By-products of sugar factories and wineries as feedstocks for erythritol generation

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Abstract

Erythritol is a polyol suitable for human nutrition which is industrially produced by microorganisms from costly glucose-rich or starch-rich feedstocks. The utilization of sugary by-products from food industries could significantly reduce the costs of the process. Erythritol production from sugarcane molasses, beet molasses and surplus grape musts was assessed, by comparing four fungal strains. Beet molasses presented toxic effects on microorganisms, which hampered erythritol production to a certain extent. Moniliella pollinis attained erythritol production yields of 0.239 ± 0.001 g/g for sugarcane molasses (106.40 ± 0.42 g/L erythritol), 0.229 ± 0.003 g/g for beet molasses (57.78 ± 1.52 g/L), 0.362 ± 0.005 g/g for red grape must (96.26 ± 1.31 g/L) and 0.383 ± 0.004 g/g for rosé grape must (92.84 ± 0.98 g/L), with nearly total sugar consumption in 6-9 days. In comparison, control fermentations with beet syrup reached erythritol yields of 0.327 ± 0.009 g/g (126.78 ± 3.21 g/L). Therefore, erythritol production could be considered as a viable route in certain food industries aiming at market diversification.

Keywords: erythritol, bioproducts, molasses, surplus grape must, food by-products
1. Introduction

Erythritol is a four-carbon polyol which is widely present in nature and can be found in seaweeds, fungi, fruits and fermented foods. Its low insulin index, its rapid absorption and excretion rates by the human organism (which makes erythritol being regarded as zero-calorie sweetener) and its safety as an additive, have contributed to its use in many food formulations (Moon et al., 2010; Rzechonek et al., 2018). In addition, erythritol has been proposed as a platform chemical (Nakagawa et al., 2020).

Erythritol is industrially produced by microorganisms. The main carbon sources for its biosynthesis are sugars, such as glucose, fructose or sucrose (Aoki et al., 1993; Hajny et al., 1964; Jeya et al., 2009), and glycerol (Cho et al., 1999a; Rymowicz et al., 2009). Under high osmotic pressure conditions (normally provoked by high substrate concentrations), these carbon sources are converted into erythritol via the pentose phosphate pathway by certain fungal strains of the genera Moniliella, Pseudozyma, Candida or Yarrowia (Rzechonek et al., 2018). The current state of erythritol production by microorganisms has been recently reviewed by several authors (Carly and Fickers, 2018; Moon et al., 2010; Regnat et al., 2018; Rzechonek et al., 2018).

When sugars are employed as raw materials for erythritol production, generally expensive glucose-rich or starch-rich feedstocks are used (Carly and Fickers, 2018). However, the utilization of sugary wastes or by-products from food industries could significantly reduce the economic costs of the process. The use of low-quality or crude glycerol as an affordable substrate for erythritol production has been described lately, attaining yields of 0.53-0.58 g/g and erythritol concentrations of up to 180 g/L depending on the initial glycerol concentration (Kobayashi et al., 2015; Mirończuk et al., 2015; Rakicka-Pustułka et al., 2020; Rakicka et al., 2017a, 2017b). In addition, agri-food wastes such as soy pulp (Liu et al., 2017b) or waste cooking oil (Kahr et al., 2011; Liu et al., 2019b, 2018a, 2017a) have been assessed for erythritol production, yielding moderate concentrations of 15-50 g/L erythritol. Nevertheless, to the best of our knowledge, sugar-rich industrial by-products (containing sucrose, glucose or fructose) have never been used as carbon sources for the direct production of erythritol. Only the utilization of sugar beet molasses has been reported as a nutritive medium for fungal biomass generation before starting the real erythritol-producing stage from concentrated glycerol (Mirończuk et al., 2015; Rakicka et al., 2017a).

Molasses is a dense, sweet syrup obtained as a by-product during the manufacture of beet or sugarcane in sugar production processes (CIBE-CEFS, 2017; Scoma et al., 2017). The world production of molasses in 2014 was estimated to be 60,965,075 t (FAOSTAT, 2020). Molasses is generally used for bioethanol fermentation and as an ingredient in animal feed (CIBE-CEFS, 2017). Although pure sugar sources are preferred, molasses has also been used as a substrate for the production of citric acid, lactic acid, amino acids, xanthan gum and butanol, among other bioproducts (Scoma et al., 2016). The main advantage of molasses over other sugar-rich feedstocks is its relatively low cost. In Spain, the price of molasses in 2016 was in the range of 124-197 €/t (MINECO, 2016). However, the use of molasses in fermentation biorefineries presents certain limitations, mainly due to its high concentration of organic matter and its high salinity, which can seriously harm microbial growth, especially because of an excess of K⁺ (Scoma et al., 2017) or, in the particular case of erythritol production, due to an inadequate C/N ratio related to an excess of proteins (Liu et al., 2020). The feasibility of erythritol production from molasses could represent a very attractive alternative for business diversification in sugar factories.

Other possible feedstocks for erythritol biosynthesis could be low-quality grape must and surplus grape must. Glucose and fructose concentrations in grape musts fluctuate depending on cultivar varieties and climate conditions, but in general their sum reaches 180-220 g/L, with typical glucose/fructose ratios of 0.91-0.99 (Amerine and Thoukis, 1958; Esteban et al., 1999). Wine production worldwide attained 29,200 ML in 2018 (OIV, 2019). Spain devoted 938,391 ha to vineyard in 2019, which makes it rank first in the world in land area planted with this crop and third as wine producer worldwide in 2018 (MAPA, 2020; OIV, 2019). During the campaign 2019, about 3,400 ML wine were produced in Spain, whereas approximately 604 ML
grape juice and must were manufactured (MAPA, 2020). The European Union has tried to avoid wine surplus through legal regulations (Council Regulation (EC) No 1493/1999; Council Regulation (EC) No 479/2008), because it causes an important commercial imbalance. This surplus is mainly managed by controlled distillation, and storage for table wine and grape juice (Corsinovi and Gaeta, 2019; Meloni and Swinnen, 2018). Recently, France suggested to the European Union the transformation of wine surplus into hand disinfectant for the COVID-19 pandemic to mitigate market problems (ICEX, 2020) and the Spanish government has approved financial aid for wine distillation, private storage and green harvesting in order to alleviate the economic shock of the pandemic in the winery sector (Spanish Royal Decree 557/2020). The use of grape musts for erythritol biosynthesis could represent a business opportunity under these circumstances.

