Assessment of vine shoots and surplus grape must for succinic acid bioproduction

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Abstract
Vine shoots and surplus grape must were assessed as feedstocks for succinic acid production with Actinobacillus succinogenes and Basfia succiniproducens. After acidic and enzymatic hydrolysis, vine shoots released 35–40 g/L total sugars. Both bacterial species produced 18–21 g/L succinic acid from this hydrolysate in 120 h. Regarding grape must fermentation, A. succinogenes clearly outperformed B. succiniproducens. Yeast extract (a source of organic nitrogen and vitamins) was the only additional nutrient needed by A. succinogenes to grow on grape must. Under mathematically optimized conditions (145.7 g/L initial sugars and 24.9 g/L yeast extract), A. succinogenes generated 88.9 ± 1.4 g/L succinic acid in 96 h, reaching a succinic acid yield of 0.66 ± 0.01 g/g and a sugar consumption of 96.64 ± 0.30%. Substrate inhibition was not observed in grape musts with 125–150 g/L initial sugars, provided that an adequate amount of yeast extract was available for bacteria. Alternative nitrogen sources to yeast extract (red wine lees, white wine lees, urea, NH₄Cl, and choline chloride) were not suitable for A. succinogenes in grape must.

Key Points
• Vine shoots and surplus grape must were assessed for succinic acid bioproduction.
• Succinic acid bioproduction was 21 g/L with vine shoots and 89 g/L with grape must.
• Fermentation was efficient at high sugar loads if organic N supply was adequate.

Keywords Succinic acid · Winery waste · Fermentation · Nitrogen source

Introduction
Succinic acid is a dicarboxylic acid with numerous industrial applications, which is also used as a platform chemical for the synthesis of other compounds (Pateraki et al. 2016a; Zeikus et al. 1999). It is mainly produced by a petrochemical route through the catalytic hydrogenation of maleic anhydride derived from butane. Nevertheless, the ability of certain rumen bacteria to produce succinic acid from a variety of carbon sources has encouraged the research on succinic acid bioproduction during the last decades (Dessie et al. 2018; Pateraki et al. 2016a; Zeikus et al. 1999).

In addition, since the United States Department of Energy classified succinic acid as an interesting building block that might be obtained from biomass in biorefineries (Aden et al. 2004), several companies have attempted its bioproduction on an industrial scale with very different fortunes (Pateraki et al. 2016a; Stylianou et al. 2021; Thuy et al. 2017). To the best of our knowledge, only Roquette, BASF, and PTT-MCC Biochem keep their facilities open for biosuccinic acid production, where mainly metabolically engineered strains are employed (BASF 2021; PTT-MCC Biochem 2021; Roquette 2021). Actinobacillus succinogenes and Basfia succiniproducens are the most efficient wild bacteria-producing succinic acid. The main drawbacks of this bioproduction process are related to feedstock prices, production of secondary metabolites, auxotrophy, pH sensitivity, NADH limitation, and product inhibition (Dessie et al. 2018).

Current commercial bioproduction of organic acids employs refined sugars, starch, or molasses as feedstocks (Jansen and van Gulik 2014). However, the use of alternative
carbon sources could reduce production costs. Accordingly, industrial, agriculture, and food by-products have been explored recently for succinic acid bioproduction (Akhtar et al. 2014; Pateraki et al. 2016a; Dessie et al. 2018). Vine shoots (Vitis vinifera L.) are the most abundant by-product from the winery industry, and they are generated during pruning activities at a rate of 1.4–2.0 t/ha (Garita-Cambronero et al. 2021a). Considering that 7,453,532 ha were devoted to vines in 2016 worldwide (OIV 2021), vine shoots constitute an interesting lignocellulosic resource in wine-producing regions. On the other hand, grape musts are an attractive sugar source due to their glucose and fructose content (about 200 g/L total). Surplus grape musts cause commercial imbalances and economic problems related to unsold wine stocks in producing countries (Hijosa-Valsero et al. 2021). In Spain, 4073 ML of wine and 796 ML of grape juice and grape must were produced in 2020 (MAPA 2020). Therefore, vine shoots and surplus grape must could be explored as feedstocks for biosuccinic acid generation, given their production volume.

The objective of this work was to assess for the first time the suitability of vine shoots and surplus grape must (winery by-products) as feedstocks for succinic acid bioproduction. Two bacterial strains—Actinobacillus succinogenes and Basfia succiniproducens—were compared in order to select the most appropriate microorganism for each feedstock. In addition, the effect of nutrient supplementation was studied with the twofold purpose of maximizing succinic acid production and discarding unnecessary nutrients. In the case of grape must, several nitrogen sources were tested as microbial nutrients for succinic acid production.

Materials and methods

Reagents

All reagents were of analytical grade. Further details about manufacturers and providers can be found in the Supplementary Information.

Feedstocks: winery surplus and by-products

Vine-shoot hydrolysates

Vine shoots were harvested in May 2019 at the experimental plots of ITACyL (Finca Zamadueñas, Valladolid, Spain). They were dried, ground, sieved, and analyzed according to Garita-Cambronero et al. (2021a). Dry vine shoots contained 32.77% glucan, 11.31% hemicellulose (49.27% total carbohydrates), 2.82% galacturonic acid, 21.30% acid-insoluble lignin, 4.34% protein, 0.52% fat, 7.91% moisture, 2.40% ashes, and 13.3 mg/g total phenolic compounds. In order to release the sugars contained in vine shoots, they were subjected to an acidic hydrolysis with 1.72% H₂SO₄ (w/w) at 134 °C for 17 min, with 10% (w/w) solids; followed by an enzymatic hydrolysis for 48 h at 50 °C, 150 rpm and pH 5.0, with the enzyme mixture CelliCTec2 (Novozymes, Bagsvaerd, Denmark) at a dose of 17.4 FPU per g biomass, as described by Garita-Cambronero et al. (2021b). Then, vine-shoot hydrolysates were vacuum-filtered (filter paper No. 1305, Filtros Anoia SA, Barcelona, Spain). The final hydrolysate was analyzed following Paniagua-García et al. (2018), and it contained 2.08 g/L cellobiose, 22.43 g/L glucose, 14.09 g/L xylene, 0.48 g/L rhamnose, 1.13 g/L arabinose (40.21 g/L total sugars), 0.11 g/L formic acid, 3.80 g/L acetic acid, 0.11 g/L levulinic acid, 0.40 g/L 5-hydroxy-methyl-furfural, 0.13 g/L furfural, and 0.60 g/L total phenolic compounds.

Grape must

Red grape must (variety Garnacha) was obtained in September 2019 from the Oenological Station of Castile and Leon—ITACyL (Rueda, Spain) and it was kept at −20 °C until use. Grape must was chemically characterized according to Paniagua-García et al. (2018). It had a density of 1.10 g/mL, and contained 125 g/L glucose, 119 g/L fructose, 0.34 g/L total Kjeldahl nitrogen, and 0.73 g/L total phenolic compounds. Its composition in terms of anions and cations is shown in Table S1.

Wine lees

Two different types of wine lees were collected at the Oenological Station of Castile and Leon—ITACyL (Rueda, Spain) in September–November 2020. Red wine lees had a density of 1.05 g/L and contained 99.3 g/L ethanol, 12.2 g/L total Kjeldahl nitrogen, and 1.51 g/L phenolic compounds. White wine lees had a density of 1.03 g/L and contained 81.8 g/L ethanol, 6.8 g/L total Kjeldahl nitrogen, and 0.43 g/L phenolic compounds. More information about their chemical characterization is given in Table S1. Wine lees were autoclaved at 121 °C for 15 min in order to inactivate residual vinification yeasts. Afterwards, they were sonicated for 30 min at 330 W and 80 kHz in an Elmasonic P 180 H ultrasound bath (Elma Schmidbauer GmbH, Singen, Germany) to provoke cell disruption and release cytoplasmic contents. These treated wine lees were used as nitrogen sources in some fermentation experiments.

