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Soybean molasses as a new and low-cost substrate for gluconic acid production by *Aspergillus niger*

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ABSTRACT

Soybean molasses (SM), a sugar-rich by-product obtained from manufacturing soy protein concentrate, is an interesting source of fermentable sugars for biotechnological processes. For the first time, SM was used as a new and low-cost substrate in *Aspergillus niger* cultures to produce gluconic acid (GA), a carboxylic acid obtained by the oxidation of glucose and with a wide range of applications. The effect of SM composition and sugar content on GA batch production was studied in a stirred-tank bioreactor using two SM samples with different total sugar concentrations of 46 % and 52 % (w/w) and different sugar profiles. GA productivity improved as the total sugar concentration increased, whereas yield was unaffected. Agitation rate increase positively affected yield and productivity in SM medium with the highest sugar concentration. Under these conditions, the highest GA titer (79 $g{\cdot}L^{-1}$) was obtained. Oxalic acid and fructose syrup were other added-value compounds found from the submerged fermentation of SM. This work proved the potential of *A. niger* in the bioconversion of SM with different compositions into GA, providing a novel strategy for low-cost and eco-friendly GA production from SM.

1. Introduction

Soybean (*Glycine max*) is one of the most important crops cultivated to attend to the increasing demand for its several applications, such as the most important source of plant-based protein. The use of soybean for human food represents 20 % of the main applications (soy milk, tofu, tempeh), 76 % is for animal feed (soybean meal) and 4 % is used to produce oil for food and chemical industries. The United States and Brazil account for about 69 % of global soy production, which attained 363 million tons in 2022 and is expected to reach 415 million tons by 2032 [1]. In the manufacturing of soy-based products, large amounts of by-products are generated, namely okara, soybean hull, soybean meal, and soybean molasses (SM) [2]. This last is the main by-product generated at the industrial processing of soybeans to produce soy protein concentrate by the extraction of defatted soybean meal and the removal of carbohydrates [3]. SM contains a high content of sugars, mainly sucrose and raffinose family oligosaccharides (RFO) – raffinose and stachyose – and small amounts of monosaccharides – glucose, fructose, and galactose. The other components of SM include phytochemicals, proteins, lipids, minerals, phenolic acids, and ashes [3–6].

In the industrial process, approximately 232 kg of SM are produced for each ton of soybean meal [3]. SM's nutritional and functional properties justify its potential to be incorporated into ruminant feed diets, replacing the content of corn grain. However, there are some limitations to including this raw material in animal feed, namely the relatively short shelf-life of SM, the low digestibility of oligosaccharides, and laxative effects on monogastric animals when SM is used in high amounts [7]. Its utilization, with or without prior pretreatments or enzymatic/acid hydrolysis, as raw material in the microbial production of bioproducts has also been reported, for instance, for the production of bioethanol [8–10], biomethane [6], butanol [11], biosurfactants [12,13], biodegradable polyesters [14], lipases and β-galactosidases [15,16], microbial lipids[17], malic acid [4], acetic acid [18], lactic acid [19], propionic acid [20], and sweetener isomaltulose [6], among others. SM was also used to produce citric acid by a mutant strain of *A. niger*, but SM favored fungus growth owing to its high protein content resulting in decreased citric acid biosynthesis [21].

Although SM has already been proposed as a substrate for the production of several metabolites, its utilization in gluconic acid biosynthesis by *A. niger* was never attempted. Gluconic acid (GA) is one of the bio-based organic acids with the most industrial relevance. Its global market size was estimated at EUR 1.31 billion in 2024 and is projected to

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reach EUR 1.86 billion by 2030, growing at a compound annual growth rate of 5.86 % over the forecast period 2024–2030 [22]. At the industrial scale, GA is produced by biotechnological processes through the oxidation of pure glucose or glucose syrup by glucose oxidase (fungi) or glucose dehydrogenase (bacteria) [23]. Since the raw materials represent most of the operating costs of metabolite production [11], it is desirable to use alternative sources of glucose. The price of SM (0.22 €/kg) [24] is close to the one of sugarcane molasses and almost six times lower than glucose syrup (1.30 €/kg) [25] making its use as a carbon source a cheap alternative to develop a new GA bioprocess. Using by-products with high wastage rates, such as SM, can be a way to increase food industry sustainability and are in line with the UN Sustainable Development Goal to ensure sustainable consumption and production patterns (SDG 12).

