et al.,
43 1997; 1998; 2000; 2003; Nykanen, 1986).

44 Aroma compounds are synthetized mainly by the *Saccharomyces cerevisiae* species during
45 food-related fermentations. However, other related species belonging to the *Saccharomyces*
46 genus, such as *Saccharomyces kudriavzevii* and *Saccharomyces uvarum*, can potentially be of
47 interest for aroma production. The phylogenetic similarities between these species and their
48 closely related *S. cerevisiae* prompted the formation of natural interspecific hybrids, which
49 are present in wine and brewing environments (Gonzalez et al., 2007; 2008). In this context,
50 the fermentative abilities of these two species, and their hybrids, have been recently
51 investigated (Arroyo-Lopez et al., 2010; Combina et al., 2012; Gonzalez et al., 2006; Lopez-
52 Malo et al., 2013; Naumov et al., 2000; 2001; Oliveira et al., 2014; Sampaio and Goncalves,
53 2008; Tronchoni et al., 2014). The above-cited studies describe significant differences in the
54 impact of these two species on the aromatic qualities of alcoholic beverages when compared

55 to *S. cerevisiae*. Specifically, *S. kudriavzevii* and *S. uvarum* have interesting oenological
56 properties which lead, for instance, to greater glycerol production or lower ethanol production
57 compared to *S. cerevisiae* (Gamero et al., 2013; Oliveira et al., 2014). The wines produced by
58 *S. uvarum* strains also have a stronger aromatic intensity than those produced by *S. cerevisiae*
59 (Coloretti et al., 2006; Eglinton et al., 2000).

60 The present study explores differences in the production of prime aroma-active acetate
61 esters and higher alcohols by *S. kudriavzevii*, *S. uvarum* and *S. cerevisiae*. As valine,
62 isoleucine, leucine and phenylalanine are the precursors of these higher alcohols, which
63 subsequently lead to acetate esters, these four amino acids were used as the sole nitrogen
64 source for the growth of these species. Next, the production of the corresponding higher
65 alcohols and esters was analysed. Ammonium and the mixture of those four amino acids were
66 also used as the nitrogen sources to better obtain a comparison of how the three
67 *Saccharomyces* species deal with nitrogen in terms of major flavour-active volatile
68 compounds formation.

69 **2. Materials and Methods**

70 *2.1. Yeast strains*

71 The yeast strains used in this work were *S. kudriavzevii* IFO 1802 (originally isolated from
72 decayed leaves in Japan (Kaneko and Banno, 1991)), *S. uvarum* CECT 12600 (isolated from
73 sweet wine in Alicante, Spain, obtained from the Spanish Type Culture Collection), and
74 commercial wine strain *S. cerevisiae* T73 (originally isolated from wine in Alicante, Spain
75 (Querol et al., 1992)).

76

77 2.2. *Cultivation to study the production of acetate esters and higher alcohols*

78 The cultivations were performed in triplicate using a synthetic medium containing 0.17%
79 YNB w/o AAs & $(NH_4)_2SO_4$ (DIFCO, USA) and 2% glucose as the carbon source. Media
80 were supplemented by different nitrogen sources. The concentration was 5 g/L when
81 $(NH_4)_2SO_4$ was used as the nitrogen source, as recommended by the provider. When
82 individual amino acids were used as the nitrogen source, the concentrations were proportional
83 to $(NH_4)_2SO_4$ (to obtain the same nitrogen content), as follows: 10 g/L leucine, 10 g/L
84 isoleucine, 12.5 g/L phenylalanine, 8.9 g/L valine (Bolat et al., 2013). The mix of these amino
85 acids was also used as the nitrogen source. In this case the total amino acids concentration
86 was 10 g/L and the proportional concentrations were 2.5 g/L leucine, 2.5 g/L isoleucine, 3 g/L
87 phenylalanine and 2 g/L valine.

88 Starter cultures were prepared by pregrowing yeast in 15-mL tubes containing 4 mL of the
89 standard complex media. Before the inoculation of the experimental culture, the grown
90 precultures were washed with water and resuspended in the same synthetic medium (with a
91 certain nitrogen source), as used in the assay. Cells were resuspended in such a volume to
92 achieve an OD_{600} of 1.7. These precultures (100 μL) were used to inoculate 1.6 mL of the
93 synthetic media. Then the initial OD_{600} was 0.1. Cultivation was performed in 96-well plates

94 with 2mL-deep wells. Wells were covered by a transparent microplate sealer (Greiner bio-
95 one, Germany) to avoid evaporation and loss of volatile flavour compounds. Cultures were
96 incubated for 5 days at 25°C. The individual 1.7-mL cultures were later transferred to 2-mL
97 tubes and were stored at -20°C for the analysis.

