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Optimization of low-cost medium for very high gravity ethanol fermentations by *Saccharomyces cerevisiae* using statistical experimental designs

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ABSTRACT

Statistical experimental designs were used to develop a medium based on corn steep liquor (CSL) and other low-cost nutrient sources for high-performance very high gravity (VHG) ethanol fermentations by *Saccharomyces cerevisiae*. The critical nutrients were initially selected according to a Plackett–Burman design and the optimized medium composition (44.3 g/L CSL; 2.3 g/L urea; 3.8 g/L $MgSO_4 \cdot 7H_2O$; 0.03 g/L $CuSO_4 \cdot 5H_2O$) for maximum ethanol production by the laboratory strain CEN.PK 113-7D was obtained by response surface methodology, based on a three-level four-factor Box-Behnken design. The optimization process resulted in significantly enhanced final ethanol titre, productivity and yeast viability in batch VHG fermentations (up to 330 g/L glucose) with CEN.PK113-7D and with industrial strain PE-2, which is used for bio-ethanol production in Brazil. Strain PE-2 was able to produce 18.6 ± 0.5% (v/v) ethanol with a corresponding productivity of 2.4 ± 0.1 g/L/h. This study provides valuable insights into cost-effective nutritional supplementation of industrial fuel ethanol VHG fermentations.

1. Introduction

Bio-ethanol is regarded as a promising alternative energy source, which is both renewable and environmentally friendly. During bio-ethanol production, the composition of media affects the physiological state and, consequently, the fermentation performance of the microorganism employed (Hahn-Hägerdal et al., 2005).

Ethanolic fermentations with very high sugar concentrations (>300 g/L) – very high gravity (VHG) fermentations – have many advantages from an industrial point of view, resulting in reduced costs because of lower energy consumption (Thomas et al., 1996). However, these fermentations are rarely fast and complete due to physiological changes in the microbial cells. The high sugar content in the fermentation medium causes an increase in the osmotic pressure, which has a damaging effect on yeast cells. *Saccharomyces cerevisiae*, the yeast commonly used for ethanolic fermentations, can ferment increased amount of sugars in the medium when all required nutrients are provided in adequate amounts (Bafrncová et al., 1999). Specific nutrients, such as nitrogen, trace elements or vitamins, are required to obtain rapid fermentation and high ethanol levels, desirable to minimize capital costs and distillation energy. On a laboratory scale, media are often supplemented with peptone and yeast extract. However, such addition is not feasible in industrial fermentation processes due to the high costs associated. Thus, it is necessary to exploit inexpensive nutrient sources to supply all nutritional requirements for yeast growth and fermentation.

Corn steep liquor (CSL), a major by-product of corn starch processing, is a low-cost source of proteins, amino acids, minerals, vitamins and trace elements and can be used as a rich and effective nutritional supplement, in particular, as replacement for yeast extract and peptone in alcoholic fermentations (Amartey and Jeffries, 1994; Kadam and Newman, 1997; Lawford and Rousseau, 1997; Seo et al., 2009; Tang et al., 2006). Moreover, the effects of metal ions on yeast cell growth and fermentation are well documented. Magnesium, calcium and zinc have been reported to influence the rate of sugar conversion and are required as cofactors for several metabolic pathways (Palukurty et al., 2008; Xue et al., 2008; Zhao et al., 2009). Furthermore, the protective effects of magnesium and calcium against ethanol stress have been extensively studied (Birch and Walker, 2000; Nabais et al., 1988). Metal ion deficiencies often occur in fermentation media (Jones and Greenfield, 1984), and studies on optimization of metal ions combinations are thus of great practical importance to improve ethanol production.

Medium optimization by the classical method of changing one variable while fixing the others at a certain level is laborious and time-consuming, especially when the number of variables is large. An alternative and more efficient approach in microbial systems is the use of statistical methods. Response surface methodology (RSM) is a commonly used method to assess the optimal fermentation conditions and also an efficient statistical technique for opti-

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mization of multiple variables with minimum number of experiments. This method has been successfully applied to optimize alcoholic fermentation (Laluce et al., 2009; Palukurty et al., 2008; Ratnam et al., 2005; Yu et al., 2009). Plackett–Burman design allows testing of the largest number of factor effects with the least number of observations, and allows random error variability estimation and testing of the statistical significance of the parameters (Plackett and Burman, 1946). The Box-Behnken is a three-level factorial design, which allows estimating and interpreting interactions between various variables at a time during the optimization process. It is suitable for exploration of quadratic responses and constructs a second-order polynomial model with very few runs (Ferreira et al., 2007).

In this study, factorial design approaches were used to develop a low-cost medium based on CSL and other inexpensive nutrient sources for high-performance batch VHG ethanolic fermentations by *S. cerevisiae*. The supplements that significantly improved ethanol production by the laboratory strain CEN.PK 113-7D were selected according to Plackett–Burman designs and the concentrations of the key nutrient factors (CSL, urea, $MgSO_4$ and $CuSO_4$) were optimized using a Box-Behnken design. Furthermore, the optimized medium was compared with a reference medium containing CSL as the sole nutrient source, using the strain CEN.PK 113-7D as well as an industrial strain (PE-2).

2. Methods

2.1. Yeasts

The laboratory *S. cerevisiae* strain CEN.PK 113-7D (van Dijken et al., 2000) was used throughout the screening and optimization experimental designs. Final comparative fermentation tests were performed also with the industrial strain PE-2 (Basso et al., 2008). Stock cultures were maintained on YPD [yeast extract 1% (w/v), bacto peptone 2% (w/v) and glucose 2% (w/v)] agar plates at 4 °C.

