

1 **Development of minimal fermentation media supplementation for ethanol production**
2 **using two *Saccharomyces cerevisiae* strains.**

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4 Alessia Tropea^{a*}, David Wilson^b, Nicola Cicero^a, Angela G. Potorti^a, Giovanna L. La Torre^a,
5 Giacomo Dugo^a, David Richardson^c and Keith W. Waldron^b

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7 ^a *"Scienze dell'Ambiente, della Sicurezza, del Territorio, degli Alimenti e della Salute"*
8 *Department, University of Messina, Messina, Italy.*

9 ^b *"Institute of Food Research", Norwich Research Park, Norwich, NR4 7UA, UK.*

10 ^c *"School of Biological Sciences", University of East Anglia, Norwich, NR4 7TJ, UK.*

11

12 atropea@unime.it

13 david.wilson@ifr.ac.uk

14 ncicero@unime.it

15 agpotorti@unime.it

16 llatorre@unime.it

17 dugog@unime.it

18 D.Richardson@uea.ac.uk

19 keith.waldron@ifr.ac.uk

20

21

22

23 * Corresponding author. Tel.: 39 090 676 5228 ; fax +39 090 676 5436

24 *E-mail address:* atropea@unime.it (A. Tropea)

25

26 **Abstract**

27 Ethanol production by fermentation is strongly dependent on media composition. Specific
28 nutrients, such as trace elements, vitamins and nitrogen will affect the physiological state and,
29 consequently, the fermentation performance of the microorganism employed.

30 The purpose of this study has been to assess the highest ethanol production by a minimal
31 medium, instead of the more complex nutrients supplementation used during alcoholic
32 fermentation. All fermentation tests were carried out using a microwell plate reader to
33 monitor the processes. Two *Saccharomyces cerevisiae* strains (NCYC 2826 and NCYC 3445)
34 were tested using three nitrogen sources, supplied with different vitamin and salts. The results
35 show that solutions made of urea phosphate, KCl, MgSO₄·7 H₂O, Ca-pantothenate, biotin
36 allowed an ethanol yield of 22.9 and 23.4 g/L for strain NCYC 2826 and NCYC 3445
37 respectively, representing 90 % and 92 % of the theoretical yield. All tests were carried out
38 using glucose as common reference carbon source.

39

40 **Keywords:** ethanol, yeast, *Saccharomyces cerevisiae*, fermentation media, alcohol
41 production, urea phosphate

42

43 **1. Introduction**

44 During ethanol production, medium composition strongly affects the physiological state and
45 the fermentation performance of the microorganism employed (Hahn-Hagerdal et al. 2005).
46 The most commonly used ethanol producer is *Saccharomyces cerevisiae* (Bai et al. 2008;
47 Pereira et al. 2010; Pereira et al. 2011) because of its good fermentative capacity, high
48 tolerance to ethanol and other inhibitors (either formed during raw-material pre-treatments or
49 produced during fermentation) and the capacity to grow rapidly under the anaerobic
50 conditions typically established in large-scale vessels (Mussatto et al. 2010). Ethanol
51 production is mainly dependent on glucose concentration (the theoretical alcohol yield
52 ranges about 0.5 g of ethanol per g of glucose) but also on specific nutrients, such as trace
53 elements (Azenha et al. 2000), vitamins (Alfenore et al. 2002) and nitrogen (Thomas and
54 Ingledeew 1990; Albers et al. 1996; Hernandez-Orte et al. 2006; Martínez-Moreno et al.
55 2012). Among thermotolerant vitamins, inositol (Kelley et al. 1988; Ding et al. 2009),
56 pantothenic acid (Taherzadeh et al. 1996) and biotin (Pejin and Razmovski 1996; Bohlscheid
57 et al. 2007) are generally required to obtain rapid fermentation and high ethanol levels, both
58 to minimize capital costs and distillation energy. Also nitrogen (N) and phosphorus (P) must

59 be included among the main nutritional requirements for yeast growth and maximum ethanol
60 production (Mukhtar et al. 2010).

61 On a laboratory scale, media are often supplemented with peptone and yeast extract or Yeast
62 Nitrogen base, but such additions are too expensive at industrial scale, so it is necessary to
63 exploit inexpensive nitrogen, vitamins and salts sources to supply all nutritional requirements
64 to yeast growth and fermentation (Azenha et al. 2000; Alfenore et al. 2002; Erdei et al.
65 2010; Carrasco et al. 2011; Izmirlioglu and Demirci 2012).