In this work, the production of erythritol from sugar-rich industrial by-products (sugarcane molasses, beet molasses and surplus grape must) is assessed for the first time. Four microbial strains were evaluated and compared in order to establish a viable procedure to obtain erythritol-rich broths in short periods (6-9 days).

2. Material and methods

2.1. Feedstock description

Four agri-food by-products were assessed for erythritol production; namely, sugarcane molasses, beet molasses, red grape must and rosé grape must. Sugarcane molasses from an ethanol plant (Azucarera del Guadalfeo, Salobreña, Spain) were provided by Vedeqsa (Terrassa, Spain) in June 2018. Beet molasses were supplied by ACOR sugar factory (Olmedo, Spain) in May 2019. Red grape must was obtained in September 2019 from the Oenological Station of Castile and Leon - ITACyL (Rueda, Spain). Rosé grape must was obtained in September 2019 from the commercial wine cellar Leyenda del Páramo (Valdevimbre, Spain). In addition, beet syrup, a sugar-rich and valuable food stream was employed as a control for erythritol production. Beet syrup was kindly provided by ACOR in November 2016. The chemical composition of these feedstocks is shown in Table 1. Although sugar contents were similar in syrup and molasses, beet syrup had higher humidity (which makes it less viscous), whereas both molasses were richer in proteins. The elevated content of ashes in molasses, especially in beet molasses (Table 1), was translated into a high concentration of anions and cations in comparison to beet syrup. It must be noted that beet molasses contained remarkably high levels of sodium and potassium. Regarding grape musts, their sugar, protein and anion contents were notably lower than those of syrup and molasses (Table 1).

| Chemical composition of the feedstocks used for erythritol production. |
|---------------------------------------------------------------|
| **Table 1.**                                                                 |
| **General components**                                                                 |
| **Beet syrup (control)** | **Sugarcane molasses** | **Beet molasses** | **Red grape must** | **Rosé grape must** |
| **Sucrose (%)** | 55.24 | 59.22 | 50.29 | n.d. | n.d. |
| **Glucose (%)** | 0.27 | 2.01 | 0.53 | 12.46 | 9.79 |
| **Fructose (%)** | 0.05 | 3.24 | 0.11 | 11.89 | 9.88 |
| **Protein (%)** | 1.09 | 3.47 | 9.28 | 0.21 | 0.20 |
| **Humidity (%)** | 40.51 | 16.95 | 17.79 | n.a. | n.a. |
| **Ashes (%)** | 0.90 | 4.28 | 9.04 | n.a. | n.a. |
| **Density (g mL⁻¹)** | 1.26 | 1.40 | 1.39 | 1.10 | 1.09 |
| **Anions and cations**                                                                 |
| **Chloride (mg/L)** | 835 | 9453 | 9929 | 29 | 34 |
| **Fluoride (mg/L)** | 400 | 1335 | 13805 | 28 | 27 |
| **Phosphate (mg/L)** | 125 | 137 | 188 | 185 | 367 |
| **Nitrate (mg/L)** | 90 | 2369 | 2890 | 1 | 2 |
| **Nitrite (mg/L)** | 70 | <5 | <50 | <1 | <1 |
| **Sulphate (mg/L)** | 1415 | 7337 | 10827 | 49 | 101 |
| **Bromide (mg/L)** | <1 | <5 | <75 | <1 | <1 |
| **Sodium (mg/L)** | 646 | 4540 | 18028 | 3 | 4 |
| **Potassium (mg/L)** | 5271 | 46546 | 46746 | 1447 | 1914 |
| **Calcium (mg/L)** | 50.15 | 6423 | 2681 | 143.5 | 173.2 |
| **Magnesium (mg/L)** | 9.33 | 701 | 49.72 | 81.88 | 2.19 |
| **Iron (mg/L)** | 7.70 | 780 | 68.46 | <0.10 | 0.27 |
| **Manganese (mg/L)** | 1.16 | 13.10 | 25.40 | 0.16 | 2.19 |
| **Zinc (mg/L)** | 0.96 | 20.67 | <10 | 0.2 | 0.46 |
| **Copper (mg/L)** | 0.63 | 8.81 | <10 | <0.04 | <0.04 |

n.d. Not detected. n.a. Not analysed.

During preliminary tests, it was observed that beet molasses, diluted at an initial sugar concentration of 300 g/L, did not allow microbial growth for erythritol production. Therefore, beet molasses were subjected to detoxification with two different approaches. In the first place, given the high potassium concentration of beet molasses, a purification step to remove cations by using the acidic ion-exchange resin Amberlite® IR-120 (Merck KGaA, Darmstadt, Germany) was assessed (Appendix A). Beet molasses with a concentration of 300 g/L total sugars were passed through the ion-exchange resin. For fermentation, these resin-treated samples were kept at approximately 300
g/L sugars or were further diluted to 200 g/L. The second approach consisted on the evaluation of different C/N ratios (since protein content in this substrate was high), both for raw beet molasses and resin-treated beet molasses. Accordingly, for initial sugar concentrations of 300 and 200 g/L, various yeast extract doses (from 0 to 5 g/L) were evaluated. The viability of erythritol production from all these samples was tested by fermentation with *M. pollinis* MUCL 40570, under the general conditions explained in section 2.2.3 (except for sugar and yeast extract concentrations).

On the contrary, beet syrup, sugarcane molasses and grape musts were used in their raw state, as they presented no toxicity for erythritol-producing microorganisms.

### 2.2. Microbial growth and erythritol production

#### 2.2.1. Strain cultivation

The strains *Moniliella pollinis* MUCL 40570 and *M. pollinis* MUCL 28141 were bought from BCCM/MUCL (Louvain-la-Neuve, Belgium), *Pseudozyma fusiformata* DSM 27425 was purchased from DSMZ (Braunschweig, Germany), and the strain *P. tsukubaensis* NRRL Y-7792 was obtained from ARS Culture Collection NRRL (Peoria, IL, USA). The fungi were cultivated in liquid media at 25 °C and 150 rpm (relative centrifugal force, RCF = 0.31) in an Infors HT Ecotron orbital shaker (Infors AG, Bottmingen, Switzerland) under aerobic conditions during 24-72 h. *M. pollinis* MUCL 40570 was grown in 20 g/L glucose, 5 g/L yeast extract and 10 g/L soy peptone; *M. pollinis* MUCL 28141 was grown in 80 g/L malt extract; whereas *P. fusiformata* DSM 27425 and *P. tsukubaensis* NRRL Y-7792 were cultivated in 10 g/L glucose, 3 g/L yeast extract, 3 g/L malt extract and 5 g/L soy peptone. Then, 1.5 ml of each of the four suspensions containing fungal cells were mixed with 0.4 ml glycerol and preserved at -80 °C.