2.2. Media and fermentations

For nutrient screening and optimization, fermentation tests were performed in basic medium (BM) consisting of 296–308 g/L glucose and 15 g/L CSL, supplemented with nutrients according to the experimental designs (Tables 2 and 4). Glucose syrup and CSL were kindly provided by a starch manufacturer (COPAM, Portugal) and autoclaved separately (121 °C, 20 min). After autoclaving, the CSL was allowed to settle for 1–2 days at 4 °C and then centrifuged (15 min at 13,100 *g*) to remove the insolubles. The packed sediment corresponded to 15% (w/w) of the whole CSL. The main composition of the centrifuged CSL (i.e. the supernatant after centrifugation) is shown in Table 1. Batch 1 and batch 2 were obtained from the same industrial plant approximately 3 months apart. The concentrated nutrient stock solutions were sterilized by filtration and added aseptically to the medium. A reference medium (RM) consisting of 290–330 g/L glucose and 100 g/L CSL, and 2YP medium consisting of 290–330 g/L glucose, 20 g/L yeast extract and 40 g/L peptone (i.e. the double of the yeast extract and peptone concentrations used in standard YP medium), were used for comparative studies. In all cases, the medium was aerated by stirring with a magnetic bar (length of 3 cm) at >850 rpm during 20 min before inoculating the fermentation flasks, reaching >95% of air saturation (approximately, 8 ppm of oxygen) (Munroe, 2006).

Table 1
Composition of the centrifuged CSL (values are average ± range of duplicate analyses).

	Batch 1	Batch 2
Density	1.15 ± 0.00	1.13 ± 0.02
Humidity (%, w/w)	65.4 ± 0.1	64.7 ± 0.0
Ash (%, w/w)	6.15 ± 0.15	5.97 ± 0.46
Free reducing sugars[a] (%, w/w)	4.09 ± 0.04	3.82 ± 0.14
Glucose[b] (%, w/w)	0.439 ± 0.014	0.408 ± 0.006
Total Kjeldahl nitrogen[c] (%, w/w)	2.25 ± 0.46	2.26 ± 0.04
Fat[d] (%, w/w)	3.19 ± 0.80	1.95 ± 0.81
pH	3.9	4.1

[a] Determined by the DNS method (Miller, 1959), using glucose as standard.
[b] Determined by HPLC.
[c] Determined using the Tecator Kjeltec 1026 system.
[d] Determined using the Tecator Soxtec HT2 system.

Yeast for inoculation was grown in 1 L Erlenmeyer flasks filled with 400 mL of medium containing 50 g/L glucose, 20 g/L peptone and 10 g/L yeast extract. After incubation at 30 °C and 150 rpm for 24–26 h (OD_{600} of 7–7.5), the cell suspension was aseptically collected by centrifugation (10 min at 7500*g*, 4 °C) and suspended in 0.9% NaCl to a concentration of 200 mg fresh yeast/mL, to minimize the transfer of nutrients from the seed culture to the fermentation medium. The yeast cells were inoculated at about 8 mg fresh yeast/mL into 40 mL of culture medium to start the fermentation.

Fermentations were done at 30 °C and 150 rpm in 100 mL Erlenmeyer flasks fitted with perforated rubber stoppers enclosing glycerol-filled air-locks to allow exhaustion of CO_2 while avoiding entrance of air. The initial pH was adjusted to 5.5 with NaOH and the final pH was higher than 3.9 in all fermentations. The progress of fermentation was followed by mass loss. Samples for analyses were taken at the beginning and end of fermentation. The fermentation experiments were stopped when the weight of the flasks did not change anymore.

2.3. Analytical procedures

Yeast growth was monitored by measuring the optical density of the culture at 600 nm (OD_{600}). Cell dry weight was determined by centrifugation (10 min at 7500*g*, 4 °C) of 20 mL of the yeast culture in a pre-weighed dried tube, washing of the pellet with 20 mL of distilled water, drying overnight at 105 °C and weighing. Glucose, ethanol and glycerol were analyzed by HPLC, using a Varian MetaCarb 87H column eluted at 60 °C with 0.005 M H_2SO_4 at a flow rate of 0.7 mL/min, and a refractive-index detector. Yeast cell number was determined with a Neubauer counting chamber and viability was determined by methylene blue staining (Mills, 1941).

2.4. Experimental design

A Plackett–Burmam design was performed to screen nine independent variables selected from the literature (Azenha et al., 2000; Kadam and Newman, 1997; Nabais et al., 1988; Palukurty et al., 2008; Wang et al., 2007; Xue et al., 2008; Zhao et al., 2009) as feasible supplements in alcoholic fermentations (g/L): CSL 25, urea 1.5, $(NH_4)_2SO_4$ 5, $MgSO_4{\cdot}7H_2O$ 5, KH_2PO_4 2, $ZnCl_2$ 0.01, $FeSO_4{\cdot}7H_2O$ 0.0072, $CuSO_4{\cdot}5H_2O$ 0.075, $CaCl_2{\cdot}2H_2O$ 0.8. For each variable, the presence and absence levels of supplements were tested (all trials were performed in duplicate), resulting in 13 independent experiments (Table 2). Significant positive variable effects were considered when the reported *p*-values were lower than 0.05.