66 The aim of this study was to assess the best yeast growth conditions to obtain the highest
67 ethanol production using the most inexpensive minimal medium. Three different N-sources,
68 urea, $(\text{NH}_4)_2\text{SO}_4$ and urea phosphate were tested observing their best yeast growth and the
69 highest ethanol production. These N-sources, chosen among the cheapest on the market, were
70 used to replace the common costly yeast extract and peptone generally used in fermentation
71 media in laboratory scale. Moreover the effect of three vitamins (biotin, inositol and Ca-
72 panthothenate), for which yeasts are non-autotrophic and therefore generally added to
73 fermentation media (Lo Curto and Tripodo 2001; Bohlscheid et al. 2007), was also tested. All
74 solutions prepared with $(\text{NH}_4)_2\text{SO}_4$ and urea as N-sources were also supplied with a standard
75 phosphate source represented by $\text{NH}_4\text{H}_2\text{PO}_4$. Solutions prepared with urea phosphate did not
76 include $\text{NH}_4\text{H}_2\text{PO}_4$, because urea phosphate can be considered as both a N- and P- source.

77 For each of the three N-sources, 16 different tests were carried out to assay how KCl and
78 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, in relationship with different vitamins, affect ethanol yield. Moreover biotin
79 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ were added in all solutions, considering respectively their well-known
80 protective effect on yeast growth and improvement of the ethanol concentration (Hu et al.
81 2003). The effect of the single compound was observed by changing one variable while
82 fixing the others.

83

84 **2. Results and Discussion**

85 *2.1 Effect of urea as main N source*

86 Table S1 concerns the results obtained when, to make up a first basic medium, urea and
87 $\text{NH}_4\text{H}_2\text{PO}_4$ were used as inexpensive N and P sources besides additional N-sources
88 respectively. It can be observed how solutions containing KCl (tests 1-4) gave rise to the
89 highest ethanol yield and at the same time the highest yeast growth for both strains.
90 Considering the vitamins contribution in relation to the presence of KCl, the lowest ethanol
91 production was obtained when inositol, Ca-panthothenate and biotin were added together in

92 the medium (test 1); the highest alcohol production by both the strains on the contrary was
93 obtained when solutions were lacking inositol (test 2).

94 A lack of Ca-panthothenate did not affect strain NCYC 2826 growth and ethanol yield (test
95 3), showing the same trend as test 1; on the contrary strain NCYC 3445 exerted a higher
96 EtOH production in comparison with test 1, but lower if compared with the solution
97 containing Ca-panthothenate instead of inositol (test 2).

98 Absence of inositol and Ca-panthothenate in test 4 gave rise to a higher EtOH yield for both
99 the strains if compared with tests 1 and 3, but it was lower if compared with test 2, when both
100 Ca-panthothenate and biotin were together in the medium.

101 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, supplied in tests 5-8, results in no further increase neither in EtOH production
102 nor in OD.

103 A good EtOH production, for both strains, was observed when the three vitamins were
104 supplied together to the medium (test 5), whereas elimination of inositol, Ca-pantothenate or
105 both vitamins did not provide any beneficial effect since ethanol yield dropped for the two
106 strains. A similar low alcohol production was obtained, for both strains, in tests 6 and 8,
107 lacking inositol alone or Ca-panthothenate and inositol respectively. Test 7, in the presence of
108 inositol and bionin, gave rise to a higher ethanol production for NCYC 3445 strain compared
109 to the other strain, even if EtOH level was lower than the theoretical one.

110 The results of the same tests, where K^+ is not supplemented, are reported in lines 9-16.

111 FE on ethanol yield in tests 9-12 was around 6-10 % for the two strains, excepting test 11
112 where the vitamin contribution was represented by inositol and biotin. In this medium strain
113 NCYC 3445 showed a higher alcohol production than strain NCYC 2826, up to 21 % FE.

114 No significant yeast growth or ethanol production were detected in tests 13-16, where
115 solutions supplied with $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ were used.

116 ***2.2 Effect of $(\text{NH}_4)_2\text{SO}_4$ as main N-source***

117 The results of tests carried out in parallel to test the effect of urea substitution with
118 $(\text{NH}_4)_2\text{SO}_4$ as main N-source and $\text{NH}_4\text{H}_2\text{PO}_4$ as P-source and in the same time secondary N-
119 source are reported in Table S2.

120 As in Table S1 these results show that solutions containing KCl but lacking $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$
121 gave rise to the highest ethanol yield and yeast growth for both strains. The two strains show
122 different behaviour **under** all conditions for this N source. Strain NCYC 3445 (test 17) grown
123 with KCl, Ca-panthothenate, inositol and biotin produced more than 18 g/L of ethanol.