#### 2.2.2. Preparation of fungal inocula

Dilute solutions of beet syrup, sugarcane molasses, beet molasses, red grape must and rosé grape must were prepared by addition of distilled water until a final concentration of 30 g/L total sugar was attained. These solutions were supplemented with 10 g/L yeast extract in the case of beet syrup and grape musts and 5 g/L yeast extract for sugarcane molasses and beet molasses. Their pH was adjusted to 5.5. Fifty millilitres of this growing medium were placed in a 100-mL Erlenmeyer flask. After sterilising the solutions, they were inoculated with a loopful of microbial cells from the cryopreserved stocks. The flasks were capped with polyurethane foam stoppers and they were incubated under aerobic conditions at 30 °C and 150 rpm (RCF = 0.31) in an Infors HT Ecotron orbital shaker during 48-72 h, until cell density reached approximately $3 \cdot 10^7$ cells/mL.

#### 2.2.3. Strain selection for erythritol production

The four fungal strains were compared in order to choose the most efficient one for erythritol production from each type of feedstock. Fermentations were carried out in 250-mL Erlenmeyer flasks containing 25 mL of feedstock (beet syrup, sugarcane molasses, beet molasses, red grape must and rosé grape must). In the case of sugarcane molasses, the initial concentration of total sugars was set to 300 g/L by addition of distilled water to the raw feedstocks; while yeast extract supplementation was set at 5 g/L. Beet molasses were diluted to a sugar concentration of 200 g/L, and yeast extract addition was adjusted to 0.67 g/L (see section 3.1). In the case of grape musts, given their lower initial sugar concentration, no dilutions with water were performed; therefore, an initial sugar concentration of about 200 g/L was used; and 6.7 g/L yeast extract were added as nitrogen source. In all cases, yeast extract values were established according to preliminary tests (data not shown). Production media were adjusted to pH 5.5 with a concentrated aqueous solution of NaOH. All media were sterilised in an autoclave at 121 °C during 20 min before inoculation. They were seeded with 3% (v/v) of fungal inoculum, they were capped with foam stoppers and were incubated under aerobic conditions at 30 °C and 200 rpm in an Infors HT Minitron orbital shaker (RCF = 0.56) during 7 days for molasses and 5 days for musts. Volume losses due to evaporation were measured at the end of the experiments, in order to calculate accurate sugar consumption values and erythritol yields. Controls were prepared with dilute beet syrup at 300 g/L sugars and 10 g/L yeast extract for sugarcane molasses and at 200 g/L sugars and 6.7 g/L yeast extract for beet molasses and grape musts. All experiments were conducted in triplicate.
2.2.4. Temporal monitoring of erythritol production

After selecting the most adequate fungal strain for each feedstock, the evolution of erythritol production over time was studied by analysing sample composition every 24 h. It was considered that the process was finished when carbon sources (sucrose, glucose or fructose) were depleted. Experiments were performed in duplicate. Nutrient addition and fermentation conditions for erythritol production were similar to those described in section 2.2.3.

2.4. Chemical analysis

The chemical composition of sugar-rich feedstocks (Table 1) was analysed according to the methods described by Paniagua-García et al. (2018).

Erythritol production broths were filtered through a nylon syringe filter with 0.20 μm pore (Agilent Technologies, Santa Clara, CA, USA) prior to analysis. The concentrations of sucrose, glucose, fructose, erythritol, mannitol, glycerol and ethanol in the samples were analysed by HPLC with an Agilent 1200 HPLC equipment (Agilent) provided with a precolumn Micro-Guard Carbo C (Biorad, Hercules, CA, USA), a column Aminex HPX-87C (Biorad) and a Refractive Index Detector (RID) G1362A (Agilent). The mobile phase was milli-Q water at a flow rate of 0.6 mL/min. The column temperature was set at 80 ºC and the injection volume was 20 µL.

Erythritol yields (Yₑ, g/g) were calculated as the ratio between the mass of erythritol produced and the mass of total sugar consumed. Sugar consumption (%) was calculated based on the mass of sugar consumed in relation to the initial mass of sugar. Total sugars were considered as the sum of sucrose, free glucose and free fructose. For both parameters (Yₑ and sugar consumption), volume losses due to evaporation were taken into account.

2.5. Statistical analysis

Comparisons among samples were carried out with one-way ANOVA and Tukey’s HSD test using the software Statistica 7 (StatSoft Inc., Tulsa, OK, USA). Differences were considered significant when p < 0.05.

2.6. Optical microscopy

Cell density was determined by counting raw samples in a Bürker chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) using a phase-contrast microscope Leica DM750 (Leica Microsystems SLU, L’Hospitalet de Llobregat, Spain). Digital photographies were taken with the software Leica Application Suite LAS EZ version 3.3.0.

3. Results and discussion

3.1. Detoxification of beet molasses

Beet molasses were diluted to a concentration of 300 g/L total sugars. A portion of this material was passed through the ion-exchange resin in order to remove potassium, whereas another portion was used untreated. For fermentation, these samples were kept at approximately 300 g/L sugars or were further diluted to 200 g/L. It was observed that potassium content in beet molasses decreased notably from 20.7 to 4.7 g/L after passing through the resin, and simultaneously total nitrogen concentration experienced a decline from 8.25 to 5.30 g/L (Table 2). Due to the acidic nature of the ion-exchange process in the resin, the effluent pH dropped to 2.75 (Table 2). Additionally, that acidity can also explain the partial hydrolysis of sucrose into glucose and fructose in the resin-treated samples (Table 2).