Furthermore, a Box-Behnken design was performed using the independent variables selected as significant in the screening design. Four replicates at the centre point level were also run to check if there was a non-linear relationship between the variables and the responses, leading to a total number of 28 trials (Table 4).

For predicting the optimal point, a second-order polynomial function was fitted to correlate the relationship between independent variables and response. X_1, X_2, X_3 and X_4 factors were correlated by the following equation:

Table 2
Experimental and predicted values of final ethanol, CO_2 produced during the initial 30 h of fermentation, final biomass and final glycerol concentrations in fermentations of BM with 308 g/L initial glucose supplemented according to the Plackett–Burman design.

Runs	CSL	Urea	$(NH_4)_2SO_4$	$MgSO_4$	KH_2PO_4	$ZnCl_2$	$FeSO_4$	$CuSO_4$	$CaCl_2$	Ethanol (g/L)		CO_2 at 30 h (g/L)		Biomass (g/L)		Glycerol (g/L)	
										Exp.[a]	Mod.[b]	Exp.[a]	Mod.[b]	Exp.[a]	Mod.[b]	Exp.[a]	Mod.[b]
1–2	0	0	0	5	0	0	0.0072	0	0.8	127.5 ± 1.1	127.4	45.4 ± 0.9	49.2	5.5 ± 0.0	5.7	8.7 ± 0.2	9.3
3–4	0	0	5	0	0	0.01	0	0.075	0.8	121.5 ± 2.5	119.1	47.6 ± 0.5	46.2	4.5 ± 0.0	4.4	8.5 ± 0.0	8.1
5–6	0	0	5	0	2	0.01	0.0072	0	0	105.0 ± 1.0	108.2	67.5 ± 0.1	70.3	5.3 ± 0.3	5.4	8.8 ± 0.4	9.6
7–8	0	1.5	0	0	2	0	0.0072	0.075	0.8	127.0 ± 2.2	128.7	46.3 ± 0.1	45.2	4.8 ± 0.3	4.6	8.7 ± 0.2	8.8
9–10	0	1.5	0	5	2	0.01	0	0	0	128.1 ± 0.6	125.7	72.6 ± 0.2	71.2	5.3 ± 0.3	5.2	10.5 ± 1.6	9.9
11–12	0	1.5	5	5	0	0	0	0.075	0	129.8 ± 1.7	132.2	43.5 ± 0.5	45.0	4.5 ± 0.0	4.6	10.0 ± 0.0	11.0
13–14	12.5	0.75	2.5	2.5	1	0.005	0.0036	0.0375	0.4	130.1 ± 0.9	125.9	70.1 ± 0.1	61.6	5.8 ± 0.3	5.6	12.0 ± 0.0	9.6
15–16	25	0	0	0	2	0	0	0.075	0	130.1 ± 1.1	129.2	62.3 ± 0.0	64.7	6.5 ± 0.0	6.7	7.8 ± 0.0	8.0
17–18	25	0	0	5	0	0.01	0.0072	0.075	0	128.6 ± 0.3	130.3	62.2 ± 0.1	61.1	6.5 ± 0.0	6.4	9.9 ± 2.1	10.0
19–20	25	0	5	5	2	0	0	0	0.8	128.0 ± 1.0	128.9	76.0 ± 0.8	73.5	6.5 ± 0.0	6.3	8.2 ± 0.0	7.9
21–22	25	1.5	0	0	0	0.01	0	0	0.8	131.2 ± 0.5	133.6	78.4 ± 0.3	79.8	6.0 ± 0.0	6.1	7.4 ± 0.0	7.8
23–24	25	1.5	5	0	0	0	0.0072	0	0	130.7 ± 1.0	129.1	77.8 ± 0.3	77.7	6.5 ± 0.0	6.4	10.1 ± 2.0	10.0
25–26	25	1.5	5	5	2	0.01	0.0072	0.075	0.8	131.4 ± 2.4	131.4	62.1 ± 0.9	65.9	4.8 ± 0.3	4.9	9.0 ± 0.2	9.6

[a] Exp.: experimental value (average ± range of duplicate independent biological experiments).
[b] Mod.: model-predicted value.

Table 3
Plackett–Burman design – effects of nutrient sources on the final ethanol titre.

Term	Coefficient	Standard error	*t*-Value	*p*-Value
CSL	3.426	0.7006	4.890	0.0002
Urea	3.122	0.7006	4.460	0.0004
$MgSO_4$	2.326	0.7006	3.320	0.0043
$ZnCl_2$	−2.269	0.7006	−3.200	0.0051
$(NH_4)_2SO_4$	−2.165	0.7006	−3.100	0.0007
KH_2PO_4	−1.626	0.7006	−2.300	0.0338
$CuSO_4$	1.497	0.7006	2.140	0.0485
$CaCl_2$	1.192	0.7006	1.700	0.1083
$FeSO_4$	−1.573	0.9376	−1.700	0.1129

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{34}X_3X_4 \quad (1)$$

In Eq. (1), Y is the predicted response corresponding to the ethanol titre at the end of the fermentation process. X_1, X_2, X_3 and X_4 are independent variables, b_0 is an offset term, b_1, b_2, b_3 and b_4 are linear effects and b_{12}, b_{13}, b_{14}, b_{23}, b_{24} and b_{34} are interaction terms.

