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# Screening of Environmental Yeasts for the Fermentative Production of Arabitol from Lactose and Glycerol

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Arabitol is a sugar alcohol, stereoisomer to xylitol, which is enlisted among the main target for biorefineries. It can serve as low calorie sweetener and as building block in the enantiopure synthesis of immunosuppressive glycolipids, herbicides, and drugs. Several studies described the fermentative production of arabitol by osmophilic yeasts, cultured with high concentrations of D-glucose. The utilization of cheaper carbon sources, such as glycerol or lactose, is of great interest for biorefinery implementation, but information on exploitation to arabitol production is still scarce. In the present study 50 yeasts belonging to 24 ascomycetous species were screened for the ability to grow and produce arabitol in presence of 80 g/L lactose or glycerol. Production from lactose was generally unsuccessful, the best producer being *Kluyveromyces lactis* WC 1401 with 0.94 g/L in 160 h. Production from glycerol was promising, with *Zygosaccharomyces rouxii* WC 1206, *Pichia guilliermondii* CBS 566, *Hansenula anomala* WC 1501, and *Candida freyschussii* ATCC 18737 yielding 3 to 4.5 g/L arabitol, with conversion yield ($Y_{P/S}$) ranging from 11 to 21.7%. Batch growth with high initial glycerol amount (160 g/L) resulted in higher production, with *H. anomala* WC 1501 yielding 10.0 g/L arabitol ($Y_{P/S}$ = 12%) in 160 h. Preliminary bioreactor fermentations with *H. anomala* WC 1501 indicated that production is not growth associated and revealed some major parameters affecting production, such as the pH and the C:N ratio, that will be the target of following studies aiming at process optimization. Cultivation under controlled oxygenation (DOT = 20%) and pH (≥ 3.0) resulted in improvement in the performance of *H. anomala* WC 1501, yielding 16.1 g/L arabitol. Cultivation in a medium with high C:N ratio, lacking inorganic nitrogen yielded 17.1 g/L arabitol. Therefore, this strain was selected for the development of a fed-batch process, aiming to improve the efficiency of the biomass, generated in the growth phase, and increasing the production in the stationary phase.

## 1. Introduction

Arabitol is a five-carbon sugar alcohol which has been attracting increasing attention as a target for biorefinery development, being enlisted among the top 12 value added chemicals derivable from biomasses (Werpy and Petersen, 2004; Erickson et al., 2013). Arabitol can find application in food industry as a low-calorie anti-cariogenic sweetener or as substrate for obtaining its stereoisomer xylitol (Loman and Ju, 2015; Li et al., 2016). Moreover, it can serve as raw material or building block in the synthesis of several enantiopure compounds, such as arabinoic and xylonic acids, immunosuppressive glycolipids, herbicides and anti-pathogenic drugs (Werpy and Petersen, 2004). Therefore, the development of sustainable microbial processes for production of arabitol, alternative to chemical reduction of arabinose, lyxose, or their corresponding lactones, has attracted increasing interest. Particular attention had been focused on production by bioconversion of arabinose, utilizing selected yeast strains, mostly belonging to the genus *Candida* (Kordowska-Wiater, 2015). Another possibility is the fermentative production with osmophilic/osmotolerant yeasts (e.g. belonging to the genera *Candida*, *Pichia*, *Debaryomyces*, and *Zygosaccharomyces*), which are known to produce arabitol naturally under certain culture conditions, usually in presence of high substrate concentration (Kordowska-Wiater, 2015). This approach is particularly interesting in the perspective of a biorefinery, if the fermentative process could be developed aiming at the valorization of wastes or cheap carbon sources. Several fermentative processes with osmophilic yeasts growing on glucose have already