98

99 *2.3. Analysis of yeast growth*

100 Growth of yeast cells was followed using a 96-well plate. Synthetic media were
101 supplemented with different nitrogen sources (ammonium or amino acids), as described
102 above. Then 100 µl of media were inoculated in a well with 2 µl of cell suspension with
103 OD_{600} = 1. Growth was monitored in a Spectrostar Nano absorbance reader (BMG Labtech,
104 Germany).

105

106 *2.4. Higher alcohols and esters determination*

107 The samples stored in the 2-mL tubes were centrifuged (13000 rpm, 2 min) and 1.5 mL of
108 the supernatant was transferred to 15-mL vials with 0.35 g of NaCl. The 20-µl volume of 2-
109 heptanone (0.005%) was added as an internal standard. Higher alcohols and esters were
110 analysed by the headspace solid phase microextraction (HS-SPME) technique using a 100-µm
111 poly-dimethylsiloxane (PDMS) fibre (Supelco, Sigma-Aldrich, Spain). Solutions were
112 maintained for 2 h at 25°C to establish the headspace-liquid equilibrium. The fibre was
113 inserted through a vial septum into the headspace and was held for 7 min. The fibre was then
114 inserted into the gas chromatograph inlet port for 4 min at 220°C with helium flow (1
115 mL/min) to desorb analytes. A Thermo Science TRACE GC Ultra gas chromatograph with a
116 flame ionization detector (FID) was used, equipped with an HP INNOWax 30 m x 0.25 m
117 capillary column coated with a 0.25-m layer of cross-linked polyethylene glycol (Agilent
118 Technologies). The oven temperature programme was: 5 min at 35°C, 2°C/min to 150°C,

119 20°C/min to 250°C and 2 min at 250°C. The detector temperature was kept constant at 300°C.
120 A chromatographic signal was recorded by the ChromQuest programme. Volatile compounds
121 were identified by the retention time for reference compounds. Quantification of the volatile
122 compounds was determined using the calibration graphs of the corresponding standard
123 volatile compounds.

124

125 *2.5. Statistical analysis*

126 The presented values are averages of biological triplicates with standard errors. The
127 differences between measured volatile compounds were determined by a one-way ANOVA,
128 followed by Tukey's HSD test (statistical level of significance was set at $P \leq 0.05$). The
129 analysis was performed using the STATISTICA 7.0 software (StatSoft, Inc., USA).

130

131 **3. Results**

132 To determine differences during the production of the major aroma-active higher alcohols
133 and esters from their corresponding precursors (branched-chain or aromatic amino acids) by
134 *S. cerevisiae* T73 strain, *S. kudriavzevii* IFO 1802 and *S. uvarum* CECT 12600, yeasts were
135 cultivated in a synthetic medium with particular nitrogen sources. Such a defined medium,
136 using a specific amino acid or ammonium as the nitrogen source, allowed us to avoid the
137 undesirable impact of other non-specific nitrogen sources. Under these conditions, growth
138 was followed and the final aroma composition was determined.

139

140 *3.1. Growth under different nitrogen sources*

141 To test whether yeasts would grow under these specific conditions, the increment of
142 populations over time was monitored (Figure 1). Although slight differences were observed
143 among species at the beginning of the exponential phases, as seen when grown with leucine or
144 valine as the nitrogen source, all the species presented a normal growth pattern, even when
145 one amino acid was used as the only nitrogen source.