A screening of vitamins (selected from the yeast mineral medium described by Verduyn et al. (1992)) was done in glucose medium containing the optimal concentration of nutrients (X_1, X_2, X_3

Table 4
Experimental values of final ethanol, CO_2 produced during the initial 38 h of fermentation, final biomass and final glycerol concentrations in fermentations of BM with 296 g/L initial glucose supplemented according to the Box-Behnken design. The final ethanol concentrations predicted by the model are also shown.

Run	Factor (g/L)				Ethanol (g/L)		CO_2 at 38 h (g/L)	Biomass (g/L)	Glycerol (g/L)
	CSL (X_1)	Urea (X_2)	$MgSO_4{\cdot}7H_2O$ (X_3)	$CuSO_4{\cdot}5H_2O$ (X_4)	Exp.[a]	Mod.[b]	Exp.[a]	Exp.[a]	Exp.[a]
1	15	0.5	5	0.075	113.9	111.5	71.0	6.0	8.0
2	15	1.5	2.5	0.075	115.7	121.0	71.7	6.5	7.3
3	15	1.5	5	0.025	124.0	120.8	88.8	7.0	8.3
4	15	1.5	5	0.125	110.6	112.8	71.2	6.0	9.1
5	15	1.5	7.5	0.075	117.2	117.0	77.5	6.5	8.8
6	15	2.5	5	0.075	129.2	128.1	86.7	7.5	9.1
7	25	0.5	2.5	0.075	123.6	122.0	81.9	7.5	8.1
8	25	0.5	5	0.025	118.2	122.2	88.4	7.0	7.7
9	25	0.5	5	0.125	110.0	109.6	77.7	6.5	8.1
10	25	0.5	7.5	0.075	113.2	114.7	80.7	6.5	7.7
11	25	1.5	2.5	0.025	135.2	133.1	95.8	8.5	8.5
12	25	1.5	2.5	0.125	118.9	116.2	86.7	6.5	8.7
13	25	1.5	5	0.075	132.5	132.7	93.2	7.5	7.9
14	25	1.5	5	0.075	132.4	132.7	93.5	8.5	8.6
15	25	1.5	5	0.075	132.0	132.7	93.5	8.0	8.6
16	25	1.5	5	0.075	133.9	132.7	92.3	8.0	8.6
17	25	1.5	7.5	0.025	123.8	124.3	93.0	8.0	7.9
18	25	1.5	7.5	0.125	112.5	112.4	85.3	6.5	8.7
19	25	2.5	2.5	0.075	134.8	133.7	96.5	8.0	8.8
20	25	2.5	5	0.025	134.4	134.1	96.2	7.5	8.8
21	25	2.5	5	0.125	122.6	120.5	89.8	6.5	9.2
22	25	2.5	7.5	0.075	126.5	128.4	94.2	7.5	8.7
23	35	0.5	5	0.075	124.0	122.8	89.1	8.5	8.2
24	35	1.5	2.5	0.075	128.6	130.7	95.8	8.5	8.3
25	35	1.5	5	0.025	135.4	134.1	98.8	7.0	8.6
26	35	1.5	5	0.125	110.2	113.8	92.3	7.0	7.9
27	35	1.5	7.5	0.075	125.5	122.1	96.5	8.0	8.6
28	35	2.5	5	0.075	131.4	131.6	99.0	7.5	8.9

[a] Exp.: experimental value.
[b] Mod.: model-predicted value.

and X_4) predicted by the Box-Behnken model (designated optimized medium – OM) and supplemented, according to a Plackett–Burman design, with (mg/L): biotin 0.1, myo-inositol 100, pantothenic acid 5, nicotinic acid 5, thiamine 5, pyridoxine 5 and p-aminobenzoic acid 1 (total number of 26 fermentation trials). Additionally, supplementation of OM with Tween 80 (2.4 ml/L), linoleic acid (60 mg/L) and ergosterol (24 mg/L) was also evaluated.

2.5. Statistical analysis

The *JMP*™ – *The Statistical Discovery Software* was used for generation and evaluation of the statistical experimental design. The optimized medium composition for ethanol production was obtained by solving the regression equation. The data from final comparative fermentations, between OM and RM were analyzed by *SigmaStat 3.10 (Systat software)*.

2.6. Determination of fermentation parameters

Ethanol conversion yield was calculated as the ratio between the maximum ethanol concentration produced and the glucose consumed (difference between the initial and residual glucose concentrations) and expressed as a percentage (%) of the theoretical conversion yield, i.e. the yield considering a production of 0.511 g of ethanol per g of glucose. Ethanol productivity was defined as the ratio between final ethanol concentration and total fermentation time (fermentation was considered to be complete when the mass loss stopped). The yeast viability was calculated as the ratio between viable (non-stained) and total cell counts.

3. Results and discussion

The development of a fermentation medium based on industrial substrates is economically desirable. CSL is a nutrient rich source and has been often used as a media supplement (Amartey and Jeffries, 1994; Jorgensen, 2009; Kadam and Newman, 1997; Lawford and Rousseau, 1997; Seo et al., 2009; Tang et al., 2006). To evaluate the feasibility of using CSL as the sole nutrient source to sustain high-performance VHG fermentation, fermentations of 300 g/L glucose solutions supplemented with CSL in concentrations ranging from 5 to 150 g/L were carried out. Increasing CSL concentration in the medium up to 110 g/L led to enhanced ethanol production. The highest ethanol production (*ca.* 125 g/L) was observed in fermentations with 75–110 g/L CSL. A slight decrease in yield occurred when CSL concentration was raised to 150 g/L (data not shown). Nevertheless, a fermentation medium with such high CSL concentration (>75 g/L) could compromise the economical viability of industrial fermentation processes, because of the high costs associated with this supplementation. Thus, the partial replacement of CSL with other inexpensive nutrient sources was studied. As the starting condition for such medium development experiments, we have used a basic medium (BM) containing 15 g/L CSL, therefore providing a minimum level of nutrients to support fermentation.