Strain cultivation

The strains Actinobacillus succinogenes DSM 22257 and Basfia succiniproducens DSM 22022 were obtained from Leibniz Institute DSMZ (Braunschweig, Germany) as
freeze-dried cultures. They were reactivated and then cryopreserved as explained in the Supplementary Information.

Working inocula were prepared by introducing a loopful of the cryopreserved solutions in a 100-mL glass bottle containing 50 mL tryptic soy broth (Becton, Dickinson and Company; Le Pont de Clai, France) at pH 7.3. Bottles were capped with a rubber septum, and gaseous CO₂ was injected inside the solution for 5 min. Then, the bottles were incubated at 37 °C and 150 rpm for 18–24 h, until an approximate cell density of 5×10⁸ cells/mL was attained.

Fermentation of vine-shoot hydrolysates

Vine-shoot hydrolysates were tested as a feedstock for succinic acid production with two different strains. In the case of *A. succinogenes* DSM 22257, the hydrolysate was enriched with a standard nutrient mix NM-A, composed of 6 g/L yeast extract, 0.2 g/L CaCl₂·2H₂O, 0.2 g/L MgCl₂·6H₂O, 1 g/L NaCl, and 3 g/L K₂HPO₄ (Ferone et al. 2017; Pateraki et al. 2016b; van Heerden and Nicol 2013); and its pH was adjusted to 6.7 with NaOH 50% w/w and NaOH 4 M. In the case of *B. succiniproducens* DSM 22022, the hydrolysates were supplemented with a standard nutrient mix NM-B, which consisted in 5 g/L yeast extract, 2 g/L (NH₄)₂SO₄, 0.2 g/L CaCl₂·2H₂O, 0.2 g/L MgCl₂·6H₂O, 2 g/L NaCl, and 3 g/L K₂HPO₄ (Cimini et al. 2019); and its pH was adjusted to 6.5. In all cases, the broths were then autoclaved at 121 °C for 15 min. In order to maintain a stable pH during the fermentation, solid MgCO₃ was added in an amount of 0.8 g MgCO₃ per 1 g of initial sugars (Zheng et al. 2009).

Fermentations were performed in 100-mL glass bottles containing 50 mL of vine-shoot hydrolysates. The samples were seeded with 5% (v/v) of the inocula described in section “Strain cultivation.” Then they were closed with rubber caps; gaseous CO₂ was injected in the bottom of the bottles for 5 min to guarantee capnophilic conditions and afterwards the bottles were incubated at 37 °C and 150 rpm in an orbital shaker (Infors AG, Bottmingen, Switzerland) for 120 h. In addition, control fermentations were performed with synthetic media containing 22 g/L glucose and 14 g/L xylose (similar to the composition of vine-shoot hydrolysates) and supplemented with NM-A for *A. succinogenes* DSM 22257 and NM-B for *B. succiniproducens* DSM 22022, respectively. All fermentations were performed in triplicate.

Preliminary fermentation of grape must

Both bacterial strains were compared in terms of their ability to produce succinic acid from red grape must. In these tests, grape must was diluted with distilled water to obtain a sugar content of 40 g/L (a value similar to that of vine-shoot hydrolysates), but also higher sugar concentrations were assessed (77, 113, and 150 g/L). For *A. succinogenes*, grape must was supplemented with NM-A and its pH was set at 6.7, whereas for *B. succiniproducens*, grape must was supplemented with NM-B and its pH was adjusted to 6.5. Samples were autoclaved at 121 °C for 15 min, and solid MgCO₃ was added as a pH buffer (0.8 g MgCO₃ per 1 g of initial sugars). Then, samples were inoculated and fermented as described in section “Fermentation of vine-shoot hydrolysates.” All fermentations were performed in triplicate for 96 h.

Improvement of succinic acid production from grape must

The previous experiments with vine shoots and grape must showed that grape must had a great potential for succinic acid production, and that *A. succinogenes* DSM 22257 had a better performance than *B. succiniproducens* DSM 22022. Therefore, the subsequent experiments were focused on the maximization of succinic acid production with *A. succinogenes* DSM 22257 from grape must.

In the first place, given the composition of grape must (rich in phosphate, K, Ca, and Mg, and short in nitrogen and NaCl; see Table S1), it was decided to determine which nutrients from the NM-A mixture were essential and which nutrients could be ruled out in order to avoid the use of unnecessary reagents during the fermentation. A Plackett–Burman experimental design with 12 runs and 5 factors (1–6 g/L yeast extract, 0–0.2 g/L CaCl₂·2H₂O, 0–0.2 g/L MgCl₂·6H₂O, 0–1 g/L NaCl, and 0–3 g/L K₂HPO₄) was performed applying the values shown in Table S2 and starting from a diluted grape must containing 77 g/L initial total sugars. The dependent variable was succinic acid concentration. Samples were fermented for 96 h using MgCO₃ as a pH buffer and according to the conditions described in section “Fermentation of vine-shoot hydrolysates” (except those regarding the standard NM-A addition).

After selecting the indispensable nutrients, their concentrations were optimized by an experimental design based on response surface methodology (RSM) in order to achieve the highest possible concentration of succinic acid. In this case, a complete factorial design, with 13 runs (4 cube points, 5 central points, and 4 axial points), 2 factors (initial sugars and yeast extract), and an alfa value of 1.41421, was performed. The concentration of initial sugars ranged from 40 g/L (grape must be diluted with water) to 243.46 g/L (undiluted grape must), whereas the concentration of yeast extract ranged from 1 to 40 g/L. The experimental conditions are provided in Table S3. Succinic acid concentration after fermentation was established as the response variable. These results were employed to create an equation to estimate the production of succinic acid from the input values of the independent variables (initial sugars and yeast extract). Then, a mathematical optimum point was calculated in order...
to maximize succinic acid. In this case, samples were also fermented for 96 h using MgCO₃ as a pH buffer and according to the conditions described in section “Fermentation of vine-shoot hydrolysates” (except those regarding nutrient addition). Control fermentations with glucose-fructose solutions similar to the optimized grape must sample were also performed for comparison purposes.

Effect of different nitrogen sources on succinic acid production from grape must

The fine-tuning of nutrient concentrations performed in section “Improvement of succinic acid production from grape must” indicated that the optimal values for succinic acid production from grape must were 145.7 g/L initial sugars and 24.9 g/L yeast extract, without any further nutrient addition. Yeast extract was replaced by alternative nitrogen sources in such a way that the total nitrogen (TN) amount added to the fermentation broth was always equivalent. Therefore, yeast extract (TN 11% w/w), red wine lees (1.22% TN), white wine lees (0.68% TN), urea (46.65% TN), NH₄Cl (26.17% TN), and choline chloride (10.03% TN) were added to diluted grape must at concentrations of 24.9, 224.5, 402.8, 5.87, 10.5, and 27.3 g/L, respectively, in order to compare the effect of various nitrogen sources on succinic acid production. In all cases, the initial sugar concentration of grape must was 145.7 g/L. Samples were autoclaved and inoculated with A. succinogenes DSM 22257 (5%, v/v), and their pH was controlled with MgCO₃. Samples were fermented in triplicate under CO₂ atmosphere for 96 h as explained in section “Fermentation of vine-shoot hydrolysates.”