**Table 2. Chemical characteristics of beet molasses samples at two dilutions with and without resin treatment.**

| Sample                      | Suc (g/L) | Glu (g/L) | Fru (g/L) | K (g/L) | TN (g/L) | pH |
|-----------------------------|-----------|-----------|-----------|---------|----------|----|
| Beet molasses, 300 g/L sugar| 299.18    | 2.34      | n.d.      | 20.7    | 8.25     | 7.15|
| Beet molasses, 200 g/L sugar| 194.66    | 1.44      | n.d.      | 13.1    | 5.14     | 7.15|
| Beet molasses, 300 g/L sugar, resin-treated | 255.15 | 29.31 | 22.73 | 4.7 | 5.30 | 2.75 |
| Beet molasses, 200 g/L sugar, resin-treated | 178.74 | 18.2 | 15.84 | 3.2 | 3.40 | 2.75 |

Notes: n.d.: not detected, Suc: sucrose, Glu: glucose, Fru: fructose, K: potassium, TN: total nitrogen.

As mentioned previously, the protein content of beet molasses is elevated (Table 1), which could cause an imbalance in C/N ratios. In order to test the adequacy of adding nitrogen sources, various levels of yeast extract were assessed. For samples containing 300 g/L initial sugars, yeast extract was added at concentrations of 0, 1 and 5 g/L; whereas for samples starting with 200 g/L sugars, yeast
The passage of beet molasses through the resin seemed to decrease the substrate toxicity, as can be seen when comparing the untreated and treated samples with a Sugar/Yeast-extract proportion of 300/5 (Figure 1). However, erythritol production in the samples with 300 g/L initial sugars was extremely low, even after resin detoxification (27 g/L erythritol in the best case). Water dilution appeared to be a more efficient detoxification method, because all the samples with an initial sugar concentration of 200 g/L were able to produce about 60 g/L erythritol (Figure 1), except that of untreated molasses with no yeast extract. This exception could indicate that in media with high concentrations of inhibitors (like untreated beet molasses), small amounts of yeast extract may improve microbial tolerance (Figure 1). Therefore, in order to reduce costs and avoid complex adsorption processes, it was decided to adopt dilution until 200 g/L sugars as the detoxification method for beet molasses; and 0.67 g/L yeast extract were added to favour erythritol production.

### 3.2. Feasibility of erythritol bioproduction from molasses and grape musts

Control fermentations at 300 g/L initial sugars highlighted that both *M. pollinis* MUCL 40570 and *M. pollinis* MUCL 28141 produced significantly higher concentrations of erythritol than the other strains (p < 0.05), while *P. fusiformata* DSM 27425 showed the poorest performance of the four (p < 0.05) (Table 3). Similar observations were applicable to sugar consumption (Table 3). This can indicate that the strains MUCL 40570 and MUCL 28141 are well adapted to media with high osmotic stress and are able to produce erythritol above 120 g/L with yields of 0.31-0.33 g/g. On the other hand, in the control medium with lower initial sugar concentration (200 g/L), the strain *M. pollinis* MUCL 40570 was significantly superior to all the others (p < 0.05) in terms of erythritol concentration (Table 3), attaining 88.8 g/L erythritol, with a total sugar consumption of 99.5% and a yield of about 0.30 g/g. It seems evident that this control medium is not adequate for erythritol production with *P. fusiformata* DSM 27425, since, even with high cellular densities, it was unable to synthesise relevant erythritol amounts (Table 3).

*Sugar cane molasses*. Erythritol concentrations and total sugar consumptions were significantly lower (p < 0.05) in this substrate than in the control for all the tested strains (Table 3, Table 4). However, the strains *M. pollinis* MUCL 40570 and *M. pollinis* MUCL 28141 attained about 80 g/L erythritol in sugarcane molasses, without significant differences between them (Table 4). The strains *P. tsukubaensis* NRRL Y-7792 and *P. fusiformata* DSM 27425 were unable to produce erythritol in this substrate, probably due to the presence of inhibitors or toxic compounds, as indicated by their low cell density in this medium (Table 4). It was observed that *M. pollinis* MUCL 40570 consumed significantly more sugars (83.7%) and reached a higher yield (0.26 g/g) than the other strains (p < 0.05) (Table 4).

*Beet molasses*. Erythritol concentrations and yields were significantly lower (p < 0.05) for beet molasses than for its respective control (beet syrup at 200 g/L sugar) with all the tested strains (Table 3, Table 4). Once more, this can indicate a toxicity effect of beet molasses on fungal cells, in spite of the dilution performed. This is especially evident in the scarcity of cells of *P. tsukubaensis* NRRL Y-7792 and *P. fusiformata* DSM 27425 in beet molasses, which are probably more sensitive to inhibitors than *M. pollinis* strains (Table 4). In fact, *P. tsukubaensis* NRRL Y-7792 and *P. fusiformata* DSM 27425 produced no erythritol at all in this feedstock, whereas *M. pollinis* MUCL
40570 and *M. pollinis* MUCL 28141 obtained about 50-60 g/L erythritol and consumed all the sugars (Table 4). In addition, the strain *Moniliella pollinis* MUCL 28141 was significantly superior to all the others (p < 0.05) in terms of erythritol production (57.8 g/L) and yield (0.23 g/g).

**Red grape must.** The strain *M. pollinis* MUCL 40570 was significantly better than the rest for erythritol generation (97.0 g/L) and sugar consumption (98.7%) (p < 0.05), obtaining a yield of 0.37 g/g (Table 4). Interestingly, the strains MUCL 40570, MUCL 28141 and even DSM 27425 produced more erythritol (p < 0.05) in this grape must than in the control (Table 3, Table 4), which indicates that red grape must could be an excellent medium for these microorganisms.

**Rosé grape must.** Similarly to red grape must, *M. pollinis* MUCL 40570 also had greater erythritol production (90.3 g/L) and total sugar consumption (99.1%) than the other strains (p < 0.05), with an erythritol yield of 0.37 g/g (Table 4) using rosé grape must as a substrate. The strains MUCL 28141 and DSM 27425 performed significantly better (p < 0.05) in this grape must than in the control (Table 3, Table 4), which highlights the adequacy of this feedstock for polyol production.