3.1. Screening of nutrient supplements that enhance VHG fermentation

Plackett–Burman design-based experiments were performed to select nutrients – metal ions, phosphate and nitrogen sources – that could replace CSL and enhance fermentation parameters. The use of additional CSL was also introduced as one of the independent variables in the screening design, since its level in BM was low. The supplements were chosen on the basis of their cost and availability with a potential industrial utilization in mind. Table 2 shows the experimental data as well the values predicted by the models constructed using four distinct response variables: final ethanol titre, CO_2 produced during the initial 30 h of fermentation, final biomass and final glycerol concentrations. Regression analysis showed that the models for ethanol titre ($R^2 = 0.85$ and adjusted $R^2 = 0.76$), CO_2 at 30 h ($R^2 = 0.93$; adjusted $R^2 = 0.89$) and biomass ($R^2 = 0.93$; adjusted $R^2 = 0.89$) are adequate, while the model for glycerol was not satisfactory. The significance of each coefficient was determined by student's *t*-test. The *p*-value was used as indicator of the statistical significance of the test. The results for the model using the final ethanol titre as the response are presented in Table 3, showing that CSL, urea and $MgSO_4$, whose probability values are below 0.01 (significance level higher than 99%), contributed significantly to enhancing the ethanol production in VHG fermentation. $CuSO_4$ had also a significant positive effect on ethanol production ($p < 0.05$, i.e. significance level >95%) and the supplementation with $CaCl_2$ showed a positive effect but its contribution was not significant (*p*-value >0.05). The others supplements ($ZnCl_2$, $(NH_4)_2SO_4$, KH_2PO_4 and $FeSO_4$) added to BM medium showed a negative effect on overall ethanol production (Table 3). The CO_2 produced during the initial 30 h of fermentation was used as a parameter to assess the initial fermentation rate. Besides CSL and urea, $ZnCl_2$ and KH_2PO_4 showed also a significant ($p < 0.01$) positive effect on the initial fermentation rate, with coefficients of 3.256 and 2.655, respectively (Table S1). Conversely, $CuSO_4$ had a strong significant ($p < 0.01$) negative effect (Table S1). Similar qualitative results were obtained when considering the CO_2 production at 48 h (Table S2). In terms of final biomass production, only CSL had a significant positive effect, while $CuSO_4$, urea, $CaCl_2$, $(NH_4)_2SO_4$ and $ZnCl_2$ had significant ($p < 0.01$) negative impacts (Table S3).

Taken together, the results of the screening experiment show that addition of more CSL had the strongest contribution to enhancing the fermentation kinetics, improving the initial fermentation rate, the final ethanol titre and the final biomass concentration. Supplementation with urea had also a positive effect on the fermentation rate and on the ethanol titre. Amino nitrogen, the principal component of CSL and urea likely explain these results. The positive effect of increased free amino nitrogen concentration has frequently been reported during optimization processes of VHG fermentation medium (see e.g. Bafrncová et al., 1999; Dragone et al., 2003; Jones and Ingledew, 1994; Ratnam et al., 2005). Besides, CSL contains many other nutrients, including trace elements and vitamins (Akhtar et al., 1997) that likely have a positive impact on fermentation. Surprisingly, supplementation with $(NH_4)_2SO_4$ had a negative impact on the final ethanol titre (Table 3) and only a slight positive effect (not statistically significant) on the initial fermentation rate (Table S1). This observation indicates that the selection of the nitrogen source for VHG media affects the overall yeast fermentation performance. Addition of $MgSO_4$ significantly increased the final ethanol titre (Table 3), which is in accordance with several reports describing a positive effect of magnesium ions on yeast ethanol tolerance and fermentation (Birch and Walker, 2000; Dombek and Ingram, 1986; Hu et al., 2003; Kadam and Newman, 1997; Wang et al., 2007). Magnesium seems to protect yeast cells during fermentation by a mechanism that results in decreased plasma membrane permeability under ethanol stress conditions (Birch and Walker, 2000; Hu et al., 2003). Supplementation with $CuSO_4$ (0.3 mM) also had a significant positive effect on the final ethanol titre (Table 3), although it affected negatively the initial fermentation rate (Table S1) and the final biomass production (Table S3). Similarly, Azenha et al. (2000) observed that addition of copper (0.1–1 mM) to synthetic medium resulted in marked increases in ethanol production by *S. cerevisiae*, although the fermentations became slower.

3.2. Optimization of medium for VHG fermentations

A three-level four-factor Box-Behnken experimental design was performed with BM supplemented with different combinations of the variables that were selected by the Plackett–Burman design as significant to enhance the final ethanol titre. Table 4 shows the experimental data and the values predicted by the model constructed using the final ethanol titre as the response variable. By applying multiple regression analysis on the experimental data, the following second-order polynomial equation giving the ethanol titre (Y) as a function of CSL (X_1), urea (X_2), $MgSO_4$ (X_3) and $CuSO_4$ (X_4) concentrations was obtained:

$$\begin{aligned} Y = {} & 132.716 + 3.701X_1 + 6.331X_2 - 3.152X_3 - 7.197X_4 \\ & - 1.955X_1X_2 - 1.156X_1X_3 + 0.523X_2X_3 - 2.956X_1X_4 \\ & - 0.924X_2X_4 + 1.249X_3X_4 - 5.606X_1^2 - 3.607X_2^2 \\ & - 4.402X_3^2 - 6.857X_4^2 \end{aligned} \quad (2)$$

For a good statistical model the R^2 value should be close to 1.0 where a value of 0.75 indicates the aptness of the model (Niladevi et al., 2009). The regression analysis of the data showed a good aptness of the proposed model with more than 93% variability in response being explained by the proposed model (R^2 value of 0.9347 and adjusted R^2 value of 0.8644). This indicated that Eq. (2) was a suitable model to describe the response of the experiment to ethanol production.