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been proposed, the ones utilizing *Zygosaccharomyces rouxii*, *Metschnikowia reukaufii*, *Kodameae ohmeri* exhibiting the highest yield (> 40% w/w) and being the most promising (Saha et al., 2007; Nozaki et al., 2003; Zhu et al., 2010). Lactose and glycerol are inexpensive carbon sources both available in large amount in industrial effluents, the former from cheese manufacturing, the latter from biodiesel industry. However, utilization of these substrates in arabitol production is still understudied and only few strains have been proposed so far. A strain of *Kluyveromyces lactis* was proposed for lactose utilization, with a production of 25 g/L and a yield of 13% w/w (Toyoda and Ohtaguchi, 2009, 2011). *Debaryomyces hansenii* and *Candida quercitrusa* were proposed for production from glycerol, the latter yielding 85 g/L after 10 days under optimized conditions (Koganti et al., 2011; Yoshikawa et al., 2014).
In the present study fifty yeasts strains were screened in order to identify potential candidate strains for the development of a fermentative process yielding arabitol from lactose and glycerol.

## 2. Materials and methods

### 2.1 Strains and culture conditions

Fifty ascomycetous yeast strains belonging to the species *Candida castellii*, *Candida freyschussii*, *Candida humilis*, *Candida maltosa*, *Candida sake*, *Hansenula anomala*, *Hansenula beijerinckii*, *Hansenula jadinii*, *Kluyveromyces bacillosporus*, *Kluyveromyces lactis*, *Kluyveromyces lodderae*, *Kluyveromyces marxianus*, *Pichia angusta*, *Pichia burtonii*, *Pichia fermentans*, *Pichia guilliermondii*, *Saccharomyces boulardii*, *Saccharomyces castellii*, *Saccharomyces cerevisiae*, *Saccharomyces dairenensis*, *Saccharomyces exiguus*, *Saccharomyces spencerorum*, and *Zygosaccharomyces rouxii* were obtained from ATCC (Manasses, VA, USA), CBS (Utrecht, the Netherlands), or from our own collection. All the strains were aerobically cultured at 30°C in YPD broth (BD Difco, Sparks, MD, USA).
To screen arabitol production on glucose, lactose, and glycerol, the yeasts were cultured at 30°C in shake flasks of MY medium, containing the following: 80 or 160 g/L carbon source, 3 g/L yeast extract (BD Difco, Sparks, MD, USA), 2 g/L $(NH_4)_2SO_4$, 3 g/L $KH_2PO_4$, 1 g/L $K_2HPO_4$, and 1 g/L MgSO4 · $7H_2O$. The cultures were inoculated 5% v/v with a 72 h seed culture grown in MY medium containing 20 g/L carbon source.
All chemicals were obtained from Sigma-Aldrich (Steinheim, Germany), unless otherwise stated.

### 2.2 Batch experiments and bioreactor operation

Batch experiments were carried out in a benchtop bioreactor (Labfors, Infors, Bottmingen, Switzerland) with 2 L of MY medium containing 160 g/L glycerol. Bioreactor cultures were inoculated 5% v/v with a 24-h seed culture grown in presence of 20 g/L glycerol. Cultures were aerated with 0.5 v/v/min filter-sterilized air and stirred from 300 to 900 rpm, with cascade regulation to keep the DOT at 20%. The pH was continuously measured and, if necessary for the specific experiment, was prevented from decreasing below 3.0 or 6.0 by automatic titration with 4 M NaOH. Foaming was kept under control through the automatic addition of Xiameter 1520 (Dow Corning, Midland, MI, USA). Samples were collected periodically to monitor the growth and to analyze glycerol and arabitol.

### 2.3 Chemical analysis

Glucose, lactose, glycerol, and arabitol in the supernatants of the cultures were quantified by HPLC with refractive index detector (1200 System, Agilent Technologies, Waldbronn, Germany) and Aminex HPX-87 H ion exclusion column. Isocratic elution was carried out at 60°C with 0.8 ml $min^{-1}$ of 5 mM $H_2SO_4$ (Raimondi et al., 2014). Arabitol identification was carried out by comparison of the retention time with that of a standard solution, without investigating its D/L configuration.
Growth was monitored by measuring the turbidity at 600 nm ($OD_{600}$). Cell counts were quantified in a Bürker chamber.