Analysis

Vine-shoot hydrolysates, grape must, and fermentation samples were centrifuged at 13,400 × g in a microcentrifuge for 3 min (Minispin, Eppendorf, Hamburg, Germany). The supernatant was filtered through a nylon syringe filter (0.20-µm pore; Agilent Technologies, Santa Clara, CA, USA) prior to analysis. Cellulose, glucose, fructose, xylose, rhamnose, arabinose, succinic acid (SA), formic acid (FA), acetic acid (AA), levulinic acid, lactic acid (LA), ethanol (E), 5-hydroxymethylfurufural (5-HMF), and furfural were quantified with an Agilent 1200 HPLC equipment (Agilent Technologies, Santa Clara, CA, USA) provided with a 300×7.8 mm i.d. cation exchange column Aminex HPX-87H (Biorad, Hercules, CA, USA) coupled to a refractive index detector (RID, G1362A, Agilent Technologies), using a mobile phase of 5 mM H₂SO₄ at a flow rate of 0.6 mL/min and a column temperature of 60 °C (Sluiter et al. 2008). The injection volume was 20 µL.

Sugar consumption during the fermentation process was expressed as a percentage. Succinic acid yield, Y (%), was calculated as the ratio between the mass of succinic acid produced (g) and the mass of total sugars consumed (g). Cell density was measured by counting bacteria in a Bürker chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) using a phase-contrast microscope Leica DM750 (Leica Microsistemas SLU, L’Hospital de Llobregat, Spain).

Statistics

Comparisons between samples were performed 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. Box-Behnken and RSM experiments were designed with Minitab 16 (Minitab Inc., State College, PA, USA). Plots and curve fitting were performed with SigmaPlot 11 (Systat Software Inc., San José, CA, USA).

Results

Feasibility of succinic acid production from vine shoots

Both A. succinogenes DSM 22257 and B. succiniproducens DSM 22022 were able to grow and produce succinic acid on vine-shoot hydrolysates, which indicates their tolerance to the inhibitors present in this hydrolysate. This fact was especially remarkable for B. succiniproducens, whose metabolite production was faster than that of A. succinogenes (Fig. 1). After 120 h of fermentation, A. succinogenes reached 21.18 ± 1.04 g/L succinic acid, whereas B. succiniproducens obtained 18.11 ± 1.26 g/L succinic acid, with sugar consumptions above 90% for both species. This implies succinic acid yields of 0.64 ± 0.04 g/g for A. succinogenes and 0.54 ± 0.03 g/g for B. succiniproducens. On the contrary, control fermentations (with glucose-xyllose solutions) attained 23.15 ± 0.18 g/L succinic acid with A. succinogenes (yield 0.70 ± 0.01 g/g) and 19.19 ± 0.60 g/L succinic acid with B. succiniproducens (yield 0.62 ± 0.01 g/g) in only 72 h, with sugar consumptions of 95–99% in both cases.

The nature of vine-shoot hydrolysates influenced the type of secondary metabolites produced by each species. Whereas A. succinogenes produced 0.14 ± 0.03 g/L lactic acid, 3.56 ± 0.31 g/L formic acid and 13.99 ± 1.12 g/L acetic acid (148:1:25:98:0; SA:LA:FA:AA:E); B. succiniproducens generated 6.83 ± 0.74 g/L lactic acid, 2.19 ± 0.26 g/L formic acid, 8.44 ± 0.24 g/L acetic acid, and 0.50 ± 0.21 g/L ethanol (36:14:4:17:1; SA:LA:FA:AA:E) after 120 h (Fig. 1). Glucose was consumed faster than xyllose by both bacterial species (Fig. 1). In fact, Salvachúa et al. (2016a) had already
observed that glucose consumption by *A. succinogenes* was faster than xylose consumption.

**Feasibility of succinic acid production from red grape must**

Given the nature of grape must, it was possible to assess several initial sugar concentrations (40, 77, 113, and 150 g/L). For both bacterial species, fermentations ended at 96 h, and it was observed that sugar consumption decreased as initial sugar concentrations augmented. As shown in Fig. 2, the highest succinic acid amount obtained by *A. succinogenes* was 43.25 ± 2.04 g/L, and it was recorded when initial sugars were at 113 g/L, consuming 62.19 ± 4.48% sugars and attaining a yield of 0.67 ± 0.02 g/g. Meanwhile, the highest succinic acid value for *B. succiniproducens* was 33.26 ± 1.06 g/L, and it occurred when initial sugars were set at 77 g/L, consuming 100% sugars and reaching a yield of 0.46 ± 0.01 g/g (Fig. 2). Succinic acid concentrations were significantly higher (*p* < 0.05) with *A. succinogenes* than with *B. succiniproducens* for all the initial sugar concentrations tested. For experiments with 113 and 150 g/L, it was observed that fructose consumption was significantly more efficient with *A. succinogenes*, whereas glucose consumption was significantly more efficient with *B. succiniproducens* (*p* < 0.05).
Under the abovementioned conditions of highest succinic acid production (initial sugar concentrations of 113 g/L for *A. succinogenes* and 77 g/L for *B. succiniproducens*), the generation of secondary metabolites was characterized by an important production of lactic acid by *B. succiniproducens*. In brief, this species produced an average of 20.80 ± 1.45 g/L lactic acid, 2.71 ± 1.12 g/L formic acid, 6.16 ± 0.27 g/L acetic acid, and 0.71 ± 0.05 g/L ethanol (47:29:4:9:1, SA:LA:FA:AA:E); whereas *A. succinogenes* produced 0.43 ± 0.11 g/L lactic acid, 5.06 ± 0.59 g/L formic acid, 5.94 ± 0.43 g/L acetic acid, and 1.46 ± 0.37 g/L ethanol (100:1:12:14:3, SA:LA:FA:AA:E) (Fig. 2). It is worth mentioning that the proportions of secondary metabolites produced from grape must by both species differed from those obtained from vine-shoot hydrolysates (section “Feasibility of succinic acid production from vine shoots”).

The best succinic acid values obtained in this work with grape must with both species (33–43 g/L) are clearly higher than those obtained with vine-shoot hydrolysates (18–21 g/L). Accordingly, grape must seems to have a greater potential as a feedstock for succinic acid production and it will be explored in more detail. Moreover, the lower production of secondary metabolites by *A. succinogenes* (especially lactic acid), together with its higher production of succinic acid, make it the species of choice for the next experiments focused on nutrient optimization with grape must.
Optimization of nutrient addition to grape must for succinic acid production

The results of the Plackett–Burman experiment revealed that yeast extract was the only nutrient in the NM-A mixture which had a significant and positive effect on succinic acid production \((p < 0.05)\), while \(K_2HPO_4\) exerted a significantly negative effect (Tables S4-S6). The rest of nutrients (\(CaCl_2\cdot2H_2O, MgCl_2\cdot6H_2O,\) and \(NaCl\)) had no statistical effect on the fermentation. Therefore, it would only be necessary to add yeast extract (or an equivalent source of organic nitrogen and vitamins) to red grape must in order to produce succinic acid with A. succinogenes DSM 22257.