146

147 *3.2. Effect of different nitrogen sources on ethyl acetate production*

148 We highlight ethyl acetate production in the analysis of the aroma compounds of the three
149 species under the above-described conditions. Each species produced approximately the same
150 amount of ethyl acetate regardless of the amino acid used as the nitrogen source (Figure 2). In
151 this comparison the lowest concentrations were produced by *S. kudriavzevii*, while *S. uvarum*
152 gave similar amounts to *S. cerevisiae*. When cultivated with either the mixture of amino acids
153 or ammonium, *S. cerevisiae* did not change ethyl acetate production. However, *S. kudriavzevii*
154 and *S. uvarum* produced significantly larger amounts of ethyl acetate when cultivated with the
155 mixture of amino acids than with individual amino acids. When ammonium was employed as

156 the nitrogen source, the ethyl acetate concentration produced by *S. kudriavzevii* was 3-fold
157 higher, and 2.5-fold higher with *S. uvarum*, than *S. cerevisiae*.
158
159 *3.3. Formation of the higher alcohols and esters derived from the corresponding amino*
160 *acid*

161 When valine was used as the sole nitrogen source, isobutanol production by *S. kudriavzevii*
162 was similar to that of *S. cerevisiae*, whereas *S. uvarum* gave much smaller amount
163 (approximately half) (Figure 3). A similar trend was observed when the mixture of amino
164 acids was used as the nitrogen source. *S. uvarum* also produced the smallest amount of
165 isobutanol when cultivated on ammonium. As expected, the highest isobutanol production
166 values for the three species were observed when precursor valine was used. Strikingly, no
167 major differences were observed during isobutyl acetate production for the different nitrogen
168 sources. In all cases, the values ranged from about 0.025 mg/l to 0.070 mg/l.
169 The phenylalanine-grown cultures of *S. kudriavzevii* exhibited the highest 2-phenylethanol
170 production, but the lowest 2-phenylethyl acetate production. On the contrary, *S. uvarum*
171 formed the largest amounts of ester, but smaller (together with *S. cerevisiae*) amounts of the
172 higher alcohol. A similar result was obtained when the nitrogen source used was the mixture
173 of amino acids, which resulted in *S. kudriavzevii* with the highest 2-phenylethanol level and *S.*
174 *uvarum* with the highest 2-phenylethyl acetate level. When ammonium was the nitrogen
175 source, 2-phenylethyl acetate was not detected in any species. Negligible concentrations of 2-
176 phenylethanol were found for *S. kudriavzevii* and *S. uvarum*, and none were seen for *S.*
177 *cerevisiae*.
178 When isoleucine was the nitrogen source, *S. cerevisiae* was the highest producer of both
179 corresponding compounds (active amyl alcohol and amyl acetate). The amyl acetate level was
180 3.7-fold and 2-fold higher than in *S. kudriavzevii* and *S. uvarum*, respectively.

181 When cultivated with leucine, the three species produced isoamyl alcohol similar
182 concentrations, while they exhibited vast differences during isoamyl acetate production. *S.*
183 *uvarum* was the highest producer of this compound. The detected concentration was 1.4-fold
184 higher compared to *S. cerevisiae* and 5-fold higher *vs. S. kudriavzevii*. Similarly, *S. uvarum*
185 exceeded the other two during isoamyl acetate production when grown with the amino acids
186 mixture. *S. kudriavzevii* dominated during isoamyl alcohol production when the mixture of
187 amino acids was used as the nitrogen source.
188
189 *3.4. Comparison of the total higher alcohols and esters produced in response to different*
190 *nitrogen sources*
191 Apart from the major volatiles deriving from the particular amino acid, which was used as
192 the nitrogen source, other higher alcohols and esters were detected. In this context, we also
193 analysed the total amounts of the higher alcohols and esters produced by *S. kudriavzevii* and
194 *S. uvarum* cultivated with different nitrogen sources. Then we compared the data to those of
195 *S. cerevisiae*. As seen in Figure 4, the highest values of the total fusel alcohols were achieved
196 by all the species when grown with the mixture of amino acids. This was not unexpected since
197 each amino acid contributes to the formation of the corresponding higher alcohol.
198 Nevertheless, the total amount of higher alcohols produced by *S. kudriavzevii* under these
199 conditions was interesting as it resulted in a 2-fold larger amount than *S. uvarum*, and in a 1.7-
200 fold larger one when compared to *S. cerevisiae*. In general, *S. kudriavzevii* exhibited the
201 highest levels of the total fusel alcohol concentrations in four of the six nitrogen sources used
202 (amino acid mixture, phenylalanine, leucine and valine), although the last two were
203 comparable with those of *S. cerevisiae*.
204 Conversely, *S. kudriavzevii* did not excel in ester formation, while *S. uvarum* showed high
205 levels of total esters, mainly when cultivated on phenylalanine and leucine. Likewise, *S.*

206 *uvarum* produced a much larger quantity of esters than the other two *Saccharomyces* species
207 when the nitrogen source was the amino acids mixture. When the amino acids mixture was
208 used as the nitrogen source, the trend of the highest total esters values was no longer
209 observed, as it was in the case of the total higher alcohols.