The analysis of variance (ANOVA) of the quadratic regression model indicated that the model was highly significant, as the F value for the model was 13.298 ($p = 0.0001$).

The results of Box-Behnken experiments showed both positive and negative dispersion of values (Table 5). The analysis of variance (ANOVA) of experimental data showed that medium supplementation with CSL, urea, $MgSO_4$ and $CuSO_4$ had a strongly linear effect on the response ($p < 0.01$, 99% significance). Increased concentrations of CSL and urea showed a positive correlation with ethanol production. Conversely, increased $MgSO_4$ and $CuSO_4$ concentrations in the medium had a negative effect on the maximum ethanol titre, possibly because CSL may already contain magnesium (Kadam and Newman, 1997) and copper ions and, consequently, low concentrations may be sufficient to complement CSL addition. Furthermore, there is a relatively narrow optimum concentration for copper and other heavy metals, which at high levels have toxic effects on yeast cells (Azenha et al., 2000).

The optimal concentrations of the four factors that maximize ethanol production were predicted using the optimization function (standard least squares numerical method) of the *JMP™ – The Statistical Discovery Software*. CSL 44.3 g/L, urea 2.3 g/L, $MgSO_4{\cdot}7H_2O$ 3.8 g/L and $CuSO_4{\cdot}5H_2O$ 0.03 g/L were chosen as the optimal concentrations (optimized medium – OM), predicting a maximum ethanol production of 139 g/L. Eight independent fermentation runs at the above optimized conditions were carried out and an average response of 130 ± 2 g/L was achieved, which reached 94% of the predicted value by the software. The good agreement between the predicted and experimental results confirmed the validity of the model.

According to the screening experiment, besides CSL and urea, $ZnCl_2$ and KH_2PO_4 also improved the initial fermentation rate significantly (Table S1), which is in agreement with previous reports on the positive effects of zinc and phosphorus in alcoholic fermentations (Xue et al., 2008; Yu et al., 2009; Zhao et al., 2009). However, supplementation of OM with 0.01 g/L $ZnCl_2$ or 2 g/L KH_2PO_4 did not have any effect on the fermentation kinetics (Fig. S1), indicating that the demand for zinc and phosphorus was already fully covered by other components, most likely CSL.

Table 5
Box-Behnken design – standardized effects of nutrient supplementation on the final ethanol titre.

Factor	Coefficient	Standard error	t-Value	p-Value
Intercept	132.72	1.5805	84.0	0.0000
CSL	3.7011	0.91250	4.06	0.0014
Urea	6.3313	0.91250	6.94	0.0000
$MgSO_4$	−3.1522	0.91250	−3.45	0.0043
$CuSO_4$	−7.1970	0.91250	−7.89	0.0000
CSL * urea	−1.9550	1.5805	−1.24	0.2380
CSL * $MgSO_4$	−1.1565	1.5805	−0.730	0.4773
Urea * $MgSO_4$	0.52250	1.5805	0.330	0.7462
CSL * $CuSO_4$	−2.9557	1.5805	−1.87	0.0841
Urea * $CuSO_4$	−0.92450	1.5805	−0.580	0.5686
$MgSO_4$ * $CuSO_4$	1.2490	1.5805	0.790	0.4436
CSL * CSL	−5.6056	1.2905	−4.34	0.0008
Urea * urea	−3.6072	1.2905	−2.80	0.0152
$MgSO_4$ * $MgSO_4$	−4.4022	1.2905	−3.41	0.0046
$CuSO_4$ * $CuSO_4$	−6.8566	1.2905	−5.31	0.0001

3.3. Screening of vitamin and lipid supplements

Supplementation of OM with vitamins (biotin, myo-inositol, pantothenic acid, nicotinic acid, thiamine, pyridoxine and p-aminobenzoic acid) (Table S4) and lipids (Tween 80, linoleic acid and ergosterol) was tested, envisioning further improvements of the fermentation rate and/or ethanol production. The positive impacts of vitamins (Alfenore et al., 2002) and lipids, particularly linoleic acid (Moonjai et al., 2002), oleic acid (the main component in Tween 80) and ergosterol (Casey et al., 1984), on yeast fermentation have been reported in the literature. Analyses of variance (ANOVA) showed that supplementation of OM with vitamins did not contribute significantly (p-value >0.05) to enhance the ethanol production in VHG fermentation (Table S5). The only vitamin showing a slightly positive effect on the final ethanol titre was biotin (coefficient of 0.378) but its contribution was not statistically significant ($p = 0.055$) (Table S5). Similarly, no improvements of final ethanol production and fermentation kinetics were observed when OM was supplemented with unsaturated fatty acids (linoleic acid or Tween 80) or ergosterol (Fig. S2). These results indicate that the yeast's requirement for vitamins and lipids was also fulfilled by CSL.