The highest succinic acid concentration observed in the Plackett–Burman experimental design was 46.37 g/L (Table S4). However, it is hypothesized that this value could be greatly improved if the concentrations of essential nutrients were optimized (namely, initial sugars and yeast extract), while ruling out unnecessary nutrients (\(K_2HPO_4, CaCl_2\cdot2H_2O, MgCl_2\cdot6H_2O,\) and \(NaCl\)). Consequently, an RSM exploring a sugar range of 40.0–243.5 g/L and a yeast extract range of 1–40 g/L was performed (Table S7). It was observed that succinic acid production and sugar consumption were close to zero for all those experiments with initial sugar concentrations above 200 g/L. The values recorded in the RSM experiment (Tables S7-S10) were employed to create the mathematical model given in Eq. (1):

\[
\text{Succinic acid} \left( \frac{g}{L} \right) = -92.9158 + 1.6914 \times 10^3 s + 5.2417 y - 0.0066s^2 - 0.1023 y^2
\]  

where \(s\) and \(y\) are the concentrations (g/L) of initial sugars and yeast extract, respectively. Predicted \(R\)-squared of the equation was 46.27%. The term \(sy\) was not used to calculate the model due to its lack of significance \((p = 0.917)\). The model suggests that succinic acid production from grape must is highly influenced by the C/N ratio, and that the highest succinic acid values are expected when sugars are in the range of 115–150 g/L and yeast extract is in the range of 20–30 g/L (Fig. 3a). The equation was used to optimize the values of the independent variables in order to maximize succinic acid production. Several working conditions were mathematically proposed (Table S11) and then checked experimentally to select the most successful one. Consequently, the model estimated that diluting grape must to 145.7 g/L initial sugars and adding 24.9 g/L yeast extract would allow obtaining 80.76 g/L succinic acid. These values were tested experimentally, and 88.93 ± 1.36 g/L succinic acid were obtained, with a sugar consumption of 96.64 ± 0.30% and a yield of 0.66 ± 0.01 g/g. Although the estimation of succinic acid was not very accurate as expected from the predicted \(R\)-squared value (i.e., the experimental value was 10% higher than the modelized response), the optimal point of the RSM represents an extraordinary improvement in comparison to the preliminary tests performed with grape must in section “Feasibility of succinic acid production from red grape must” (88.93 g/L versus 43.25 g/L succinic acid).

The mathematical model for sugar consumption based on RSM results is shown in Fig. 3b, and further details are provided in Tables S12-S14.

Time evolution of succinic acid production under optimal conditions

After establishing the optimal nutrient composition for grape must, control fermentations with pure glucose and fructose were also performed to evaluate the ability of A. succinogenes DSM 22257 to grow under similar initial sugar concentrations in the absence of the rest of constituents of grape must. Thus, aqueous solutions containing 72.4 g/L glucose, 73.7 g/L fructose (145.7 g/L total sugars), and 24.9 g/L yeast extract were fermented with and without the addition of NM-A salts \((0.2 g/L CaCl_2\cdot2H_2O, 0.2 g/L MgCl_2\cdot6H_2O, 1 g/L NaCl,\) and 3 g/L \(K_2HPO_4\)).

The highest succinic acid production after 96 h corresponded to grape must \((85.37 ± 0.69 g/L succinic acid),\) followed by the control solution with NM-A salts \((62.24 ± 2.96 g/L succinic acid),\) whereas the control solution without salts was not fermentable \((3.15 ± 4.28 g/L succinic acid);\) with significant differences \((p < 0.05)\) between the three treatments. It is therefore evident that succinic acid production is unviable in the absence of NM-A salts in control solutions; in contrast to what is observed with grape must, whose natural content of anions and cations seems to provide the required mineral nutrients. It is remarkable that metabolite ratios were different in grape must and in the control with NM-A nutrients (Fig. 4). Whereas in the grape must 85.37 ± 0.69 g/L succinic acid, 0.66 ± 0.05 g/L lactic acid, 7.68 ± 0.68 g/L formic acid, 13.32 ± 0.38 g/L acetic acid, and 0.51 ± 0.31 g/L ethanol \((169:1:15:26:1, SA:LA:FA:AA:E)\) were produced after 96 h; 62.24 ± 2.96 g/L succinic acid, 12.42 ± 14.07 g/L lactic acid, 8.71 ± 0.26 g/L formic acid, 10.51 ± 0.30 g/L acetic acid, and 1.45 ± 0.36 g/L ethanol were obtained in the control solution \((43:9:6:7:1, SA:LA:FA:AA:E);\) which is due to the different chemical compositions of both samples, as explained in section “Preliminary assessment of vine shoots and grape must.” The dissimilarity in lactic acid production between both samples is especially remarkable. According to the proposed metabolic pathways for A. succinogenes, succinic acid production is influenced by CO2 pressure, substrate composition and concentration, and oxidation–reduction conditions (Dessie et al. 2018; Zeikus et al. 1999). In a literature review, Pateraki et al. (2016a) indicated that the presence of reducing substances or an adequate balance of
reducing and oxidizing substances could provoke the preferential shift of carbon sources to succinate production instead of lactate production. Grape must is a complex matrix, and its chemical composition could hypothetically favor succinate over lactate generation. In addition, as highlighted by Brink and Nicol (2014), secondary metabolites such as FA and AA were produced only during the exponential growth phase of microorganisms, while succinic acid was produced during the exponential and stationary phases (Fig. 4). It was considered that fermentations had finished after 96 h, reaching sugar consumptions of 93.96 ± 1.63% in the case of grape must and 91.52 ± 7.55% in the case of the control solution with NM-A salts (Fig. 4). In both cases, the curves for succinic acid production were adjusted to the Hill equation with four parameters ($r^2 > 0.999$), as shown in Table S15.

Comparison of nitrogen sources for succinic acid production from grape must

After 96 h of grape-must fermentation, 84.83 ± 2.15 g/L, 3.06 ± 2.58 g/L, 3.50 ± 5.09 g/L, 2.33 ± 0.12 g/L, 0.07 ± 0.12 g/L, and 0 ± 0 g/L succinic acid were obtained with yeast extract, red wine lees, white wine lees, urea, NH$_4$Cl, and choline chloride, respectively (Fig. S1). Succinic acid production was clearly and statistically higher ($p < 0.05$) with yeast extract than with any of the other nitrogen sources tested with grape must. Sugar consumption was nil with NH$_4$Cl and choline chloride (Fig. S1). In addition, cell density was statistically similar ($p > 0.11$) and about $10^9$ cell/mL for yeast extract, red wine lees, white wine lees, whereas this value was about $10^8$ cells/mL for urea, NH$_4$Cl, and choline chloride. The presence of low concentrations of ethanol in the fermentation broths supplemented with wine lees is due to the natural occurrence of this alcohol in lees and not to the fermentative activity of Actinobacillus succinogenes DSM 22257 (Fig. S1).

Discussion

Preliminary assessment of vine shoots and grape must

The amounts of succinic acid produced by both bacterial species (18–21 g/L) from vine-shoot hydrolysates were relatively low. This is partly due to the initial sugar concentration of this feedstock (roughly 36 g/L), which is a limiting factor to get higher succinic acid values. Similarly, the tested bacterial strains obtained 18–24 g/L succinic acid from surplus grape must with comparable initial sugar concentrations to vine-shoot hydrolysates (40 g/L sugar) (Fig. 2). The strategy of concentrating vine-shoot hydrolysates by evaporation to obtain higher sugar amounts before fermentation does not seem energetically advisable. In fact, Filippi et al. (2022) concentrated a hydrolysate of grape pomace and grape stalk to reach an initial sugar value of 55.7 g/L, whose fermentation with Actinobacillus succinogenes DSM 22257 led to barely 24.9 g/L succinic acid. However, the development of membrane-based upstream and downstream processes could make it economically feasible to increase sugar concentrations or recover succinic acid from low-concentration broths, respectively, in the near future (García-Aguirre et al. 2020; Zaman et al. 2020), which may be applicable to vine-shoot hydrolysates. On the other hand, the carbohydrate-rich nature of grape must allowed that succinic acid concentrations raised to 33–43 g/L when sugar concentrations in grape must were increased to 77–113 g/L (Fig. 2), thus hinting a possibility of improvement.