### 2.4 Statistical analysis

All values are means of three separate experiments. ANOVA followed by Tukey post hoc comparisons, was applied for the analysis of independent measures. Means differences were considered statistically significant for $P < 0.05$.

## 3. Results and discussion

### 3.1 Screening of arabitol production on lactose and glycerol

Arabitol production by 50 mesophilic ascomycetous yeasts was compared in glucose, lactose, and glycerol. Based on literature evidence (Kordowska-Wiater, 2015), indicating that arabitol production is enhanced by

osmotic stress, the strains were first cultured in presence of 20 g/L carbon source, then transferred to flasks with 80 g/L (Table 1).

*Table 1: Growth, substrate consumption (ΔS), and product generation (ΔP) of 50 yeasts after 160 h of growth in MY medium containing 80 g/L glucose, lactose, and glycerol. $Y_{P/S}$ was calculated for the strains which yielded at least 0.5 g/L arabitol. - indicates ΔS < 1 g/L; -- indicates ΔP < 0.1 g/L. Values are means, n = 3, SD always < 7%.*

| | Glucose | | | | Lactose | | | | Glycerol | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $OD_{600}$ | ΔS g/L | ΔP g/L | $Y_{P/S}$ % | $OD_{600}$ | ΔS g/L | ΔP g/L | $Y_{P/S}$ % | $OD_{600}$ | ΔS g/L | ΔP g/L | $Y_{P/S}$ % |
| *C. castellii* ATCC 22945 | 4.4 | - | -- | | | | | | 28.0 | 30.9 | -- | |
| *C. freyschussii* ATCC 18737 | 22.3 | 29.0 | 2.1 | 7.3 | | | | | 30.3 | 25.2 | 3.1 | 12.3 |
| *C. humilis* WC 1105 | 20.5 | > 79 | 1.2 | 1.5 | | | | | 46.0 | 61.3 | -- | |
| *C. maltosa* ATCC 20275 | 13.7 | 79.0 | 0.5 | | 0.8 | - | -- | | 28.6 | 22.3 | -- | |
| *C. sake* ATCC 28138 | 16.1 | 30.0 | -- | | 0.4 | 6.7 | -- | | 32.0 | 13.1 | -- | |
| *H. anomala* WC 1501 | 22.3 | 36.4 | 1.1 | 3.0 | 0.4 | - | -- | | 21.2 | 20.8 | 4.5 | 21.7 |
| *H. beijerinckii* CBS 2565 | 1.7 | - | -- | | 1.9 | - | -- | | 4.6 | 5.6 | -- | |
| *H. jadinii* CBS 1600 | 20.0 | 76.5 | 2.3 | 3.0 | 0.6 | - | -- | | 45.3 | 70.7 | 2.1 | 3.0 |
| *K. bacillosporus* ATCC 200960 | 10.2 | > 79 | 1.4 | 1.7 | 0.8 | - | -- | | 29.6 | 22.0 | -- | |
| *K. lactis* MW 270-7B | 4.9 | - | -- | | 3.7 | 2.8 | 0.9 | 33.7 | 4.0 | 3.3 | 0.1 | |
| *K. lactis* MW L9 | 4.9 | 5.4 | 0.1 | | 0.2 | - | -- | | 3.4 | 3.6 | -- | |
| *K. lactis* WC 1401 | 4.8 | 18.2 | 0.4 | | 0.2 | - | -- | | 4.2 | 2.6 | -- | |
| *K. lactis* WC 1403 | 5.2 | 15.8 | 0.4 | | 0.2 | - | -- | | 3.8 | 3.1 | -- | |
| *K. lactis* WC 1406 | 5.2 | 5.5 | 0.1 | | 6.1 | 79.7 | -- | | 4.2 | 2.9 | -- | |
| *K. lactis* WC 1410 | 20.4 | > 79 | 0.3 | | 0.9 | - | -- | | 24.3 | 18.3 | -- | |
| *K. lactis* WC 1412 | 5.5 | 3.3 | 0.1 | | 0.2 | - | -- | | 3.0 | 1.9 | -- | |
| *K. lodderae* ATCC 6308 | 20.3 | 77.3 | -- | | 0.9 | - | -- | | 19.0 | 19.3 | -- | |