210 **4. Discussion**

211 According to previous studies, *S. kudriavzevii* and *S. uvarum* show differences in several
212 oenological traits, including the production of volatile aroma compounds during wine
213 fermentation (Gamero et al., 2013; 2014). To further examine these differences in detail, we
214 explored the responses to nitrogen sources compared to higher alcohols and acetate esters
215 production. Indeed, the cultivation conditions used in our assays differed from typical
216 fermentation processes. Yet, unlike complex media, the use of defined synthetic media
217 allowed us to explore more precisely how the three species varied in the formation of the
218 higher alcohols and acetate esters deriving from the corresponding amino acids and
219 ammonium.

220 As the results show, the employment of individual amino acids as the nitrogen source led
221 to a much higher production of the measured volatiles than with the other two nitrogen
222 sources (amino acids mixture and ammonium) in all the species. Some exceptions were found
223 during the production of isoamyl alcohol and isobutyl acetate. The largest amounts of isoamyl
224 alcohol were obtained when produced from the amino acids mixture (by *S. kudriavzevii* and *S.*
225 *cerevisiae*). Isobutyl acetate formation did not differ significantly when related to the nitrogen
226 sources. This is surprising if we consider the significantly higher isobutanol (isobutyl acetate
227 precursor) production with valine than with the other two nitrogen sources. This result
228 indicates differences in the metabolism of isobutanol, particularly its subsequent esterification
229 compared to the other higher alcohols.

230 The lowest concentrations of volatile compounds (with no particular differences found
231 among species) were detected when ammonium was the nitrogen source. This result agrees
232 with the observation published by Vuralhan et al. (2003; 2005), in which no activities of the
233 2-oxo-acid decarboxylase involved in the higher alcohol production pathway were detected in

234 the cultures grown on ammonium used as the nitrogen source. Correspondingly, no or very
235 low concentrations of higher alcohols were detected.
236 Relatively large differences were found among species in terms of concentrations of the
237 higher alcohols and acetate esters deriving from their precursor. *S. uvarum*, for instance,
238 surpassed the other two species for 2-phenylethyl acetate production. This result is consistent
239 with previously reported conclusions, which indicated that good 2-phenylethyl acetate
240 production was a typical trait of *S. uvarum* (Antonelli et al., 1999; Gamero et al., 2013;
241 Masneuf-Pomarede et al., 2010).
242 When we summarised and compared the amounts of the total higher alcohols and total
243 esters produced by the three species, *S. uvarum* also showed reasonable ester formation,
244 whereas *S. kudriavzevii* seemed to prefer the production of higher alcohols under these
245 conditions. These differences could have been caused by distinct regulation mechanisms,
246 different gene expression or diverse enzyme activities. For instance, remarkable differences
247 among these species have been observed in the expression levels of those genes involved in
248 the production of flavour compounds during winemaking (Gamero et al., 2014). The genes
249 that codify permeases, transaminases and other enzymes involved in amino acids metabolism
250 were up-regulated in *S. uvarum* compared to *S. kudriavzevii*. *S. kudriavzevii* showed an up-
251 regulation of *ATF2*. This gene, together with *ATF1*, encodes alcohol acetyl transferases,
252 which catalyse the esterification of higher alcohols by acetyl coenzyme A. It has been
253 previously shown in *S. cerevisiae* that Atf2p plays a minor role in esters formation compared
254 to Atf1p (Verstrepen et al., 2003). In *S. kudriavzevii* and/or *S. uvarum*, these data suggest that
255 the roles of Atf1p and Atf2p, and their substrate specificities, might be distinct from *S.*
256 *cerevisiae*. Similar interspecific differences have also been observed between two
257 subgenomes (*S. cerevisiae*-derived and *S. eubayanus*-derived) in lager-brewing hybrid *S.*
258 *pastorianus* by Bolat and coworkers (Bolat et al., 2013). These authors described different

259 roles for two 2-oxo-acid decarboxylase isoenzymes (involved in higher alcohols production),
260 encoded by these two subgenomes. These different roles were based on distinct enzyme
261 characteristics. The *S. cerevisiae* allele was preferentially involved in the production of the
262 higher alcohols derived from the amino acids contained in wort. In contrast, the allele from
263 the *S. eubayanus* subgenome was involved in the formation of the higher alcohols derived
264 from the amino acids synthesised *de novo* by the yeast.