The different sugar composition between vine-shoot hydrolysates and grape must could have contributed to their different behaviors in succinic acid production and secondary metabolites profile even in the case where their initial sugar concentrations were similar (~40 g/L). Vine-shoot hydrolysates contain mainly glucose and xylene, whereas grape must contains glucose and fructose. In fact, metabolite ratios in Actinobacillus succinogenes and B. succiniproducens depend on the type of sugars fermented (Salvachúa et al. 2016b), their concentrations (Babaei et al. 2019), their proportions (Cimini et al. 2019; Ferone et al. 2018), and the complexity of the broth (Leung et al. 2012; Pateraki et al. 2016b). Furthermore, Actinobacillus succinogenes is known to produce lower succinic acid amounts from xylose than from other sugars, such as glucose, fructose, or lactose (Chen et al. 2011). In addition, glucose and fructose offer similar succinic acid yields (Liu et al. 2008) and they are fermented simultaneously by Actinobacillus succinogenes (Gunnarsson et al. 2014).

Nutrient optimization for grape must

By means of statistical tools, it was possible to simplify the NM-A medium for grape must, thus discarding the addition of conventional Actinobacillus succinogenes mineral nutrients, such as K$_2$HPO$_4$, CaCl$_2$
$_2$H$_2$O, MgCl$_2$·6H$_2$O, and NaCl, whereas yeast extract was maintained. This can be explained by the chemical composition of grape must (Table S1), which contains phosphate, K, Ca, and Mg in apparently sufficient amounts, but only 0.34 g/L TN. Actinobacillus succinogenes is an auxotrophic species that needs vitamins (especially biotin) and fatty acids, which are normally supplemented through yeast extract (Dessie et al. 2018; Pateraki et al. 2016a).

The RSM unveiled interesting relationships between variables. Cell density was negatively correlated to initial sugar concentrations ($r^2 = 0.6978$; Table S7). This agrees partly with literature data stating that cell growth of Actinobacillus succinogenes begins to diminish from 60 to 100 g/L glucose and...
that it is totally inhibited with about 160 g/L (Dessie et al. 2018; Pateraki et al. 2016a). However, as opposed to the trend observed before nutrient optimization, where sugar consumption declined sharply when initial sugars were above 100 g/L (Fig. 2a), the RSM results have shown that sugar consumption can be almost complete even at substrate concentrations of 150 g/L, provided that appropriate yeast extract amounts are supplemented (Table S7). This implies that the growth of A. succinogenes under high sugar concentrations in batch fermentations can be ameliorated by an adequate balance of organic nitrogen, thus increasing succinic acid production in a remarkable way. Although succinic acid production from broths with 120–150 g/L sugars had already been reported (Li et al. 2018; Thuy et al. 2017), to the best of our knowledge, this is the first time that the influence of nitrogen dosing on substrate inhibition is described for A. succinogenes. A wide spectrum of yeast extract supplementation can be found in scientific literature, ranging from 5 g/L (Ferone et al. 2019a) to 30 g/L (Li et al. 2010), but without establishing any global relationships between yeast extract and substrate (i.e., sugar) consumption.

The beneficial effect of the specific nutrient fine-tuning by RSM on grape must is reflected in Fig. 4. The better performance of grape must in comparison with the control solution with NM-A salts is presumably due to the fact that nutrient concentrations (initial sugars and yeast extract) had been optimized specifically for grape must, whereas the NM-A salts solution can be considered as a non-optimized broth. However, the moderate-high succinic acid values attained with the control solution supplemented with NM-A salts indicate that A. succinogenes DSM 22257 can cope successfully with high sugar concentrations. In both cases, succinic acid production was negligible during the first 24 h (Fig. 4), probably due to the adaptation of bacteria to a medium with such a high substrate concentration. The curves of succinic acid and bacterial cell density followed parallel trends but with a time gap: whereas cell density started to rise at $t = 24$ h; succinic acid did at $t = 48$ h (Fig. 4). This succinic acid lag phase could be shortened by applying certain techniques, such as cell immobilization (Brink and Nicol 2014; Li et al. 2018), large inoculation volumes (Wan et al. 2008), continuous fermentation (Ferone et al. 2019b), or microbial adaptation to high sugar concentrations (Ahmad et al. 2021).

**Influence of nitrogen sources on succinic acid production from grape must**

In the present work, yeast extract outperformed the other nitrogen sources for the production of succinic acid with A. succinogenes DSM 22257 from surplus grape must. The addition of wine lees to grape must resulted in an inefficient succinic acid bioproduction (Fig. S1). Wine lees contain yeast cellular debris, and they have been successfully employed as organic nitrogen sources for succinic acid production (Filippi et al. 2022). Similar sources of residual fungal biomass, such as spent yeast hydrolysate, dry yeast cells, or Aspergillus autolyse, have been assessed for succinic acid production with A. succinogenes, observing a decrease of 0–40% in succinic acid concentrations or an increase in fermentation times in comparison with commercial yeast extract (Chen et al. 2011; Du et al. 2007; Li et al. 2011; Liu et al. 2008; Sawisit et al. 2012). On the other hand, Jiang et al. (2010) reported that spent brewer’s yeast hydrolysate had to be supplemented with vitamins in order to successfully replace yeast extract when producing succinic acid with A. succinogenes (Jiang et al., 2010).

Contrarily to what was observed in the present work, urea and inorganic salts, such as KNO$_3$, NH$_4$Cl, and (NH$_4$)$_2$SO$_4$, have been proved to allow succinic acid production with A. succinogenes from agri-food feedstocks, although implying a production decrease of 40–60% compared to yeast extract (Liu et al. 2008; Shen et al. 2016a). In contrast, the inorganic salt (NH$_4$)$_2$HPO$_4$ had excellent results for the fermentation of cassava root (Thuy et al. 2017). Moreover, some vegetable-origin feedstocks have been used for succinic acid production without the addition of any nitrogen source, thanks to their intrinsic protein content, such as certain fruit juices, juice of oil palm leaves or waste wheat bread (Leung et al. 2012; Ferone et al. 2019c; Tan et al. 2016).

**Feasibility of succinic acid bioproduction from winery surplus and by-products**

Table 1 summarizes literature data on batch fermentation processes to obtain succinic acid from industrial, agriculture and food-related feedstocks. Vine-shoot hydrolysates are in the low range of the rank regarding succinic acid production. This is in agreement with the results observed for other woody materials, such as grape pomace and grape stalk (Filippi et al. 2022) or poplar wood (Pennacchio et al. 2018), whose succinic acid bioproduction reached 16–25 g/L (Table 1). Apart from the relatively low product concentrations obtained (about 20 g/L succinic acid), the physicochemical and enzymatic pretreatment of vine shoots implies energy and material costs that hinder its profitability. However, as discussed in section “Preliminary assessment of vine shoots and grape must,” vine shoots could become an interesting feedstock for succinic acid bioproduction if adequate and cost-efficient concentration and recovery techniques are developed.