In the current study, two SM samples (SM-1 and SM-2) of different sugars composition, without prior hydrolysis and pretreatments, were used as carbon sources to assess their suitability to the batch production of GA by *A. niger*. As SM is a new substrate for GA production, Fig. 1 represents the catabolism of sugars present in SM by *A. niger* and further bioconversion of free glucose and glucose released from hydrolysis of sucrose and RFO into GA.

Firstly, (1) stachyose is hydrolyzed by α-galactosidase (mainly present in extracellular medium), which cleaves in the α-1,6 linkages, obtaining one unit of D-galactose and raffinose [26,27]. Hereafter, (2) raffinose is broken down into (3) D-galactose and sucrose or (4) fructose and melibiose (composed of monomers of galactose and glucose). Sucrose is degraded in the β-1,2 linkage by invertase, producing glucose and fructose [28–30]. Thus, (5) free glucose and monomers of glucose released from sucrose and RFO hydrolysis can be converted into GA by *A. niger* glucose oxidase (GOx), which is mainly located in cell walls and extracellular medium. GOx specifically oxidizes β-D-glucose into glucono-δ-lactone and hydrogen peroxide. Further, glucono-δ-lactone can be hydrolyzed spontaneously or by the enzyme lactonase into GA, which can be used as a carbon source and metabolized by the pentose phosphate pathway [23,31].

Since GOx uses molecular oxygen as the direct electron acceptor, dissolved oxygen in the culture medium is crucial in the GA biosynthesis pathway [32–34], particularly in viscous media. Thus, the effect of an agitation rate of 400 and 600 rotations per minute (rpm) on GA production from SM in a stirred tank bioreactor was also evaluated. The feasibility of using SM as an alternative and sustainable raw material to obtain high GA titer is of great interest for the industrial production of GA and SM valorization under a biorefinery and circular bioeconomy perspective.