| *K. marxianus* WC 1400 | 23.3 | 77.0 | 0.1 | | 5.3 | 79.0 | -- | | 23.1 | 18.3 | -- | |
| *K. marxianus* WC 1402 | 23.9 | 64.8 | 0.1 | | 23.8 | > 79 | -- | | 22.6 | 21.3 | -- | |
| *K. marxianus* WC 1405 | 20.4 | > 79 | -- | | 19.7 | 6.8 | -- | | 22.8 | 26.3 | -- | |
| *K. marxianus* WC 1408 | 20.7 | 72.9 | 0.1 | | 24.9 | 70.5 | -- | | 24.6 | 6.9 | -- | |
| *K. marxianus* WC 1409 | 26.9 | > 79 | 0.2 | | 21.2 | 79.1 | -- | | 26.6 | 8.4 | -- | |
| *K. marxianus* WC 1411 | 24.3 | > 79 | 0.1 | | 0.7 | - | -- | | 23.3 | 19.5 | -- | |
| *P. angusta* WC 1502 | 29.0 | > 79 | 0.7 | 0.9 | 1.4 | - | -- | | 34.9 | 26.5 | -- | |
| *P. burtonii* CBS 2452 | 19.1 | 77.7 | 3.0 | 3.9 | | | | | 11.6 | 21.4 | -- | |
| *P. fermentans* WC 1507 | 22.5 | 31.4 | 0.1 | | | | | | 44.8 | 52.9 | 0.2 | |
| *P. guilliermondii* CBS 566 | 31.8 | 77.5 | 0.2 | | 4.8 | - | -- | | 37.0 | 39.4 | 4.3 | 11.0 |
| *S. boulardii* WC 1019 | 10.3 | > 79 | -- | | | | | | 20.9 | 18.3 | -- | |
| *S. castellii* ATCC 76901 | 4.4 | > 79 | -- | | | | | | | | | |
| *S. cerevisiae* BY4741 | 2.6 | 20.1 | -- | | | | | | 14.4 | 11.2 | -- | |
| *S. cerevisiae* BY4742 | 2.9 | 20.9 | -- | | | | | | 1.2 | - | -- | |
| *S. cerevisiae* WC 1053 | 10.4 | > 79 | -- | | | | | | 12.0 | 2.3 | -- | |
| *S. dairenensis* WC 1052 | 13.2 | > 79 | -- | | | | | | | | | |
| *S. exiguus* WC 1049 | 4.9 | 12.5 | -- | | | | | | 4.3 | 2.1 | -- | |
| *S. exiguus* WC 1050 | 10.6 | > 79 | -- | | | | | | 5.5 | 4.2 | -- | |
| *S. spencerorum* ATCC 200069 | 7.4 | 28.9 | -- | | | | | | 11.6 | 14.5 | -- | |
| *S. spencerorum* ATCC 60635 | 12.4 | > 79 | -- | | | | | | 27.5 | 32.5 | -- | |
| *T. delbrueckii* WC 1507 | 2.9 | > 79 | -- | | | | | | 0.4 | - | -- | |
| *Z. rouxii* WC 1200 | 11.2 | > 79 | 14.0 | 17.7 | | | | | 18.5 | 21.1 | 0.4 | |
| *Z. rouxii* WC 1201 | 17.9 | > 79 | 10.4 | 13.2 | | | | | 15.0 | 13.2 | 0.4 | |
| *Z. rouxii* WC 1202 | 13.2 | > 79 | 7.3 | 9.2 | | | | | 5.7 | 4.5 | 0.8 | 17.2 |
| *Z. rouxii* WC 1203 | 15.5 | > 79 | 10.2 | 12.9 | | | | | 8.0 | 9.9 | 0.7 | 6.5 |
| *Z. rouxii* WC 1204 | 15.1 | > 79 | 14.9 | 18.8 | | | | | 7.9 | 11.8 | 1.5 | 12.4 |
| *Z. rouxii* WC 1206 | 13.8 | > 79 | 7.8 | 9.8 | | | | | 10.7 | 21 | 4.1 | 19.3 |
| *Z. rouxii* WC 1207 | 15.7 | > 79 | 6.6 | 8.4 | | | | | 11.7 | 3.1 | 0.2 | |
| *Z. rouxii* WC 1208 | 4.5 | 2.8 | 0.1 | | | | | | 0.3 | - | -- | |
| *Z. rouxii* WC 1209 | 9.7 | > 79 | 7.6 | 9.7 | | | | | 12.5 | 9.6 | 0.6 | 6.5 |
| *Z. rouxii* WC 1210 | 9.7 | 63.0 | 4.7 | 7.4 | | | | | 0.2 | - | -- | |
| *Z. rouxii* WC 1211 | 11.9 | 78.2 | 4.5 | 5.7 | | | | | 16.4 | 14.6 | 0.4 | |
| *Z. rouxii* WC 1212 | 11.7 | 58.8 | 4.4 | 7.5 | | | | | 18.5 | 9.6 | 1.5 | 15.5 |