On the other hand, grape must presented promising results as a feedstock, with succinic acid values in the range of 84–89 g/L. These high values are related to the initial sugar concentrations employed (145.7 g/L sugars), which did not provoke substrate inhibition thanks to an adequate
### Table 1  Batch fermentations for succinic acid using industrial, agriculture, and food-related feedstocks

| Strain/Genotype | Substrate | Initial sugars (g/L) | Nitrogen source(s) | Salts (a) | \( \text{SA} \) (g/L) | \( \text{FA} \) (g/L) | AA (g/L) | PyA (g/L) | PrA (g/L) | LA (g/L) | E (g/L) | Y (g/g) | \( \Delta S \) (%) | t (h) | Reference |
|----------------|-----------|----------------------|-------------------|-----------|----------------|----------------|---------|-----------|-----------|----------|--------|--------|-----------------|------|-----------|
| A. succinogenes DSM 22257 | Jerusalem artichoke (Helianthus tuberosus) hydrolysate | 76.5 | - | - | 47.9 | - | - | - | - | - | 0.67 | - | 95 | 48 | Gunnarsson et al., 2014 |
| A. succinogenes GXAS157 | Duckweed (Ludisia discolor) hydrolysate | 64.4 | CSL | K\(_2\)HPO\(_4\), Na\(_2\)HPO\(_4\), H\(_2\)O, Mg\(_{6}\)Cl\(_2\), NaCl | 57.85 | - | - | - | - | - | - | 0.89 | - | 90 | 48 | Shen et al., 2016 |
| A. succinogenes ATCC 55618 | Corn stover hydrolysate | 80 | YE, CSL | Na\(_2\)HPO\(_4\), K\(_2\)HPO\(_4\), sodium acetate, NaCl, Mg\(_{6}\)Cl\(_2\), H\(_2\)O, CaCl\(_2\)| 43 | - | - | - | - | - | - | 0.74 | - | 145 | 70 | Salvadó et al., 2016 |
| A. succinogenes CGMCC 1716 | Corn fiber hydrolysate | 70 | YE | KH\(_2\)PO\(_4\), Mg\(_{6}\)Cl\(_2\), H\(_2\)O, CaCl\(_2\), NaCl | ~48 | - | - | - | - | - | - | - | - | 48 | Chen et al., 2011 |
| A. succinogenes CGMCC 1716 | Corn fiber hydrolysate | 70 | YE | Yeast cells | ~47 | - | - | - | - | - | - | - | 0.68 | - | 70 | Chen et al., 2011 |
| A. succinogenes ATCC 55618 | Corn stover hydrolysate | 789 | YE | Na\(_2\)HPO\(_4\), H\(_2\)O, Na\(_2\)HPO\(_4\), biotin | 70.6 | 0.3 | 2.8 | - | 3 | - | - | 0.88 | - | 101 | 40 | Gunter et al., 1996 |
| A. succinogenes CGMCC 1716 | Corn stover hydrolysate | 100 | YE, CSL | KH\(_2\)PO\(_4\), Mg\(_{6}\)Cl\(_2\), H\(_2\)O, CaCl\(_2\), NaCl | 67.53 | - | - | - | - | - | - | - | 97 | 70 | Li et al., 2011 |
| A. succinogenes CGMCC 1716 | Corn stover hydrolysate | 58 | YE | Na\(_2\)HPO\(_4\), H\(_2\)O, K\(_2\)HPO\(_4\), H\(_2\)O | 45.5 | -1 | -5 | - | - | - | - | 0.81 | - | 100 | 48 | Zheng et al., 2009 |
| A. succinogenes CCTCC M 2011399 | Sugarcane juice | 70 | - | K\(_2\)HPO\(_4\), Na\(_2\)HPO\(_4\), H\(_2\)O, Mg\(_{6}\)Cl\(_2\), H\(_2\)O, NaCl | 57.11 | - | -7 | - | - | - | - | - | 95 | 48 | Shen et al., 2016 |
| A. succinogenes CGMCC 1593 | Sugarcane molasses (inverted) | 644 | YE | Na\(_2\)HPO\(_4\), H\(_2\)O, Na\(_2\)HPO\(_4\), biotin | 46.4 | ~2 | -7 | - | - | - | - | 0.79 | ~90 | 48 | Liu et al., 2008 |
| A. succinogenes DSM 22257 | Sugarcane molasses (ultrafiltration) | 60 | YE, CSL | NaCl, Mg\(_{6}\)Cl\(_2\), H\(_2\)O, MnSO\(_4\), Na\(_2\)HPO\(_4\), K\(_2\)HPO\(_4\), Na\(_2\)HPO\(_4\), K\(_2\)HPO\(_4\) | 64.1 | - | - | - | - | - | - | - | 28 | Cao et al., 2018 |
| A. succinogenes ATCC 55618 | Wheat flour hydrolysate | 70 | YE | Na\(_2\)HPO\(_4\), H\(_2\)O, Na\(_2\)HPO\(_4\), NaCl, Mg\(_{6}\)Cl\(_2\), H\(_2\)O, CaCl\(_2\) | 35.6 | - | - | - | - | - | 0.82 | ~61 | ~65 | Du et al., 2007 |
| A. succinogenes DSM 22257 | Grape pomace and grape stalk hydrolysate | 555 | Wine lees | Na\(_2\)HPO\(_4\), H\(_2\)O, Na\(_2\)HPO\(_4\), NaCl, Mg\(_{6}\)Cl\(_2\), H\(_2\)O, CaCl\(_2\), H\(_2\)O | 24.9 | 7.2 | 9.1 | - | - | - | - | 0.59 | ~91 | 40 | Filippi et al., 2022 |
| A. succinogenes DSM 22257 | Alga (Saccharina latissima) hydrolysate | ~55 | YE | K\(_2\)HPO\(_4\), Mg\(_{6}\)Cl\(_2\), CaCl\(_2\), NaCl | 36.8 | 1.4 | 7.9 | - | - | 1 | - | 100 | 48 | Martins et al., 2016 |
Table 1 (continued)