### Table 3. Erythritol production in control solutions (dilute beet syrup) with four different strains (average ± standard deviation). Fermentation times were 7 days for samples with 300 g/L initial sugars, and 5 days for samples with 200 g/L initial sugars.

| Initial sugar (g/L) | Strain          | Cells mL⁻¹ (x 10⁹) | Glucose (%)  | Fructose (%) | Total (%)  | Concentration (g/L) | Yield (g/g) |
|---------------------|-----------------|--------------------|--------------|--------------|------------|---------------------|-------------|
| 300                 | MUCL 40570      | 2.68 ± 0.19        | 97.7 ± 0.0   | 98.3 ± 0.1   | 98.0 ± 0.0 | 126.78 ± 3.21       | 0.327 ± 0.009 |
|                     | MUCL 28141      | 1.95 ± 0.36        | 98.0 ± 0.3   | 97.3 ± 1.5   | 97.6 ± 0.9 | 124.11 ± 4.88       | 0.311 ± 0.010 |
|                     | NRRL Y-7792     | 1.41 ± 0.71        | 65.3 ± 2.5   | 22.0 ± 13.7  | 43.6 ± 5.7 | 64.05 ± 3.08        | 0.403 ± 0.065 |
|                     | DSM 27425       | 1.83 ± 0.38        | 24.0 ± 5.5   | 17.2 ± 5.5   | 20.6 ± 5.4 | 9.87 ± 5.49         | 0.120 ± 0.049 |
| 200                 | MUCL 40570      | 2.29 ± 0.36        | 99.5 ± 0.2   | 99.5 ± 0.2   | 99.5 ± 0.2 | 88.79 ± 3.11        | 0.299 ± 0.013 |
|                     | MUCL 28141      | 1.82 ± 0.42        | 98.8 ± 0.1   | 98.8 ± 0.1   | 98.8 ± 0.1 | 76.84 ± 2.52        | 0.259 ± 0.011 |
|                     | NRRL Y-7792     | 2.15 ± 0.62        | 63.0 ± 2.9   | 16.1 ± 1.8   | 39.5 ± 0.6 | 43.71 ± 3.70        | 0.406 ± 0.027 |
|                     | DSM 27425       | 1.22 ± 0.19        | 18.3 ± 4.0   | 12.5 ± 3.1   | 15.4 ± 0.5 | 2.23 ± 1.28         | 0.053 ± 0.030 |

### Table 4. Erythritol production from molasses and grape musts with four different strains (average ± standard deviation). Fermentation times were 7 days for molasses and 5 days for musts.

| Sugar source         | Strain          | Cells mL⁻¹ (x 10⁹) | Glucose (%)  | Fructose (%) | Total (%)  | Concentration (g/L) | Yield (g/g) |
|----------------------|-----------------|--------------------|--------------|--------------|------------|---------------------|-------------|
| Sugarcane molasses   | MUCL 40570      | 1.49 ± 0.62        | 76.2 ± 2.6   | 91.1 ± 1.7   | 83.7 ± 2.1 | 86.88 ± 5.96        | 0.263 ± 0.012 |
| (300 g/L)            | MUCL 28141      | 1.30 ± 0.21        | 81.4 ± 1.3   | 75.5 ± 1.4   | 78.4 ± 1.3 | 79.47 ± 3.61        | 0.241 ± 0.009 |
|                      | NRRL Y-7792     | 0.02 ± 0.00        | 4.1 ± 1.7    | 5.3 ± 2.0    | 4.7 ± 1.8  | 0.00 ± 0.00         | 0.000 ± 0.000 |
|                      | DSM 27425       | 0.02 ± 0.00        | 4.7 ± 1.1    | 5.3 ± 1.2    | 5.0 ± 1.1  | 0.00 ± 0.00         | 0.000 ± 0.000 |
| Beet molasses        | MUCL 40570      | 2.28 ± 0.39        | 100 ± 0      | 100 ± 0      | 100 ± 0    | 49.52 ± 0.76        | 0.199 ± 0.004 |
| (200 g/L)            | MUCL 28141      | 2.05 ± 0.18        | 100 ± 0      | 100 ± 0      | 100 ± 0    | 57.78 ± 1.52        | 0.229 ± 0.003 |
|                      | NRRL Y-7792     | 0.06 ± 0.03        | 2.9 ± 2.5    | 0 ± 2.2      | 0.6 ± 2.4  | 0.00 ± 0.00         | 0.000 ± 0.000 |
|                      | DSM 27425       | 0.04 ± 0.00        | 2.5 ± 2.9    | 0.0 ± 2.5    | 0.2 ± 2.7  | 0.00 ± 0.00         | 0.000 ± 0.000 |
| Red must             | MUCL 40570      | 2.53 ± 0.11        | 100 ± 0      | 97.3 ± 0.7   | 98.7 ± 0.3 | 96.95 ± 0.71        | 0.372 ± 0.002 |
| (225 g/L)            | MUCL 28141      | 1.66 ± 0.47        | 100 ± 0      | 82.5 ± 3.6   | 91.4 ± 1.8 | 87.40 ± 3.23        | 0.363 ± 0.004 |
|                      | NRRL Y-7792     | 3.92 ± 1.13        | 40.4 ± 4.1   | 9.1 ± 0.8    | 25.0 ± 2.5 | 13.72 ± 0.50        | 0.222 ± 0.025 |
|                      | DSM 27425       | 1.49 ± 0.06        | 48.1 ± 1.6   | 12.5 ± 0.8   | 30.6 ± 1.0 | 29.32 ± 1.38        | 0.382 ± 0.010 |
| Rosé must            | MUCL 40570      | 3.31 ± 0.46        | 100 ± 0      | 98.3 ± 0.1   | 99.1 ± 0.0 | 90.33 ± 0.85        | 0.375 ± 0.003 |
| (208 g/L)            | MUCL 28141      | 1.49 ± 0.18        | 100 ± 0      | 85.6 ± 2.0   | 92.8 ± 1.0 | 84.06 ± 1.42        | 0.374 ± 0.004 |
|                      | NRRL Y-7792     | 3.60 ± 1.14        | 46.8 ± 1.2   | 10.2 ± 0.5   | 28.5 ± 0.8 | 13.41 ± 0.21        | 0.204 ± 0.009 |
|                      | DSM 27425       | 2.31 ± 0.31        | 60.6 ± 2.0   | 13.2 ± 0.2   | 36.9 ± 1.1 | 27.04 ± 1.00        | 0.318 ± 0.001 |
3.3. Strain screening and selection