On glucose, the best arabitol producers were found within the species *Z. rouxii* in agreement with literature information (Saha et al., 2007). With only one exception, the strains of *Z. rouxii* consumed most of glucose

and produced up to 14.9 g/L arabitol, with product/substrate yield ($Y_{P/S}$) ranging from 5.7 to 18.8%. Few arabitol producers were found in species other than *Z. rouxii* (e.g. *C. freyschussii* ATCC 18737, *H. anomala* WC 1501, H. jadinii CBS 1600, and *P. burtonii* CBS 2452) although with a production always $\leq$ 3.0 g/L and $Y_{P/S} \leq 7.3\%$.

A limited number of strains was capable of growth with 20 g/L lactose and even fewer with 80 g/L, among them those belonging to *K. lactis* and *K. marxianus*. Overall, the screening for an arabitol producer from lactose was unsuccessful, despite it was reasonable to expect some good candidate within *K. lactis*, based on literature data (Toyoda and Ohtaguchi, 2009). Only *K. lactis* MW 270-7B was found to produce arabitol, even though in little concentration (0.9 g/L) and at the expense of a minor substrate consumption.

With only few exceptions, most strains grew well in 20 g/L glycerol and were transferred to 80 g/L glycerol. Arabitol was found in the supernatant of 16 strains, which had been identified as producer also on glucose or lactose: *C. freyschussii* ATCC 18737, *H. anomala* WC 1501, H. jadinii CBS 1600, *P. fermentans* WC 1507, *P. burtonii* CBS 2452, *K. lactis* MW 270-7B, and 10 strains of *Z. rouxii*. The best producers were *H. anomala* WC 1501, *P. guilliermondii* CBS 566, *Z. rouxii* WC 1206, *C. freyschussii* ATCC 18737 which yielded 4.5, 4.3, 4.1, and 3.1 g/L arabitol, respectively, with $Y_{P/S}$ ranging from 11.0 to 21.7%, while all the other strains yielded less than 2.1 g/L. For *H. anomala* WC 1501, *P. guilliermondii* CBS 566, *C. freyschussii* ATCC 18737, production was significantly higher ($P < 0.05$) on glycerol than on glucose, while the opposite behavior was observed for *Z. rouxii* WC 1206 and all the other strains of it species.