| Strain          | Substrate                          | Initial sugars (g/L) | Nitrogen source (a) | Salts (b) | SA (g/L) | FA (g/L) | AA (g/L) | PyA (g/L) | PrA (g/L) | LA (g/L) | E (g/L) | Y (g/g) | ΔS (%) | t (h) | Reference                                      |
|-----------------|------------------------------------|----------------------|---------------------|-----------|----------|----------|----------|-----------|-----------|----------|---------|--------|--------|-------|------------------------------------------------|
| A. succinogenes | Pineapple juice                    | 56.2                 | -                   | -         | 37       | 1.2      | 6.1      | -         | -         | -        | 1.00    | 65     | -168   | 65    | Ferone et al., 2019b                           |
| A. succinogenes | Lemon-lime softdrink (hydrolysate) | 36.1                 | YE                  |           | 40.4     | 3.1      | 7.5      | -         | -         | -        | 1.11    | 100    | -120   | communication omitted                        |
| A. succinogenes | Almond syrup (hydrolysate)         | 42.4                 | YE                  |           | 45.3     | 2.1      | 7.5      | -         | -         | -        | 1.10    | 95     | -130   | 95    | Ferone et al., 2019b                           |
| A. succinogenes | Cassava root hydrolysate           | 150                  | (NH2)2HPO4          | -         | 93.34    | -0      | -20      | -         | -55       | -        | 0.77    | -      | 50     | Thuy et al., 2017                              |
| A. succinogenes | Alga (Laminaria digitata) hydrolysate | 45                   | YE                  |           | 33.78    | -4      | -8       | -         | -         | -4       | -0.1    | 0.63   | 48     | Alvarado-Morales et al., 2015                   |
| A. succinogenes | Waste wheat bread hydrolysate      | 40                   | -                   | -         | 47.3     | 0        | 0.2      | 0         | -         | -        | 1.16    | 100    | 60     | Leung et al., 2012                             |
| A. succinogenes | Grape-shoot hydrolysate            | 36.1                 | YE                  |           | 21.18    | 3.56     | 13.99    | -         | 0.14      | -        | 0.64    | 91.24  | 120    | This work                                     |
| A. succinogenes | Corn stover hydrolysate            | 145.7                | YE                  |           | 88.93    | 7.3      | 12.7     | -         | 0.48      | 0.84     | 0.66    | 96.64  | 96     | This work                                     |
| B. succiniciproducens | Corn stover hydrolysate          | 60                   | YE, CSL, (NH4)2SO4 |           | 30.6     | -        | -        | -         | -         | -        | -       | -      | 72     | Salvadó et al., 2016b                          |
| B. succiniciproducens | Corn stover hydrolysate          | 20–23                | YE, (NH4)2SO4       |           | 16.5     | -        | -        | -         | -         | -0.75    | 100    | 30     | Pernacchio et al., 2018                        |
| B. succiniciproducens | Spent sulphite liquor (diluted, ultrafiltered) | 18                  | YE                  |           | 8.2      | -        | -        | -         | -         | -        | 0.52    | -      | -15    | Pateraki et al., 2016b                         |
| E. coli SD121 (modified) | Licorice waste (Glycyrrhiza uralensis)  | 44                   | YE, tryptone, (NH4)2SO4·7H2O |           | 36.55    | -        | 4        | -         | -         | 0.83     | -        | 0.31    | 84     | Wang et al., 2011                             |
| E. coli MG-PYC (modified) | Sugarcane molasses             | 58                   | YE                  |           | 35       | -        | -        | -         | -         | -        | 0.69    | -88    | 60     | Wang et al., 2018                              |
| E. coli KJ122-pKJSUC-24T (modified) | Sugarcane molasses             | -                    | Beta inc. (NH4)2HPO4·NH4H2PO4 |           | 55.8     | 4.97     | -        | -         | -         | 0.96     | -4.97   | -      | 72     | Chan et al., 2012                              |
organic nitrogen balance. The highest succinic acid values obtained so far from agriculture or food-related feedstocks in batch fermentations correspond to cassava root hydrolysate (Thuy et al. 2017), corn stover hydrolysate (Guettler et al. 1996; Li et al. 2011), and ultrafiltered sugarcane molasses (Cao et al. 2018), reaching 64–93 g/L succinic acid. In fact, the fermentation of grape must has provided the second highest value of succinic acid reported in literature for this type of feedstocks in batch processes (Table 1). Furthermore, grape must does not need any pretreatment before fermentation, apart from diluting with water, which simplifies the process. As explained in section “Optimization of nutrient addition to grape must for succinic acid production,” grape must needs 24.9 g/L yeast extract to maximize succinic acid production. The market price of bulk yeast extract in Spain is 7 €/kg (7.6 $/kg) (Biospringer, Lesaffre Ibérica SA, personal communication, April 2022), but yeast extract could also be replaced by cheaper organic nitrogen sources, such as spent yeast hydrolysate or a combination of spent yeast with vitamins (see section “Influence of nitrogen sources on succinic acid production from grape must”). In any case, Eq. (1) enables the calculation of alternative working points with lower yeast extract concentrations which would still provide acceptable succinic acid values (Fig. 3). On the contrary, A. succinogenes requires almost no external nutrient addition when grown on cassava root hydrolysate or waste bread hydrolysate (Leung et al. 2012; Thuy et al. 2017), which makes these feedstocks really attractive for succinic acid production, provided that they are not detracted from human nutrition (Table 1). Moreover, engineered Escherichia coli strains are able to produce succinic acid with very low nutrient requirements (Chan et al., 2012; Jampatesh et al. 2019; Khor et al. 2016; Sawisit et al. 2015, 2018). All this makes surplus grape must a promising feedstock for succinic acid production.

Biosuccinic acid market price is estimated at 3–9 $/kg depending on its purity and application (Cok et al. 2014). In 2015, the fossil-based equivalent was valued at around 2.50 $/kg (E4tech et al. 2015). Bulk grape must for human consumption was sold in Spain for 0.42 €/L (0.46 $/L) (VINE-TUR 2021), but the internal price of surplus grape must in a wine-producing company is assumed to be much lower. The described optimal method to obtain succinic acid from diluted grape must would imply a maximum feedstock cost (grape must and yeast extract) of 4.79 € (5.20 $) per 1 kg of succinic acid in the fermentation stage, which could be reduced considerably if yeast extract is replaced by spent yeast hydrolysate or another alternative nutrient, and, especially, if non-commercial surplus grape must is employed. Although operation, recovery, and purification costs can currently account for 50–80% of the overall process cost (García-Aguirre et al. 2020), technological advances in the bioindustrial sector are expected to lessen this figure.
Table 2 Fed-batch, immobilization, and other alternative fermentation types for succinic acid using industrial, agriculture, and food-related feedstocks