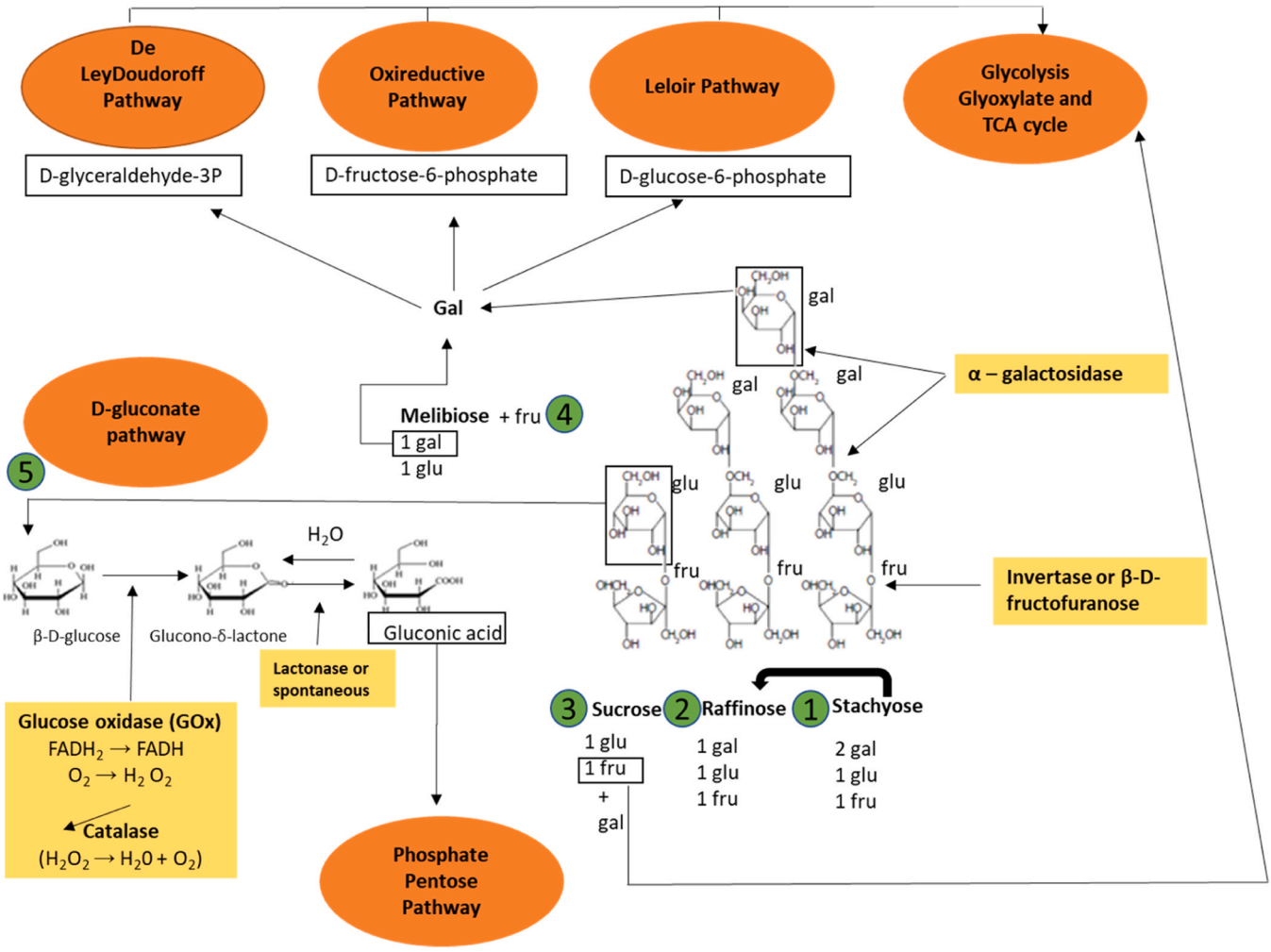

Fig. 1. Sugars and gluconic acid metabolic networks of *A. niger*. The names of the sugar metabolic pathways are shown in orange circles and enzymes are represented in yellow squares. Part of the Leloir pathway, the oxidoreductive pathway, and the non-phosphorylated De LeyDoudoroff pathway in *A. niger* are represented (glu: glucose; fru: fructose; gal: galactose). The Leloir pathway converts D-galactose into D-glucose-6-phosphate, which subsequently enters glycolysis, and then TCA cycle or glyoxylate cycles. The oxido-reductive pathway converts D-galactose into D-fructose-6-phosphate, which posteriorly can also enter glycolysis. In the non-phosphorylated De LeyDoudoroff pathway, galactose is converted into D-glyceraldehyde-3 P.

2. Materials and methods

2.1. Microorganism

Aspergillus niger MUM 92.13 was supplied by Micoteca of Universidade do Minho (MUM, Braga, Portugal) and used for the inoculation of soybean molasses media. The fungus was grown on Potato Dextrose Agar (PDA) Petri dishes for 5 days at 30 °C and stored at 4 °C for a maximum of 1 month.

2.2. Spores suspension and pre-inoculum preparation

Firstly, a spore suspension of *A. niger* MUM 92.13 was prepared by adding a sterile peptone solution (1 $g{\cdot}L^{-1}$ peptone and 0.1 $g{\cdot}L^{-1}$ Tween 80) to 5-days-grown culture on PDA Petri dishes. The suspension was used to inoculate 62.5 mL of preculture medium (25 $g{\cdot}L^{-1}$ glucose, 3 $g{\cdot}L^{-1}$ yeast extract, 3 $g{\cdot}L^{-1}$ malt extract, and 5 $g{\cdot}L^{-1}$ peptone), in a 250-mL Erlenmeyer flask, at an initial spores concentration of $2{\times}10^6$ $spores{\cdot}mL^{-1}$. After 24 h of incubation at 28 °C and 200 rpm in an orbital shaker, the preculture was used to inoculate soybean molasses medium in the stirred tank bioreactor.

2.3. Soybean molasses (SM)

Two SM samples were kindly supplied by two different soybean processing companies from two different countries: SM-1 (Brazil) and SM-2 (Finland). The samples were kept in 1-L polyethylene flasks and frozen at - 20 ºC until use.