In the case of molasses, the aim was to use a broth with an initial sugar concentration of 300 g/L because of two reasons. In the first place, numerous research works indicate that erythritol-producing fungi, such as *Moniliella*, *Pseudozyma*, *Torula* or *Starmerella magnoliae*, tolerate glucose concentrations about 300 g/L, attaining erythritol yields of 0.23-0.60 g/g in model solutions (Burschäpers et al., 2002; Cho et al., 1999a, 1999b; Hajny et al., 1964; Jeya et al., 2009; Koh et al., 2003; Lin et al., 2010, 2001; Oh et al., 2001; Ryu et al., 2000). Secondly, if there is no substrate inhibition or excessive osmotic pressure, elevated initial sugar concentrations normally result in higher product concentrations, which can be translated into lower purification costs (Moon et al., 2010; Troostembergh et al., 2001). Working at 300 g/L total sugars was possible with sugarcane molasses, but beet molasses had to be further diluted to 200 g/L total sugars because of toxic effects on the employed fungi. Regarding grape musts, as their raw composition consisted of barely 200-230 g/L total sugars, they were used as they stood. Anyway, grape musts resulted in a better feedstock for erythritol production than sugarcane molasses, in spite of their lower sugar content (Table 4); probably due to the presence of inhibitory substances in molasses.

In the four raw substrates, the proportions between sugar concentration and total nitrogen concentration (C/N) were 116, 34.3, 622 and 599 for sugarcane molasses, beet molasses, red grape must and rosé grape must, respectively. After yeast extract addition, these C/N ratios changed to 96, 33.9, 206 and 193, in that same order. It has been reported that C/N ratios (sugar/total nitrogen) in the range of 150-180 are ideal for erythritol production (Liu et al., 2020; Oh et al., 2001). In this work, it was possible to obtain erythritol from an *a priori* unsuitable feedstock like beet molasses (whose nitrogen content was too high), although with some limitations. As mentioned in section 3.1, the effect of yeast extract addition could go beyond C/N ratio modification, and may help fungi to cope with inhibitors. In fact, yeast extract is a source of vitamins and it has been suggested that vitamins could play a role in erythritol biosynthesis (Rzechonek et al., 2018). However, the combination of yeast extract with cheaper nitrogen sources in different proportions should be explored in the future in order to reduce costs.

![Figure 2](image.png)

Figure 2. Optical microscope photographs taken in bright field with an augmentation of 400 times. a) *M. pollinis* MUCL 40570 in sugarcane molasses, b) *M. pollinis* MUCL 28141 in beet molasses, c) *P. tsukubaensis* NRRL Y-7792 in red grape must, d) *P. fusiformata* DSM 27245 in rosé grape must.
A relevant fact corroborated in the present work is the feasibility of using the feedstock itself (supplemented with yeast extract) to prepare microbial inocula. The four tested strains were generally able to proliferate in media made of dilute molasses or dilute grape must and then be used to successfully inoculate the fermentation broth. Beet molasses had already been employed to propagate erythritol-producing fungi (Mironczuk et al., 2015; Rakicka et al., 2017a), but in order to ferment a different feedstock afterwards. The advantage of employing the same substrate for the propagation phase and the fermentation phase is the cost reduction on reagents, because normally inocula are prepared in high-cost rich nutritive media.

Figure 2 shows some example photographies of the assessed fungal strains during the transformation of feedstocks to obtain erythritol. For three of the studied feedstocks (sugarcane molasses, red grape must and rosé grape must), M. pollinis MUCL 40570 offered the best results for erythritol production (Table 4). The strain M. pollinis MUCL 40570 (= MUCL 11525 = CBS 461.67) was isolated from a pollen sample of a honeycomb in the USA, and, according to Dooms et al. (1971), it corresponds to the strain I2A described by Hajny et al. (1964), which obtained erythritol yields of 0.35-0.40 g/g on various types of sugars. Meanwhile, the strain M. pollinis MUCL 28141 is a mutant of the previous strain. Different companies have published patents where mutant fungi are obtained to produce higher amounts of erythritol (Cho et al., 1999b; Troostembergh et al., 2001). However, in the particular case described herein, the mutant strain MUCL 28141 only outperformed the wild strain MUCL 40570 when working with beet molasses. On the other hand, the strain P. tsukubaensis NRRL Y-7792 was originally isolated from a flower in mount Tsukuba (Japan). It obtained moderate erythritol concentrations in control media (44-64 g/L) and low values in grape musts (13-14 g/L), but was not capable of growing in sugarcane molasses and beet molasses. On the contrary, other strains from the same species have been reported as erythritol superproducers, such as P. tsukubaensis KN75 (= KCCM 10356), which obtained 245 g/L erythritol from a solution containing 300 g/L initial glucose, with a yield of 0.61 g/g (Jeya et al., 2009). Nevertherless, to the best of our knowledge, this strain is not commercially available. Finally, the strain P. fusiformata DSM 27245 was isolated from floral nectar in Tenerife (Spain). Its performance was moderate in grape musts (27-30 g/L erythritol), but it failed in molasses and syrup. This is the first time that this species is tested for erythritol production.

Figure 3. Evolution of erythritol production for each feedstock with M. pollinis MUCL 40570 (or MUCL 28141 in the case of beet molasses). a) Sugarcane molasses, b) beet molasses, c) red must, d) rosé must.
According to the results obtained, M. pollinis MUCL 40570 was selected to perform further tests with sugarcane molasses, red must and rosé must; whereas for beet molasses M. pollinis MUCL 28141 was of choice.

3.4. Erythritol production kinetics

Short biological processes are preferable in biorefineries in order to decrease energetic costs. The evolution of erythritol production was monitored every 24 h with the four tested feedstocks, using M. pollinis MUCL 40570 for sugarcane molasses, red grape must and rosé grape must; and M. pollinis MUCL 28141 for beet molasses (Figure 3).

In sugarcane molasses, the growth of M. pollinis MUCL 40570 was fast after inoculation (Figure 3a). During the first 24 h, fungal biomass increased more than 20-fold, consuming only 10.6% of initial sugars. Thereafter, cells started to hydrolyse sucrose into glucose and fructose at a high pace, thus resulting in an almost total sucrose hydrolysis at 72-96 h. This provoked the simultaneous augmentation of glucose and fructose concentrations in the broth. Erythritol production began after 24 h of fermentation and its concentration increased smoothly, reaching 106.40 ± 0.42 g/L at 216 h after inoculation, with 100 ± 0.0% glucose consumption, 99.34 ± 0.15% fructose consumption and 99.67 ± 0.07% total sugar consumption; and with an erythritol yield of 0.239 ± 0.001 g/g (Figure 3a). In addition, ethanol and small amounts of glycerol and mannitol were generated during the transformation of sugarcane molasses (Appendix A).

