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

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Abstract

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

The purpose of this study has been to assess the highest ethanol production by a minimal medium, instead of the more complex nutrients supplementation used during alcoholic fermentation. All fermentation tests were carried out using a microwell plate reader to monitor the processes. Two *Saccharomyces cerevisiae* strains (NCYC 2826 and NCYC 3445) were tested using three nitrogen sources, supplied with different vitamin and salts. The results show that solutions made of urea phosphate, KCl, $MgSO_4 \cdot 7\ H_2O$, Ca-panthothenate, biotin allowed an ethanol yield of 22.9 and 23.4 g/L for strain NCYC 2826 and NCYC 3445 respectively, representing 90 % and 92 % of the theoretical yield. All tests were carried out using glucose as common reference carbon source.

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

1. Introduction

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

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

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

The aim of this study was to assess the best yeast growth conditions to obtain the highest ethanol production using the most inexpensive minimal medium. Three different N-sources, urea, $(NH_4)_2SO_4$ and urea phosphate were tested observing their best yeast growth and the highest ethanol production. These N-sources, chosen among the cheapest on the market, were used to replace the common costly yeast extract and peptone generally used in fermentation media in laboratory scale. Moreover the effect of three vitamins (biotin, inositol and Ca-panthothenate), for which yeasts are non-autotrophic and therefore generally added to fermentation media (Lo Curto and Tripodo 2001; Bohlscheid et al. 2007), was also tested. All solutions prepared with $(NH_4)_2SO_4$ and urea as N-sources were also supplied with a standard phosphate source represented by $NH_4H_2PO_4$. Solutions prepared with urea phosphate did not include $NH_4H_2PO_4$, because urea phosphate can be considered as both a N- and P- source.

For each of the three N-sources, 16 different tests were carried out to assay how KCl and $CuSO_4 \cdot 5H_2O$, in relationship with different vitamins, affect ethanol yield. Moreover biotin and $MgSO_4 \cdot 7H_2O$ were added in all solutions, considering respectively their well-known protective effect on yeast growth and improvement of the ethanol concentration (Hu et al. 2003). The effect of the single compound was observed by changing one variable while fixing the others.

2. Results and Discussion

2.1 Effect of urea as main N source

Table S1 concerns the results obtained when, to make up a first basic medium, urea and $NH_4H_2PO_4$ were used as inexpensive N and P sources besides additional N-sources respectively. It can be observed how solutions containing KCl (tests 1-4) gave rise to the highest ethanol yield and at the same time the highest yeast growth for both strains. Considering the vitamins contribution in relation to the presence of KCl, the lowest ethanol production was obtained when inositol, Ca-panthothenate and biotin were added together in

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

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

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

$CuSO_4 \cdot 5H_2O$, supplied in tests 5-8, results in no further increase neither in EtOH production nor in OD.

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

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

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

No significant yeast growth or ethanol production were detected in tests 13-16, where solutions supplied with $CuSO_4 \cdot 5H_2O$ were used.

2.2 Effect of $(NH_4)_2SO_4$ as main N-source

The results of tests carried out in parallel to test the effect of urea substitution with $(NH_4)_2SO_4$ as main N-source and $NH_4H_2PO_4$ as P-source and in the same time secondary N-source are reported in Table S2.

As in Table S1 these results show that solutions containing KCl but lacking $CuSO_4 \cdot 5H_2O$ gave rise to the highest ethanol yield and yeast growth for both strains. The two strains show different behaviour under all conditions for this N source. Strain NCYC 3445 (test 17) grown with KCl, Ca-panthothenate, inositol and biotin produced more than 18 g/L of ethanol.

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

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

$CuSO_4 \cdot 5H_2O$ supplementation (test 21-24) did not appear to be beneficial since ethanol production by strain NCYC 2826 and OD_{600} dropped, especially if inositol was absent or biotin was the only vitamin. Strain NCYC 3445 was able to grow in the media containing KCl, $CuSO_4 \cdot 5H_2O$, inositol, Ca-panthothenate and biotin, producing 11.68 g/L of ethanol (test 21). Also in this case absence of inositol, Ca-panthothenate or both of them caused EtOH yield reduction (tests 22-24).

The removal of KCl from media generally caused a diminution in ethanol yield and yeast growth for both the strains (testes 25-32). In comparison with the results obtained from tests carried out using urea as main N-source (Table S1), use of $(NH_4)_2SO_4$ was followed by a higher capacity in alcohol production from both yeast strains. The highest ethanol production for strain NCYC 2826 was around 6g/L, reaching a 23% FE, in absence of $CuSO_4 \cdot 5H_2O$, inositol and Ca-panthothenate (test 26); whereas strain NCYC 3445 reached a 25% FE in testes 27 and 31. In this case the media composition was the same with the exception of absence/presence of $CuSO_4 \cdot 5H_2O$.

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

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

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

less than the one obtained in solution 34; whereas for strain NCYC 3445 a 49% of FE was lost. In media supplemented with $CuSO_4 \cdot 5H_2O$ (tests 37-40) a relevant decrease in ethanol production can be observed. The highest EtOH yield was around 5.8 g/L for strain NCYC 2826 when the medium was supplemented with the three vitamins (test 37). Strain NCYC 3445 shown in this medium a higher capacity in alcohol production in comparison with the other strain, reaching a FE of 30%. Anyway a better response was given by the strain NCYC 3445 when the vitamins added were Ca-panthothenate and biotin (test 38), with an ethanol yield of 8.3 g/L. Subtraction of Ca-panthothenate, alone or with inositol, was followed by a further decreasing in EtOH production for both strains (tests 39-40).

KCl elimination from media (testes 41-48) was always followed by a decrease of yeast growth and ethanol production. FE for strain NCYC 2826 was up to 11% (test 41) when the medium was supplemented by the three vitamins. It dropped down to 10% (test 45) when the same medium was supplied by $CuSO_4 \cdot 5H_2O$. Again, when lacking inositol, or Ca-panthothenate or both, the FE decreased down to 2%. The highest FE for strain NCYC 3445 was up to 9% when the three vitamins were added to reaction media (tests 42,43) and increased up to 14% when $CuSO_4 \cdot 5H_2O$ and these vitamins were added to the medium (test 45). In all the other trials (testes 46-48) alcohol concentration dramatically drops after $CuSO_4 \cdot 5H_2O$ addition for both strains.

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

When the media were supplied with all the substances tested, with the exception of $CuSO_4 \cdot 5H_2O$ (tests 1, 17, 33), strain NCYC 2826 was not affected by using different N-sources since ethanol production was in any trial the same. On the contrary strain NCYC 3445 achieved the highest ethanol yield when $(NH_4)_2SO_4$ was used as N-source (test 17, Table S2).

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

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

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

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

3. Experimental

See supplementary materials

4. Conclusion

The research allowed us to propose a minimal, low-cost solution for supplementing waste media, among the many tested, for ethanol production. It was made of urea phosphate salt 2.3 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 mg/L. Both the yeast strains tested, *S. cerevisiae* NCYC 2826 and *S. cerevisiae* NCYC 3445 demonstrate the highest ethanol production in this medium, 22.9 g/L (90% FE) and 23.4 g/L (92% FE) respectively.

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

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

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

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

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See supplementary materials

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

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