124 However the absence in the media of either inositol, Ca-panthothenate or both of them
125 affected growth and ethanol yield for strain NCYC 3445 (tests 18-20). In fact FE dropped
126 down to 43%.

127 The highest EtOH production reached by strain NCYC 2826 was 13.33 g/L when the only
128 vitamin supplement was biotin (test 20); this can be compared with the lower alcohol
129 concentration recorded in tests 17-19 with different vitamin combinations. When the three
130 vitamins were added to the media EtOH yield was 12.57 g/L, with a FE of 49 % (test 17); a
131 decrease of 5% (test 18) and 13 % (test 19) in FE when inositol and Ca-panthothenate are
132 absent.

133 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ supplementation (test 21-24) did not appear to be beneficial since ethanol
134 production by strain NCYC 2826 and OD_{600} dropped, especially if inositol was absent or
135 biotin was the only vitamin. Strain NCYC 3445 was able to grow in the media containing
136 KCl, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, inositol, Ca-panthothenate and biotin, producing 11.68 g/L of ethanol
137 (test 21). Also in this case absence of inositol, Ca-panthothenate or both of them caused
138 EtOH yield reduction (tests 22-24).

139 The removal of KCl from media generally caused a diminution in ethanol yield and yeast
140 growth for both the strains (testes 25-32). In comparison with the results obtained from tests
141 carried out using urea as main N-source (Table S1), use of $(\text{NH}_4)_2\text{SO}_4$ was followed by a
142 higher capacity in alcohol production from both yeast strains. The highest ethanol production
143 for strain NCYC 2826 was around 6g/L, reaching a 23% FE, in absence of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$,
144 inositol and Ca-panthothenate (test 26); whereas strain NCYC 3445 reached a 25% FE in
145 testes 27 and 31. In this case the media composition was the same with the exception of
146 absence/presence of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$.

147 ***2.3 Effect of urea phosphate as main N and P-sources***

148 In Table S3 the results of the tests carried out using urea phosphate as the only source of N
149 and P are reported. The results appear to be similar to those previously reported though slight
150 differences between the two strains are observed.

151 Also in this case solutions containing KCl (33-36) gave rise to the highest ethanol yield and
152 yeast growth for both strains. The presence of the three vitamins together or the addition only
153 of inositol and biotin (tests 33 and 35 respectively) allowed both the strains to reach the same
154 FE. On the contrary a different behaviour can be noticed when inositol is lacking. The
155 presence of Ca-panthothenate and biotin (test 34) gave rise to the highest EtOH production by
156 strain NCYC 2826 and NCYC 3445, up to 22.9 g/L and 23.4 g/L respectively. When biotin
157 was the only vitamin added to the medium (test 36), the FE for strain NCYC 2826 was 15 %

158 less than the one obtained in solution 34; whereas for strain NCYC 3445 a 49% of FE was
159 lost. In media supplemented with $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (tests 37-40) a relevant decrease in ethanol
160 production can be observed. The highest EtOH yield was around 5.8 g/L for strain NCYC
161 2826 when the medium was supplemented with the three vitamins (test 37). Strain NCYC
162 3445 shown in this medium a higher capacity in alcohol production in comparison with the
163 other strain, reaching a FE of 30%. Anyway a better response was given by the strain NCYC
164 3445 when the vitamins added were Ca-pantothenate and biotin (test 38), with an ethanol
165 yield of 8.3 g/L. Subtraction of Ca-pantothenate, alone or with inositol, was followed by a
166 further decreasing in EtOH production for both strains (tests 39-40).

167 KCl elimination from media (testes 41-48) was always followed by a decrease of yeast
168 growth and ethanol production. FE for strain NCYC 2826 was up to 11% (test 41) when the
169 medium was supplemented by the three vitamins. It dropped down to 10% (test 45) when the
170 same medium was supplied by $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. Again, when lacking inositol, or Ca-
171 pantothenate or both, the FE decreased down to 2%. The highest FE for strain NCYC 3445
172 was up to 9% when the three vitamins were added to reaction media (tests 42,43) and
173 increased up to 14% when $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and these vitamins were added to the medium (test
174 45). In all the other trials (testes 46-48) alcohol concentration dramatically drops after
175 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ addition for both strains.

176 Optimization of nutrients for ethanol production by yeast has been extensively studied
177 (Kadam and Newman 1997; Azenha et al. 2000; Alfenore et al. 2002; Slininger et al. 2006;
178 Wang et al. 2006; Bohlscheid et al. 2007). These works were focused on vitamin
179 supplementation, nitrogen sources or mineral effect on yeast fermentation. The present work,
180 as previously pointed out, was aimed to make up the minimal medium producing the highest
181 ethanol concentration and in the same time to elucidate how some media components such as
182 vitamins, minerals as well as nitrogen sources can play an important role linked both by their
183 presence and also by their interactive effects.