During the kinetic studies of beet molasses, a deficient fermentation was observed with M. pollinis MUCL 28141. Accordingly, the experiment was conducted twice to check its repeatability. In addition, it was also performed with M. pollinis MUCL 40570 to discard any problem with the strain MUCL 28141. However, in all three cases, long lag phases were observed at the beginning or in the middle of the fermentation, thus disturbing erythritol production (only one of the experiments is shown in Figure 3b). This fact could be related to the inhibitory effects of beet molasses on fungal cells, in spite of the efforts to reduce its toxicity and their apparent success (Figure 1, Table 4). Beet molasses are known to cause toxic effects on fungi and bacteria (Chen et al., 2017; Roukas, 1998). Consequently, further research is needed to determine which factors are detrimental to erythritol synthesis and process repeatability, and whether nutrient supplementation could be beneficial to reduce the toxicity of beet molasses.

For red must and rosé must, the evolutions of erythritol production with M. pollinis MUCL 40570 followed parallel trends (Figure 3 c,d). In both cases, fungal biomass developed fast after inoculation and it attained the stationary phase in about 24 h (Figure 3 c,d). During this brief time, erythritol production was almost negligible (below 4 g/L), because sugars were being used for cellular growth and reproduction. After that initial stage, erythritol concentration increased sharply, reaching a plateau at 120-144 h. During the first 72 h, fructose consumption was apparently faster than that of glucose; however, glucose was fully depleted after 96 h, whereas fructose was not exhausted until 144 h after inoculation (Figure 3 c,d). Red must reached a maximum erythritol concentration of 96.26 ± 1.31 g/L in 144 h, with 100 ± 0.0% glucose consumption, 99.24 ± 0.10% fructose consumption and 99.63 ± 0.05% total sugar consumption, attaining an erythritol yield of 0.362 ± 0.005 g/g. On the other hand, rosé grape must obtained a maximum erythritol concentration of 92.26 ± 1.31 g/L in 144 h, with 100 ± 0.0% glucose consumption, 98.99 ± 0.02% fructose consumption and 99.50 ± 0.01% total sugar consumption, reaching an erythritol yield of 0.383 ± 0.004 g/g. Apart from erythritol, the activity of M. pollinis MUCL 40570 in musts generated low quantities of other metabolites, such as ethanol and glycerol, whose concentrations varied over time (Appendix A).

3.5. Erythritol production from industrial and agri-food by-products

Recently, crude glycerol and food wastes have been evaluated as feedstocks for erythritol production (Table 5). Most works are focused on crude glycerol and waste cooking oil, where erythritol concentrations of 12-180 g/L and yields of 0.35-0.74 g/g have been obtained (Table 5). On the contrary, the assessment of carbohydrate-containing by-products (such as those related to fruits, corncob, soy or cane processing) is less common, and erythritol productions of 3-26 g/L have been reported (Table 5).
Table 5. Literature values of erythritol production from various wastes and by-products.

| Substrate                                      | Carbon-source concentration (g/L) | Reactor type a | Working volume (L) | Strain                                      | Erythritol (g/L) | Substrate consumption (%) | Yield (g/g) | Time (h) | Reference                                      |
|------------------------------------------------|-----------------------------------|----------------|--------------------|---------------------------------------------|-----------------|---------------------------|-------------|----------|-----------------------------------------------|
| Crude glycerol (palm oil biodiesel)            | 200                               | B              | 0.5                | M. megachiliensis SN-G24 FERM BP-1430 (mutant) | 32              | 30                        | 0.58        | 144      | (Kobayashi et al. 2015)                      |
| Crude glycerol (beef tallow)                   | 200                               | B              | 0.5                | M. megachiliensis SN-G24 FERM BP-1430 (mutant) | 27              | 37                        | 0.35        | 168      | (Kobayashi et al. 2015)                      |
| Crude glycerol (biodiesel)                     | 232                               | B              | 0.05               | Y. lipolytica A16 (mutant)                   | 109.2           | 100                       | -           | 132      | (Yang et al. 2016)                          |
| Crude glycerol (biodiesel)                     | 150                               | B-2            | 2                  | Y. lipolytica A1B pAD-UTGUT1 (recombinant)   | 82.2            | 100                       | 0.55        | 92       | (Rakicka et al. 2017a)                      |
| Crude glycerol (biodiesel)                     | 300                               | C              | 3.5                | Y. lipolytica Wratislavia K1                | 162             | 93                        | 0.58        | -        | (Rakicka et al. 2017b)                      |
| Crude glycerol (soap)                          | 300                               | C              | 3.5                | Y. lipolytica Wratislavia K1                | 116             | 100                       | 0.38        | -        | (Rakicka et al. 2017b)                      |
| Crude glycerol (refinery)                      | 300                               | FB             | 2                  | Y. lipolytica MK1 (mutant)                  | 165             | 100                       | 0.53        | 146      | (Rakicka-Pustulka et al. 2020)              |
| Waste cooking oil                              | 20                                | B              | 0.05               | Y. lipolytica M53 (mutant)                  | 12              | -                         | -           | 120      | (Liu et al. 2017b)                         |
| Waste cooking oil                              | 30                                | B              | 3                  | Y. lipolytica M53 (mutant)                  | 22.1            | 73                        | 0.74        | 72       | (Liu et al. 2017a)                         |
| Waste cooking oil                              | 30                                | B              | 0.05               | Y. lipolytica M53 (mutant)                  | 21.8            | 85                        | -           | 168      | (Liu et al. 2018a)                         |
| Waste cooking oil                              | 30                                | B              | 0.05               | Y. lipolytica M53 (mutant)                  | 20.4            | 97                        | 0.61        | 144      | (Liu et al. 2019b)                         |
| Waste cooking oil                              | 60                                | B              | 3                  | Y. lipolytica M53 (mutant)                  | 49.1            | 94                        | -           | 168      | (Liu et al. 2019b)                         |
| Pineapple peel residues                        | 20                                | B              | 0.05               | Y. lipolytica M53 (mutant)                  | 6               | -                         | -           | 120      | (Liu et al. 2017b)                         |
| Corncob                                        | 20                                | B              | 0.05               | Y. lipolytica M53 (mutant)                  | 3               | -                         | -           | 120      | (Liu et al. 2017b)                         |
| Sugarcane bagasse                              | 20                                | B              | 0.05               | Y. lipolytica M53 (mutant)                  | 5               | -                         | -           | 120      | (Liu et al. 2017b)                         |
| Apple pomace                                   | 20                                | B              | 0.05               | Y. lipolytica M53 (mutant)                  | 5               | -                         | -           | 120      | (Liu et al. 2017b)                         |
| Okara (soybean residue)                        | 20                                | B              | 0.05               | Y. lipolytica M53 (mutant)                  | 7.2             | -                         | -           | 120      | (Liu et al. 2017b)                         |
| Okara (soybean residue)                        | 30                                | B-2            | 0.05               | Y. lipolytica M53 (mutant)                  | 14.2            | -                         | -           | 120      | (Liu et al. 2017b)                         |
| Xylose mother liquor (corncobs)                | 217                               | B              | 3                  | Aureobasidium pullulans CGMCC3.0837 ER 35 (mutant) | 26.4            | 59 c                      | 0.12        | 144      | (Guo et al. 2016)                          |
| Beet syrup (control)                           | 300                               | B              | 0.025              | M. pollinis MUCL 40570                      | 126.8           | 98.0                      | 0.33        | 168      | This work                                   |
| Beet syrup (control)                           | 200                               | B              | 0.025              | M. pollinis MUCL 40570                      | 88.8            | 99.5                      | 0.30        | 120      | This work                                   |
| Sugarcane molasses                             | 300                               | B              | 0.025              | M. pollinis MUCL 40570                      | 106.4           | 99.7                      | 0.24        | 216      | This work                                   |
| Beet molasses                                  | 200                               | B              | 0.025              | M. pollinis MUCL 28141 (mutant)             | 57.8            | 100                       | 0.23        | 168      | This work                                   |
| Red must                                       | 225                               | B              | 0.025              | M. pollinis MUCL 40570                      | 96.3            | 99.6                      | 0.36        | 144      | This work                                   |
| Rosé must                                      | 208                               | B              | 0.025              | M. pollinis MUCL 40570                      | 92.8            | 99.5                      | 0.38        | 144      | This work                                   |