265 It is obvious that the amino acid metabolism is controlled by a complex regulation system.
266 The comparative analysis performed of three different species grown with distinct nitrogen
267 sources revealed remarkable differences during the production of aroma-active higher
268 alcohols and acetate esters. Our results indicate that despite *S. kudriavzevii* and *S. uvarum*
269 being closely related to *S. cerevisiae*, the amino acid metabolism and subsequent production
270 of flavour-active higher alcohols and acetate esters differed among these species. This
271 knowledge can provide new possibilities for yeast-based applications in fragrance, flavour
272 and food industries.

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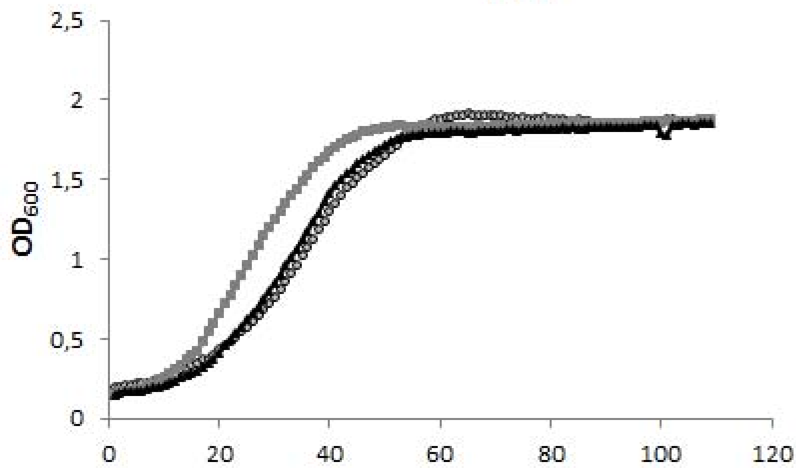
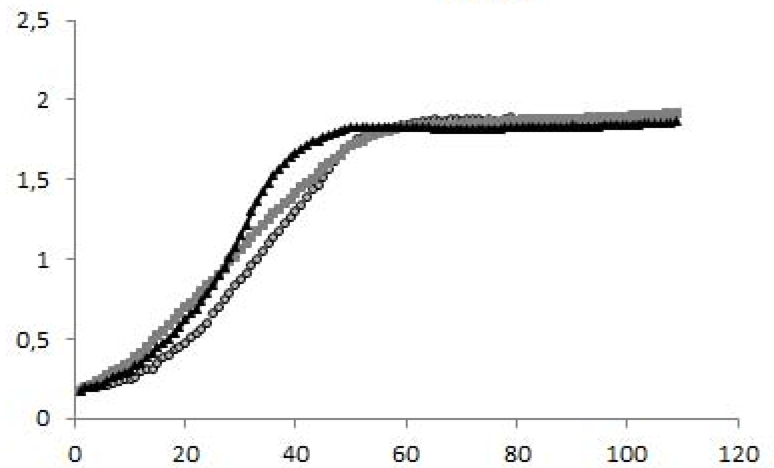
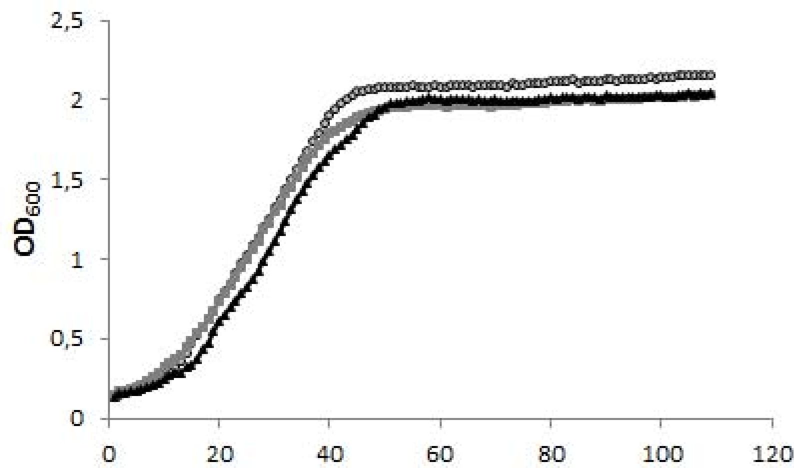
369
370