184 When the media were supplied with all the substances tested, with the exception of
185 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (tests 1, 17, 33), strain NCYC 2826 was not affected by using different N-
186 sources since ethanol production was in any trial the same. On the contrary strain NCYC
187 3445 achieved the highest ethanol yield when $(\text{NH}_4)_2\text{SO}_4$ was used as N-source (test 17,
188 Table S2).

189 Though inositol has been reported to influence favourably ethanol production in *S. cerevisiae*,
190 playing an important role in ethanol tolerance (Kelley et al. 1988; Ding et al. 2009), in our
191 study this vitamin seems unable to affect final ethanol yield. The highest yields were up to

192 22.9 g/L and 23.4 g/L for strains NCYC 2826 and NCYC 3445 respectively (tests 2, 34) if
193 inositol lacks. Also biotin and Ca-panthothenate seem to be essential to reach the highest
194 alcohol concentration for both the yeasts.

195 This study pointed out the crucial role played in all media by KCl. Addition of this salt was
196 essential for both the strains to grow and produce ethanol. This can be explained in that the
197 cellular volume, turgor, electrical membrane potential and ionic strength depend mostly on
198 intracellular K^+ concentration (Yenush et al. 2005; Navarrete et al. 2010). Moreover K^+ , as
199 other metal ions like Mg^{2+} , Ca^{2+} and Zn^{2+} , can increase the rate of glycolysis and,
200 subsequently, the conversion of pyruvate to ethanol (Wills 1990).

201 When $CuSO_4 \cdot 5H_2O$ was included in media only strain NCYC 3445 was able to produce
202 alcohol from KCl-containing solutions, using $(NH_4)_2SO_4$ as N-source, a behaviour similar in
203 all solutions. No growth or ethanol production was recorded for strain NCYC 2826.
204 Generally $CuSO_4 \cdot 5H_2O$ did not result in either an increase in EtOH production or in EY or
205 OD. This was apparent for both strains when the three vitamins were supplied to the media.
206 This can be explained by the possible toxicity of copper, depending on the *S. cerevisiae* strain
207 and the growth conditions (Sarais et al. 1994; Pearce and Sherman 1999).

208

209 **3. Experimental**

210 See supplementary materials

211

212 **4. Conclusion**

213 The research allowed us to propose a minimal, low-cost solution for supplementing waste
214 media, among the many tested, for ethanol production. It was made of urea phosphate salt 2.3
215 g/L; KCl 0.2 g/L; $MgSO_4 \cdot 7 H_2O$ 3.8 g/L; Ca-panthothenate 0.0833 mg/L; biotin 0.0833
216 mg/L. Both the yeast strains tested, *S. cerevisiae* NCYC 2826 and *S. cerevisiae* NCYC 3445
217 demonstrate the highest ethanol production in this medium, 22.9 g/L (90% FE) and 23.4 g/L
218 (92% FE) respectively.

219 This study reveals how it is possible to improve ethanol production by *Saccharomyces* strains
220 through a minimal medium supplementation and suggests also its use at industrial scale,
221 instead of the more complex nutrient addition reported in literature, reducing the cost of the
222 fermentation process. **Several studies had been reported about starchy or lignocellulosic**
223 **feedstock for bioethanol production, and to get the higher alcohol production these substrates**
224 **had been supplemented with salt, vitamins (Nigam, 1999; Nigam, 2000; Jørgensen, 2009;**
225 **Carrasco et al., 2011) and also with peptone and yeast extract (Santos et al., 2012; Van Eylen**

226 et al, 2011; Erdei et al., 2010; Arapoglou et al., 2010; Tian et al., 2009). Among the several
227 compounds usually used for supplementing media fermented for ethanol production the
228 nutrient solution pointed out in this study can be added to starchy or lignocellulosic
229 fermentable substrate for enhancing ethanol production and fermentation rate and
230 consequently reducing the industrial cost.

231 Finally, comparing the results obtained using three different N-sources, a different behaviour
232 of the two strains was observed. This can suggest the importance of further screening on
233 other *Saccharomyces* strains.

234 Further studies to improve ethanol yield using other simple media on other *Saccharomyces*
235 strains are in progress.

236

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239

240 **See supplementary materials**

241 Supplementary materials relating to this article, including further details of materials and
242 methods, is available online.

243

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