3.4. VHG fermentations with the optimized medium using laboratory and industrial strains

The significance of this medium optimization process was studied in VHG fermentations with the laboratory strain CEN.PK 113-7D and with the industrial strain PE-2, which has been isolated from bio-ethanol production facilities in Brazil and is currently one of the most widely used strains in Brazilian distilleries (Argueso et al., 2009; Basso et al., 2008). Hence, several fermentations of glucose (290–330 g/L) to ethanol were performed to compare the optimized medium (OM) with a reference medium (RM) that contained 100 g/L CSL as the sole nutrient source (Table 6). Fermentation progression is illustrated by the CO_2 production (mass loss) profiles shown in Fig. 1. Under the oxygen-limiting conditions used in this study, the kinetics of ethanol production closely followed the pattern of CO_2 evolution. Under VHG conditions, the final ethanol titres in RM were higher (relative increase *ca.* 6%) than those in standard double-strength YP medium (2YP), although the initial fermentation rates were similar in these media (Fig. 1).

With strain CEN.PK 113-7D, the maximum ethanol concentration in fermentations with approximately 290–300 g/L initial glucose was significantly higher ($n = 8$, p-value <0.01) in OM (130 g/L) than in RM (120 g/L), corresponding to a relative increase of 8%. This clear improvement in ethanol production was not evident

Table 6
VHG fermentations with approximately 300–330 g/L initial glucose with the RM and the OM by strains CEN.PK 113-7D and PE-2. Values are average ± standard deviation of eight biological replicates for the CEN.PK 113-7D data and four biological replicates for PE-2, except for the yeast viability values which are average ± range of biological duplicates.

Strain	CEN.PK 113-7D		PE-2	
Medium	RM	OM	RM	OM
Initial glucose (g/L)	290 ± 4	297 ± 6	322 ± 4	316 ± 14
Maximum ethanol concentration (g/L)	120 ± 3	130 ± 2	130 ± 0	147 ± 4
Maximum ethanol titre (%, v/v)	15.2 ± 0.4[a]	16.5 ± 0.3[b]	16.5 ± 0.0[c]	18.6 ± 0.5[d]
Residual glucose (g/L)	17.6 ± 5.0	4.3 ± 2.8	13.5 ± 0.1	0.6 ± 1.0
Final glycerol (g/L)	10.3 ± 0.5	9.9 ± 0.5	10.8 ± 0.1	13.3 ± 0.9
Final biomass (g/L)	7.0 ± 0.8	7.5 ± 0.8	9.8 ± 0.8	9.6 ± 0.6
Ethanol yield (% of the theoretical value)	86 ± 2	87 ± 3	81 ± 0	93 ± 5
Final ethanol productivity (g/L/h)	1.74 ± 0.15	1.87 ± 0.21	1.67 ± 0.00	2.41 ± 0.10
Yeast viability (%)	64 ± 7[e]	85 ± 3[f]	43 ± 4[g]	89 ± 2[h]

Maximum ethanol titre: a,b – differ significantly ($p < 0.01$) as determined by Tukey test ($n = 8$); c,d – differ significantly ($p < 0.01$) as determined by Tukey test ($n = 4$). Yeast viability: e,f; g,h – differ significantly ($p < 0.05$) as determined by Tukey test ($n = 2$).

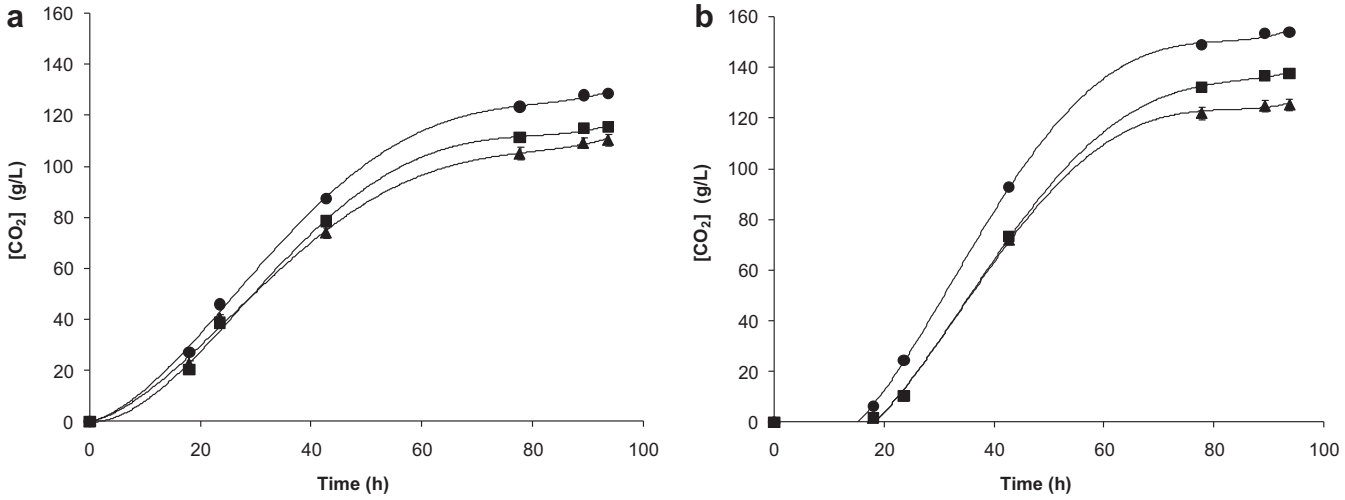

Fig. 1. Profiles of CO_2 production by strains CEN.PK 113-7D (a) and PE-2 (b) in fermentations of 325 g/L initial glucose using the following media: 2YP (▲); RM (■); OM (●).