*H. anomala* WC 1501, *C. freyschussii* ATCC 18737, *P. guilliermondii* CBS 566, and *Z. rouxii* WC 1206 were cultured in flasks of MY medium containing 160 g/L glycerol, in order to augment the osmotic stress and to evaluate whether arabitol production could be improved. Doubling the concentration of glycerol significantly reduced arabitol production by *Z. rouxii* WC 1206 to 0.6 g/L ($P < 0.05$), whereas it lead to higher concentration ($P < 0.05$) in all the other strains, particularly in *H. anomala* WC 1501. *H. anomala* WC 1501, *C. freyschussii* ATCC 18737, and *P. guilliermondii* CBS 566, yielded 10.0, 7.2 and 4.8 g/L arabitol after 160 h of cultivation, with $Y_{P/S}$ yield of 15.6, 11.4, and 9.1%, respectively. The species *C. freyschussii* and *P. guilliermondii* are described herein for the first time as capable of producing arabitol from glycerol. *P. guilliermondii* was reported to transform L-arabinose to L- arabitol (Saha and Boothast, 1996), therefore the data here reported could extend the range application of this yeast to both fermentative and biotransformation processes. However, *H. anomala* WC 1501 seemed the most promising for the fermentative production of arabitol from glycerol and was selected for a deeper investigation of the production kinetics and the factors affecting the process. The ability of *H. anomala* was already pointed out in a previous screening (Yoshikawa et al., 2014), although it has never been investigated and optimized so far.

### 3.2 Batch fermentations of *H. anomala* WC 1501

Bioreactor batch fermentations of *H. anomala* WC 1501 were carried out with 160 g/L glycerol, in order to study the kinetics of growth and production (Figure 1). The culture grew with a specific rate of 0.2 $h^{-1}$ for 20 h, then it get slower and progressively entered into the stationary phase at approx. 40 h. Growth was accompanied by a drastic pH drop (from 6.2 to 2.2 between 16 and 20 h), a progressive increase in the oxygen demand, resulting in increasing the stirring to keep the DOT at 20%, a partial consumption of glycerol (approx. 20 g/L at 24 h), and a minor production of arabitol (0.5 g/L at 24 h). During the stationary phase, glycerol continued to be consumed, although with reduced oxygen demand, while arabitol progressively accumulated. After 160 h, 58 g/L glycerol were consumed and 8.1 g/L arabitol were obtained, with a $Y_{P/S}$ of 15.6% and a volumetric productivity of 0.05 g/L/h. These data clearly indicate that arabitol production by *H. anomala* WC 1501 is not growth associated and is the result of a secondary utilization of the excess of carbon source. Therefore the medium composition, and in particular the balance between carbon and the other nutrients, is crucial for the optimization of the production. The conditions under which the cultured is kept during the stationary phase are another factor to be investigated, in order to improve the efficiency of the biomass generated during the growth phase.

A previous study reported that arabitol production by *D. hansenii* was associated to acidic conditions, while pH > 3.5 resulted in drastically worse yield and productivity (Koganti and Ju, 2013). In order to determine how the pH affected production by *H. anomala* WC 1501, batch fermentations were carried out with automatic titration preventing the pH from dropping below 3 and 6. Compared with uncontrolled cultures, glycerol was consumed at a greater extent ($P < 0.05$) and yielded a greater amount of arabitol ($P < 0.05$) at the end of the fermentation (160 h). At pH = 3, 152 g/L glycerol were consumed and 16.1 g/L arabitol were obtained, with a $Y_{P/S}$ of 10.6% and a volumetric productivity of 0.10 g/L/h. At pH = 6, 99.7 g/L were consumed and 13.2 g/L arabitol were produced, with $Y_{P/S}$ of 13.2% and a volumetric productivity of 0.08 g/L/h. This results suggest that, unlike *D. hansenii*, for *H. anomala* the optimal conditions in terms of $Y_{P/S}$ and productivity have to be searched within a range of higher pH values.