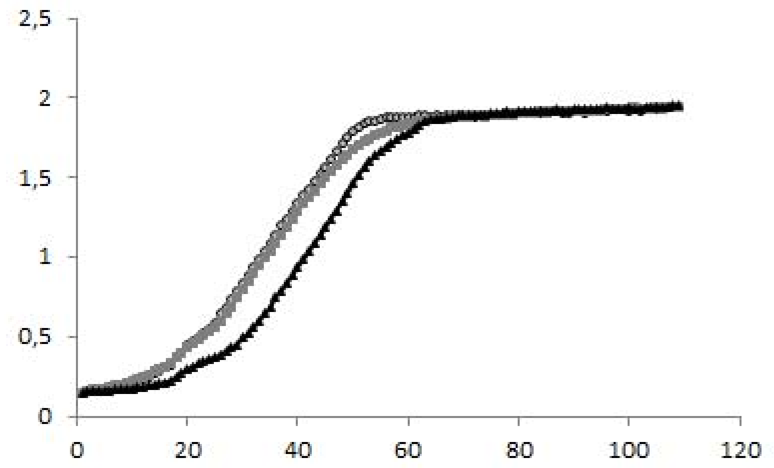
371

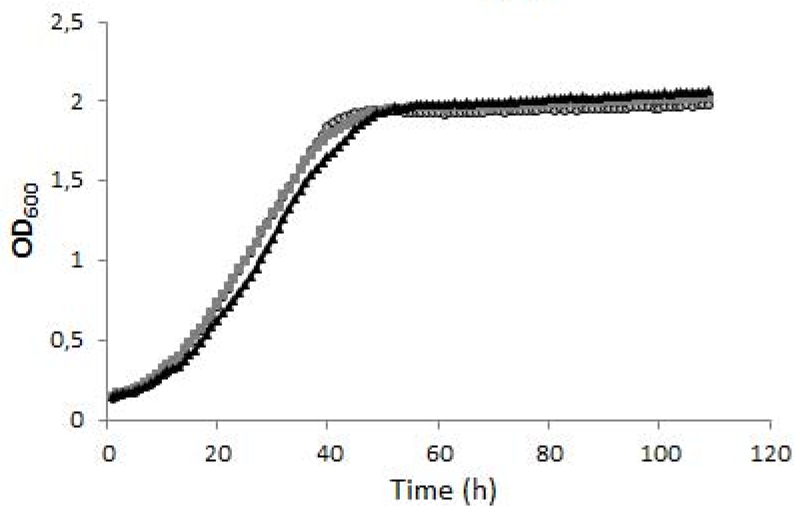
372 Fig. 1. Growth of *S. cerevisiae* T73, *S. kudriavzevii* IFO 1802, and *S. uvarum* CECT 12600
373 with the indicated amino acids (and ammonium) as the nitrogen source (MIX – amino acid
374 mixture).
375
376 Fig. 2. Ethyl acetate produced by *S. cerevisiae* T73, *S. kudriavzevii* IFO 1802, and *S. uvarum*
377 CECT 12600 depending on the nitrogen source. The statistically significant differences
378 among the species were determined independently for each nitrogen source and are indicated
379 by labels above the columns.
380
381 Fig. 3. Production of the higher alcohols and esters derived from the stated amino acid and a
382 comparison of these higher alcohol and ester when produced from the amino acids mixture
383 (MIX) or ammonium sulphate used as the nitrogen source. The statistically significant
384 differences among the species were determined independently for each nitrogen source and
385 are indicated by labels beside the columns.
386
387 Fig. 4. Total amount of the higher alcohols (A) and esters (B) produced by *S. cerevisiae* T73,
388 *S. kudriavzevii* IFO 1802, and *S. uvarum* CECT 12600 depending on the nitrogen source used.
389 The statistically significant differences among the species were determined independently for
390 each nitrogen source and are indicated by labels above the columns.
391