in the final biomass concentration obtained in RM and OM (Table 6). Supplementation with optimal nutrients concentrations resulted also in higher fermentation rate (Fig. 1a) and a higher overall ethanol productivity (relative increase of 7%) (Table 6). With *ca.* 300 g/L initial glucose, only 4.3 g/L of residual glucose remained unconsumed. However, when the initial glucose concentration was raised close to 330 g/L, the fermentation stopped when the sugar residual was still 45 g/L (data not shown), possibly due to ethanol inhibition or a synergistic effect between high concentrations of ethanol and glucose in the late stages of fermentation (see Casey and Ingledew, 1986 and references therein). Furthermore, this increase in the initial glucose concentration resulted in a considerable decrease in the fermentation rate (slower fermentation) and, consequently, in overall ethanol productivity, probably due to the increased osmotic pressure (higher content of sugar) at the beginning of the fermentation process (Pratt et al., 2003).

With strain PE-2, the maximum ethanol concentration in fermentations of 300–330 g/L initial glucose was also significantly higher ($n = 4$, p-value <0.01) in OM (147 g/L) compared to RM (130 g/L), corresponding to a relative increase of 13% (Table 6). The fermentation rate was also enhanced by optimal supplementation (Fig. 1b), contributing to a relative increase of 44% in the overall ethanol productivity (Table 6).

The influence of the medium optimization process on yeast viability was also investigated. Near the end of fermentations, i.e. at the point in which an ethanol titre of 15.0 ± 0.4% (v/v) was reached, the viability of strain CEN.PK 113-7D has improved from 64% in RM to 85% in OM (Table 6). Similarly, when the ethanol titre reached 16.8 ± 0.3% (v/v), the viability of PE-2 cells improved from 43% in RM to 89% in OM (Table 6). For both strains, the increases in cell viability were statistically significant at the 95% level ($n = 2$, p-value <0.05). The higher viability possibly resulted from a higher ethanol tolerance reflecting the fact that medium supplementation with metal ions, principally magnesium, exhibited protective effect against ethanol toxicity (Xue et al., 2008).

Strain CEN.PK 113-7D was unable to ferment more than 300 g/L glucose, while the results indicate that the industrial strain PE-2 was less sensitive to osmotic pressure and ethanol stress, being able to consume 330 g/L glucose and produce a higher ethanol titre (Table 6).

Rapid fermentations and high final ethanol titres are highly desired by the ethanol industry. Ethanol concentrations as high as the maximum of 18.6 ± 0.5% (v/v) obtained in this work with the strain PE-2 have only rarely been reported in the literature. Moreover, the corresponding productivity of 2.4 ± 0.1 g/L/h and ethanol yield of 93 ± 5% (Table 6) are very interesting for batch VHG fermentation. To our knowledge, the highest ethanol titres (20.6–23.8%, v/v) were obtained in fermentations of VHG wheat mashes at temperatures not higher than 27 °C (Jones and Ingledew, 1994; Thomas et al., 1993; Thomas and Ingledew, 1992). However, at 30 °C the ethanol titre decreased to about 18% with a productivity of *ca.* 2.6 g/L/h, using supplementation with urea (0.96 g/L) (Jones and Ingledew, 1994). Alfenore et al. (2002) reported a final ethanol titre of 19%

(v/v) and a productivity of 3.3 g/L/h, but these authors have used an aerated (0.2 vvm) glucose fed-batch process with an exponential vitamin feeding strategy. Most recently, Seo et al. (2009) reported an ethanol concentration of 160 g/L (i.e. 20.3%, v/v) with an overall productivity of *ca.* 2.0 g/L/h, obtained in a glucose fed-batch process with 0.13 vvm aeration.

Although our optimization process was conducted with a laboratory strain, similar enhancement of VHG fermentation was observed with an industrial strain, suggesting that OM can be useful to test and compare the fermentative capacity of different strains. In fact, there is a lack of suitable media to test yeast strain performance under VHG conditions, since standard laboratory media that have been designed for cultivation with much lower sugar concentrations are nutrient-limited for VHG fermentations (Fig. 1). The OM described here is based on low-cost substrates and is adequate to test the limits of yeasts in terms of sugar consumption and ethanol production.

Our results show that the screening and optimization methodologies described here were effective not only in lowering the amount of CSL needed (44.3 g/L CSL in OM) to sustain fast and complete VHG fermentation, but also in significantly improving the kinetics of VHG fermentations, permitting to reach higher final ethanol titres and productivities.

4. Conclusions

A response surface methodology was successfully employed to optimize a VHG fermentation medium based in CSL and other low-cost nutrients for the efficient production of ethanol from glucose by *S. cerevisiae*. Using the optimized medium composition (g/L: CSL 44.3, urea 2.3, $MgSO_4 \cdot 7H_2O$ 3.8 and $CuSO_4 \cdot 5H_2O$ 0.03), industrial strain PE-2 was able to ferment up to 330 g/L glucose and produce 18.6% (v/v) ethanol, with a batch productivity of 2.4 g/L/h and an ethanol yield of 93%. The screening and optimization methodologies described here represent valuable tools for the development of cost-effective industrial fermentation media.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2010.04.082.

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