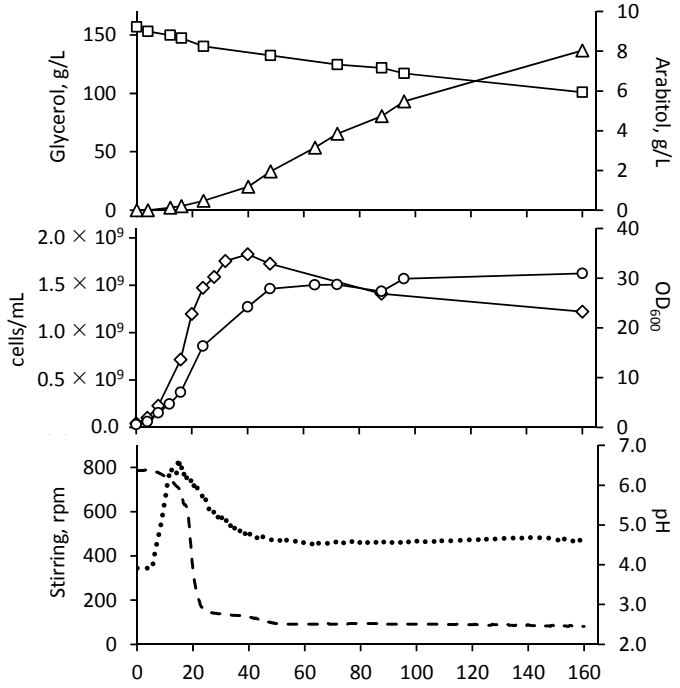


*Figure 1: Timecourse of a representative batch fermentation of H. anomala WC 1501 with uncontrolled pH. Symbols: □, glycerol; △, arabitol; ○, turbidity; ◇, cell counts; dashed line, pH; dotted line, stirring.*

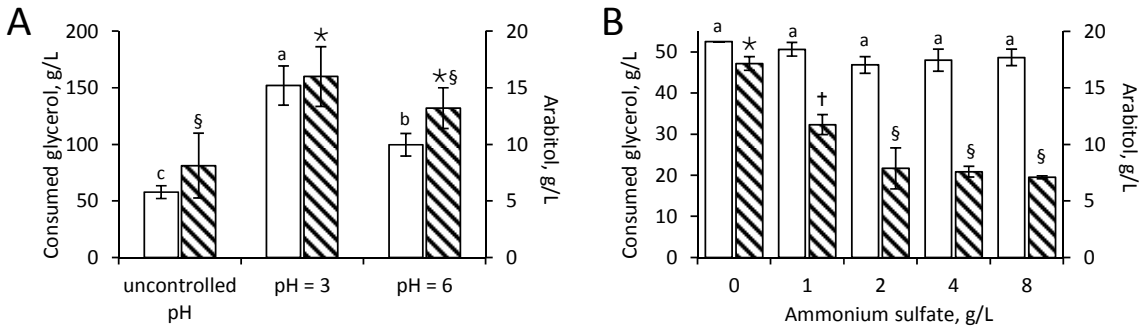


*Figure 1: Effect of the pH (A) and ammonium sulfate (B) on glycerol consumption (white bars) and arabitol production (dashed bars) after 160 h of cultivation. Values are means ± SD, n = 3, within each series, values with a common symbol or letter are not significantly different (P > 0.05).*

Since arabitol production was not growth associated and occurred mostly during the stationary phase when the culture was in presence of an excess of carbon and was likely limited by the nitrogen source, batch fermentations were carried out in MY medium containing different concentrations of ammonium sulfate (0, 1, 2, 4, and 8 g/L). After 160 h of incubation, the 2, 4, and 8 g/L ammonium sulfate yielded the same arabitol production of 7-8 g/L. A significant increase in arabitol production was observed with 1 and especially with 0 g/L ammonium sulfate. In these conditions, 11.7 and 17.1 g/L were obtained. These results indicate that production is not only positively influenced by the concentration of the carbon source, impacting the osmotic pressure of the broth, but is also affected by the carbon:nitrogen ratio of the nutrients.

## 4. Conclusions

The screening of the yeasts failed to obtain any good candidate yeast for producing arabitol from lactose, but identified *H. anomala* WC 1501 as a good candidate for production from glycerol. Preliminary batch fermentation experiments indicated that production is not growth associated and revealed some major

parameters affecting production, such as the glycerol concentration, the C:N ratio, and the pH. These parameters will be the target of following studies aiming at optimizing the conditions for producing arabitol from glycerol in a fed-batch process.

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