| Strain                  | Substrate                              | Initial sugars (g/L) | Nitrogen source(a) | Salts(b)                                                                 | Fermentation type(c) | SA (g/L)(d) | FA (g/L) | AA (g/L) | PyA (g/L) | LA (g/L) | E (g/L) | Y (g/g) | t (h) | Reference               |
|-------------------------|----------------------------------------|----------------------|--------------------|----------------------------------------------------------------------------|----------------------|-------------|-----------|-----------|-----------|----------|---------|--------|------|-------------------------|
| A. succinogenes         | Corn stover hydrolysate                | 40                   | YE                 | NaH2PO4·2H2O, K2HPO4·3H2O                                                  | FB                   | 53.2        | ~0        | ~5        | -         | -        | -       | 0.825  | 44   | Zheng et al., 2009      |
| A. succinogenes         | Sugarcane juice                        | 70                   | -                  | K2HPO4, NaH2PO4·H2O, MgCl6·H2O, CaCl2·2H2O, NaCl                          | FB                   | 62.1        | -         | ~7        | -         | -        | -       | 48     | Shen et al., 2016a      |
| A. succinogenes         | Sugarcane molasses (inverted)          | 35                   | YE                 | Na2HPO4·12H2O, NaH2PO4·2H2O, NaCl, MgCl2, CaCl2                           | FB                   | 55.2        | ~3        | ~7        | -         | -        | -       | 48     | Liu et al., 2008        |
| A. succinogenes         | Grape pomace and grape stalk hydrolysate | 40.1                 | Wine lees          | NaH2PO4·H2O, NaHPO4·NaCl, MgCl6·H2O, CaCl2·2H2O                           | FB                   | 37.2        | 6         | 9.2       | -         | -        | -       | 47     | Filippi et al., 2022    |
| A. succinogenes         | Cassava root hydrolysate               | 70                   | (NH4)2HPO4         | -                                                                            | FB                   | 151.4       | -         | -         | 1         | -        | -       | ~1.5   | 48   | Thuy et al., 2017       |
| A. succinogenes         | Sugarcane molasses (ultrafiltered)     | 60                   | YE, CSL            | NaCl, MgCl2·6H2O, CaCl2·2H2O, MnCl2, sodium acetate, Na2HPO4·NaH2PO4·K2HPO4 | B, I                 | 45.6        | -         | -         | -         | -        | -       | 0.76   | 24   | Cao et al., 2018        |
| A. succinogenes         | Corn stover hydrolysate                | -                    | CSL                | Sodium acetate, K2HPO4·12H2O, NaH2PO4·2H2O, MgCl2, CaCl2                  | B, SSF               | 47.4        | ~2        | ~10       | ~2        | -        | -       | 0.72   | 48   | Zheng et al., 2010      |
| B. succiniciproducens   | Spent sulphite liquor (diluted, nanofiltered) | 18                   | YE                 | NaH2PO4·H2O, NaHPO4·NaCl, MgCl6·H2O, CaCl2                               | FB                   | 33.8        | -         | -         | -         | -        | -       | 0.58   | ~70  | Pateraki et al., 2016b  |
| Corynebacterium glutamicum 534 (mutant) | Cassava bagasse hydrolysate         | 35                   | -                  | NaCl                                                                       | B, I                 | 22.4        | -         | 9.2       | -         | ~3.7     | -       | 0.648  | 54   | Shi et al., 2014        |
| E. coli SD121 (modified) | Corn stover hydrolysate                | 44                   | YE, tryptone, (NH4)2SO4·7H2O                                      | MgSO4·7H2O, NaH2PO4·12H2O, NaH2PO4·2H2O, MgCl2, CaCl2                 | FB                   | 57.8        | -         | 8.17      | -         | -        | 1.62    | 0.87   | 72   | Wang et al., 2011       |
| E. coli MG-PYC (modified) | Licorice waste (Glycyrrhiza uralensis) hydrolysate | 45                   | YE                 | MgSO4·K2HPO4·KH2PO4·NaCl, NaCl, MnCl2, CaCl2                              | FB                   | 65         | -         | -         | -         | -        | -       | 0.89   | 128  | Wang et al., 2018       |
| E. coli KJ122 (modified) | Cassava pulp hydrolysate               | -                    | Betaine, (NH4)2HPO4·NH3H2PO4                                      | KCl, MgSO4·7H2O, FeCl3·6H2O, CoCl2·6H2O, CuCl2·2H2O, ZnCl2·2H2O, Na2MoO4·2H2O, MnCl2·4H2O | B, SSF               | 80.86       | -         | ~8        | -         | -        | -       | 0.703  | 96   | Sawisri et al., 2015    |
| Strain          | Substrate                      | Initial sugars (g/L) | Nitrogen source(a) | Salts(b)                                                                 | Fermentation type(c) | SA (g/L)(d) | FA (g/L) | AA (g/L) | PyA (g/L) | LA (g/L) | E (g/L) | Y (g/g) | t (h) | Reference                          |
|----------------|--------------------------------|----------------------|--------------------|--------------------------------------------------------------------------|----------------------|-------------|-----------|-----------|-----------|-----------|---------|---------|-------|-----------------------------------|
| E. coli KJ122  | Cassava pulp hydrolysate       | -                    | Betaine, (NH₄)₂HPO₄, NH₄H₂PO₄ | KCl, MgSO₄·7H₂O, FeCl₃·6H₂O, CoCl₂·6H₂O, CuCl₂·2H₂O, ZnCl₂, Na₂MoO₄·2H₂O, H₂BO₃, MnCl₂·4H₂O | FB, SSF              | 98.63       | -         | -5        | -         | -         | 0.716   | 96      | Sawisit et al., 2015             |
| E. coli KJ122  | Rice straw hydrolysate         | -                    | Betaine, (NH₄)₂HPO₄, NH₄H₂PO₄ | KCl, MgSO₄·7H₂O, FeCl₃·6H₂O, CoCl₂·6H₂O, CuCl₂·2H₂O, ZnCl₂, Na₂MoO₄·2H₂O, H₂BO₃, MnCl₂·4H₂O | B, SSF               | 69.8        | -         | -17       | -         | -         | 0.83    | 120     | Sawisit et al., 2018             |
| E. coli KJ122  | Rice straw hydrolysate         | -                    | Betaine, (NH₄)₂HPO₄, NH₄H₂PO₄ | KCl, MgSO₄·7H₂O, FeCl₃·6H₂O, CoCl₂·6H₂O, CuCl₂·2H₂O, ZnCl₂, Na₂MoO₄·2H₂O, H₂BO₃, MnCl₂·4H₂O | FB, SSF              | 103.1       | -         | -10       | -         | -         | 0.84    | 120     | Sawisit et al., 2018             |
| E. coli AS1600a| Rice straw hydrolysate         | -                    | Betaine, (NH₄)₂HPO₄, NH₄H₂PO₄ | KCl, MgSO₄·7H₂O, FeCl₃·6H₂O, CoCl₂·6H₂O, CuCl₂·2H₂O, ZnCl₂, Na₂MoO₄·2H₂O, H₂BO₃, MnCl₂·4H₂O | B, SSF               | 85.6        | -         | -11       | -         | -         | 0.90    | 144     | Jampatesh et al., 2019           |
| Y. lipolytica  | Crude glycerol                | 120                  | YE, peptone        | -                                                                          | B, I                 | 53.6        | -         | -         | -         | -         | 0.45    | 37      | Li et al., 2018                  |
| Y. lipolytica  | Crude glycerol                | 120                  | YE, peptone        | -                                                                          | FB, I                | 209.7       | -         | -3        | -         | -         | 322     | Li et al., 2018                  |
| Y. lipolytica  | Municipal organic waste       | 120                  | YE                 | -                                                                          | FB                   | 54.4        | -         | 3         | -         | -         | 0.44    | 66      | Stylianou et al., 2021           |

(a) CSL, corn steep liquor, YE, yeast extract. (b) The pH-buffer MgCO₃ is not included as a nutritive salt. (c) B batch, FB fed-batch, I immobilization, SSF simultaneous saccharification and fermentation. (d) SA succinic acid, FA formic acid, AA acetic acid, PyA pyruvic acid, LA lactic acid, E ethanol, Y succinic acid yield.
Therefore, succinic acid bioproduction from grape must could become profitable in the future depending on the evolution of market prices, available technologies, and legislative framework.

Although sugar consumption was almost total with grape must (>96%), succinic acid yields (0.66 g/g) were far below the theoretical yields of 1.12 g/g from glucose or 1.31 g/g from xylose g/g (Bradfield and Nicol, 2014, 2016). This is quite common in batch fermentations (Table 1), because carbon is used not only for succinic acid production, but also for cell growth and for the generation of secondary metabolites. In order to avoid these drawbacks and increase succinic acid production, alternative techniques, such as fed-batch fermentation, continuous fermentation, simultaneous saccharification and fermentation, or cell immobilization, have been assessed with industrial, agriculture, and food-related feedstocks, as shown in Table 2. The highest succinic acid concentrations have been reported with cassava root hydrolysate and crude glycerol in fed-batch mode, attaining 150–210 g/L succinic acid (Li et al. 2018; Thuy et al. 2017). Cell immobilization and continuous fermentation can lead to higher cell concentrations, higher succinic acid concentrations, higher yields, shorter lag phases, shorter fermentation times, lower concentrations of secondary metabolites and therefore higher productivities (Ferone et al., 2019c; Pat eraki et al. 2016a). Succinic acid bioproduction from alternative feedstocks also needs the development of adequate and economical downstream processes for product recovery and purification (García-Aguirre et al. 2020).

Italy, France, and Spain were the three major wine producers worldwide in 2019 (FAOSTAT 2022). Quality wines in the European Union are protected by Designation of Origin marks, and most vineyards are concentrated in certain geographical regions. Hence, the production of wine, winery surplus, and winery by-products is also located in particular regions, which could encourage the establishment of a centralized biorefinery plant for winery surplus and by-products in each wine-producing region, thus managing the logistics of feedstock collection and transport within a radius of about 100 km.

Accordingly, further research on genetic engineering, cell cultivation, nutrient optimization, fermentation technologies, and recovery processes could make grape must a suitable feedstock for industrial succinic acid bioproduction in the future.

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Author contribution MHV: investigation, formal analysis, writing—original draft, AIPG: resources. RDA: Project administration.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval The manuscript does not contain experiments using animals. The manuscript does not contain human studies.

Competing interests The authors declare no competing interests.

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