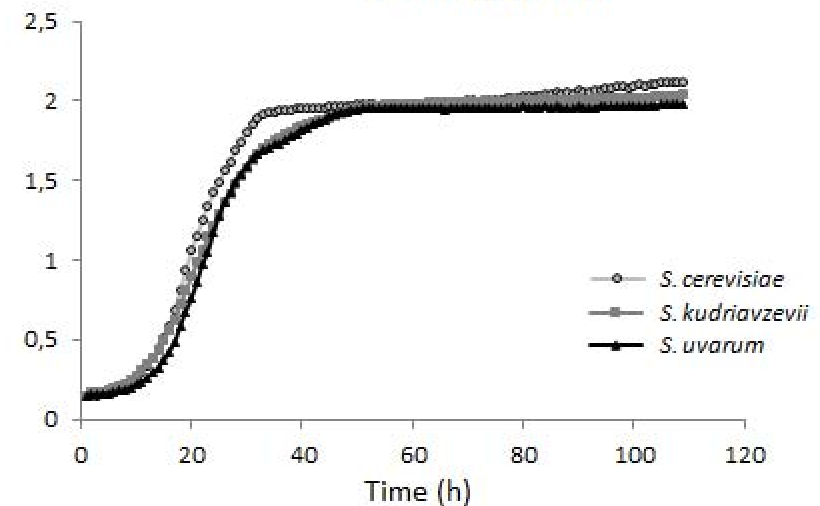

Val
2,5
2
1,5
1
0,5
0
OD$_{600}$
0
20
40
60
80
100
120

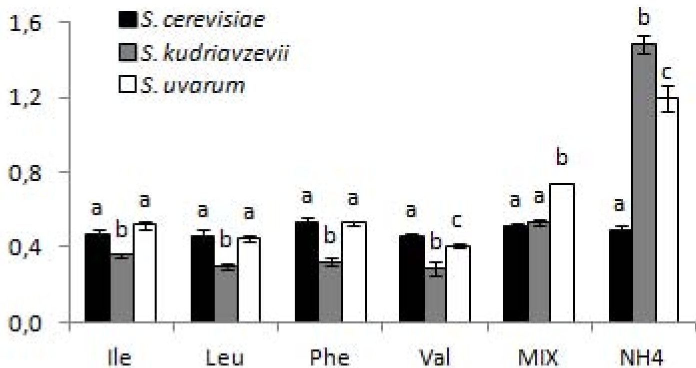

Phe
2,5
2
1,5
1
0,5
0
0
20
40
60
80
100
120


Ile
2,5
2
1,5
1
0,5
0
OD$_{600}$
0
20
40
60
80
100
120

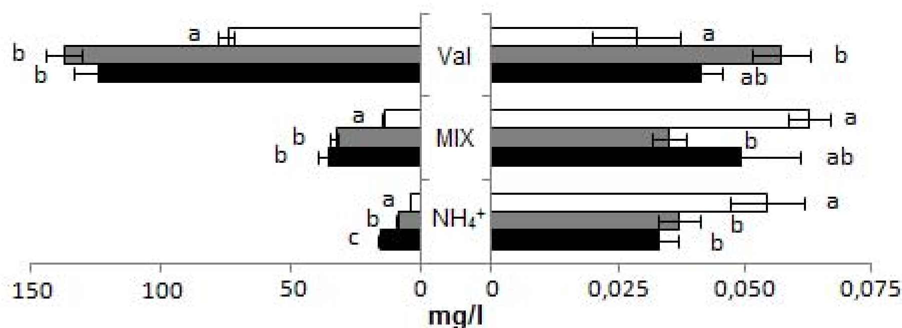

Leu
2,5
2
1,5
1
0,5
0
0
20
40
60
80
100
120


MIX
2,5
2
1,5
1
0,5
0
OD$_{600}$
0
20
40
60
80
100
120
Time (h)