Notes: a) B: batch, B-2: batch with two stages, FB: fed batch, C: continuous. b) Xylose mother liquor contained 126 g/L xylose, 47 g/L arabinose and 44 g/L glucose. c) 58% xylose, 22% arabinose, 100% glucose.
Liu et al. (2019a, 2018b) have also explored the feasibility of solid-state fermentation with food wastes, such as okara (soybean residue), sesame meal, soya bean cake, rape seed cake and peanut press cake, obtaining 100-200 mg erythritol per gram of solid biomass.

It must be noted that maximum erythritol theoretical yields are different for hexoses and glycerol. Stoichiometrically, one mole of erythritol is produced from one mole of hexose, whereas two moles of erythritol are produced from three moles of glycerol (Nakagawa et al., 2020). This implies theoretical yields of 0.678 g/g from hexoses and 0.884 g/g from glycerol. Nevertheless, the highest erythritol yields obtained experimentally in small-scale batch reactions from pure glucose solutions are in the range of 0.456-0.497 g/g for wild strains (Hajny et al., 1964; Jeya et al., 2009) and up to 0.595 g/g for mutant strains (Lin et al., 2010). In addition, a yield of 0.61 g/g has been reported in an industrial-scale reactor working with pure glucose (Jeya et al., 2009). This indicates that the erythritol values obtained in this work with sugarcane molasses, beet molasses and grape musts (58-106 g/L erythritol, 0.23-0.38 g/g) are moderate-high, considering the most common yields reported for pure glucose solutions and the concentrations attained with industrial and food by-products (Table 5).

4. Conclusions

Sugarcane molasses and surplus grape musts are suitable feedstocks for erythritol bioproduction, thus providing alternatives for business diversification in sugar factories and large wine cooperatives and cellars. Before its application at industrial scale, the evaluation of cheaper nitrogen sources, the need for supplementing other nutrients, the effect of sugar inversion, the possibility of cell immobilisation, the use of fed-batch and continuous reactors, or the utilization of modified or alternative strains should be considered. On the other hand, the use of beet molasses still requires a deep optimisation step in order to solve repeatability problems before a reliable method is proposed.

Acknowledgements: We would like to thank Azucarera del Guadalfeo (Salobreña, Spain), Vedeqsa (Terrassa, Spain), Sociedad Cooperativa General Agropecuaria ACOR (Valladolid, Spain), Estación Enológica de Castilla y León – ITACyL (Rueda, Spain) and Leyenda del Páramo (Valdeviebre, Spain) for generously providing the feedstocks. Authors thank R. Antón del Río, N. del Castillo Ferreras and G. Sarmiento Martínez for their technical help.

Funding: This study was funded by the European Regional Development Fund (ERDF) within the programme Interreg V-A España-Portugal (POCTEP) 2014-2020 through the project BIOVINO [0688_BIOVINO_6_E].

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APPENDIX A

By-products of sugar factories and wineries as feedstocks for erythritol generation

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Detoxification of beet molasses with adsorption resins

Beet molasses were diluted with water to achieve a total sugar concentration of 300 g/L. The acidic ion-exchange resin Amberlite® IR-120 (Merck KGaA, Darmstadt, Germany) was packed into a 120-mL glass column and it was washed with 1 L of milli-Q water at a flow rate of 10 mL/min and afterwards with 100 mL of an aqueous solution of 6% H₂SO₄ (w/w) at 2 mL/min. The column was left overnight soaked in this acidic solution, and then a further 50 mL of 6% H₂SO₄ (w/w) were passed at 2 mL/min. Then, the resin was washed again with 1 L of milli-Q water at 10 mL/min. After that, 120 mL of beet molasses passed through the column at a rate of 2 mL/min (discarding the first 30 mL), thus obtaining a sample with pH ~2.5.

Metabolites generated during erythritol production

Figure A.1. Minor metabolites generated during erythritol production. a) Sugarcane molasses, b) beet molasses, c) red must, d) rosé must. Note: The release of gaseous CO₂ was not monitored.