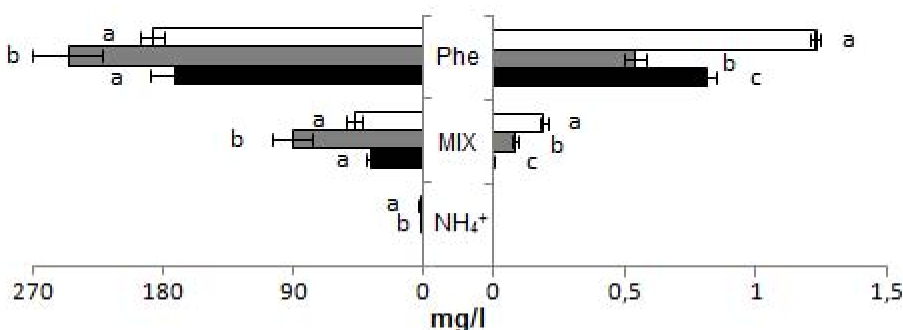

Ammonium
2,5
2
1,5
1
0,5
0
0
20
40
60
80
100
120
Time (h)
S. cerevisiae
S. kudriavzevii
S. uvarum



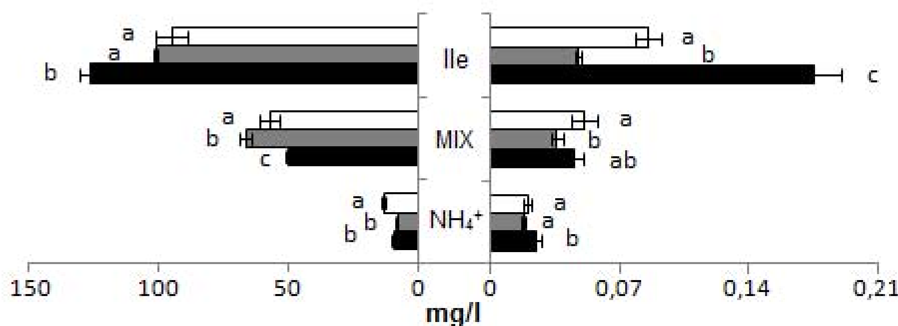
S. cerevisiae
S. kudriavzevii
S. uvarum
mg/L
1,6
1,2
0,8
0,4
0,0
a
b
c
Ile
Leu
Phe
Val
MIX
NH4



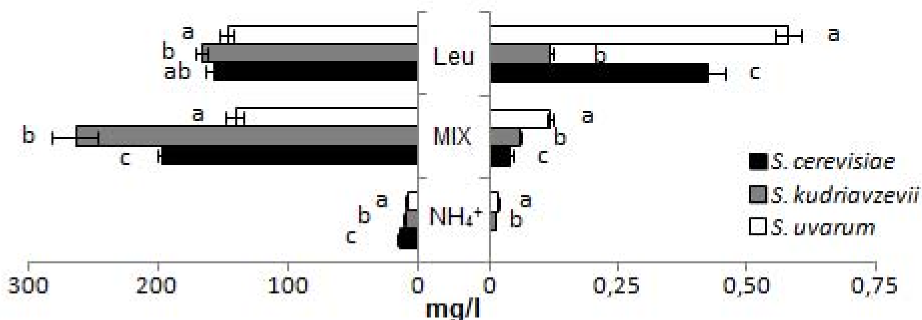
Isobutanol
Isobutyl acetate
Val
MIX
NH₄⁺
a b b a b ab a b b a b ab a b c a b b
150 100 50 0 0 0,025 0,050 0,075
mg/l



2-Phenylethanol
2-Phenylethyl acetate
Phe
MIX
NH₄⁺
a b a a b c a b a a b c a b
270 180 90 0 0 0,5 1 1,5
mg/l



Active amyl alcohol
Amyl acetate
Ile
MIX
NH₄⁺
a a b a b c a b ab a b c a a b a b b
150 100 50 0 0 0,07 0,14 0,21
mg/l



Isoamyl alcohol
Isoamyl acetate
Leu
MIX
NH₄⁺
a b ab a b c a b c a b c a b c a b
300 200 100 0 0 0,25 0,50 0,75
mg/l
S. cerevisiae
S. kudriavzevii
S. uvarum

A

mg/l

420
315
210
105
0

a b b a a b b a b a a b a b c a b c

Ile Leu Phe Val MIX NH4

B

S. cerevisiae
S. kudriavzevii
S. uvarum

mg/l

1,5
1,0
0,5
0,0

a b c a b c a b c a b b a b c a a b

Ile Leu Phe Val MIX NH4