2.4. GA production from SM

A 2-L lab-scale stirred tank bioreactor (STR) (BIOLAB, B. Braun, Germany) filled with 0.5 L of SM-1 or SM-2-based media was used to evaluate the GA production by *A. niger* MUM 92.13. As the free glucose concentrations of both SM were very distinct, different dilution factors in both SM were performed to evaluate GA production from the same initial free glucose concentrations. SM-1 was diluted 1:10 and 1:4.6, resulting in the final concentrations of 100 $g{\cdot}L^{-1}$ (46 $g{\cdot}L^{-1}$ total sugars, 14 $g{\cdot}L^{-1}$ glucose) and 217 $g{\cdot}L^{-1}$ (100 $g{\cdot}L^{-1}$ total sugars, 28 $g{\cdot}L^{-1}$ glucose) of SM-1 in the medium. SM-2 was diluted 1:3.5 and 1:1.8, whereby the concentrations 290 $g{\cdot}L^{-1}$ (150 $g{\cdot}L^{-1}$ total sugars, 14 $g{\cdot}L^{-1}$ glucose) and 560 $g{\cdot}L^{-1}$ (290 $g{\cdot}L^{-1}$ total sugars, 28 $g{\cdot}L^{-1}$ glucose) of SM-2 were reached in the medium. Both SM media were also composed of 3.5 $g{\cdot}L^{-1}$ $CaCO_3$ and 1 % (v/v) of silicone oil as an antifoam agent. The bioreactor with the medium was sterilized in an autoclave at 115 °C for 30 min before the inoculation. The batch experiments were conducted at 28 ºC for 72 h at 400 rpm (rotation per minute) of agitation rate and 1 vvm (volume of air per volume of liquid per minute) of aeration rate. The GA production under 600 rpm of agitation rate and 1 vvm of aeration rate was also investigated in experiments with more concentrated SM (217 $g{\cdot}L^{-1}$ for SM-1 and 560 $g{\cdot}L^{-1}$ for SM-2). The medium pH was measured with a probe (405-DPAS-SC-K8S/325, Metler Toledo, Urdorf, Switzerland) and kept constant at 6.5 ± 0.5 by the addition of NaOH 3 M through peristaltic pumps. A polarographic oxygen probe (InPro6820/12/320 T-type, Metler Toledo, Greisensee, Switzerland) and the respective meter (type 170) were used to measure the dissolved oxygen concentration in the medium.

2.5. Estimation of k_La

The k_La (volumetric mass transfer coefficient) values were measured using the static gassing-out technique, as mentioned in Wise [35] and Stanbury and Whitaker [36], before starting the experiments in the STR bioreactor at 400 rpm and 600 rpm with SM media without cells.

2.6. Analytical methods

Culture samples were collected twice per day to measure GA and sugars. After centrifugation of the culture samples at 9000 rpm for 10 min, the supernatant was separated from mycelium and stored at -20 ºC until the analysis.

Determination of sugars and organic acids by high-performance liquid chromatography (HPLC). An HPLC with an Aminex HPX-87 H column (300 mm×7.8 mm, 8 μm particle size) at 25 ºC coupled with UV detector (210 nm) and refractive index (RI) detectors was used to measure GA, glucose, and sucrose of fermentation samples and both molasses. The mobile phase was sulfuric acid 0.1 M at 0.5 $mL{\cdot}min^{-1}$ of flow rate. The other sugars - galactose, fructose, stachyose, and raffinose - were quantified in both molasses samples as well as at the beginning and the end of the experiments by HPLC equipped with a RI detector, an Asahipak NH2P-50 G 4 A, 4.6 mm × 10 mm (Shodex) pre-column, and an Asahipak NH_2P-50 4E column, 4.6 × 250 mm, 5 μm particle size (Shodex) at 30 °C. The mobile phase was a mixture of acetonitrile in pure water (70:30, v/v), containing 0.04 % ammonium hydroxide in water at a flow rate of 1 $mL{\cdot}min^{-1}$.

Quantification of total phenolic compounds. Total phenolic compounds were determined by the Folin–Ciocalteau method (Commission Regulation (EEC) No.2676/90), and the calibration curve was constructed with gallic acid as standard. The results of total phenolic compounds (TPC) are expressed in g gallic acid equivalents (GAE) per L.

Determination of total nitrogen, protein, and ashes of SM samples. Total nitrogen concentration was measured spectrophotometrically using the commercial kit LCK 338 (Hach-Lange GmbH, Germany). The crude protein was calculated by multiplying the total nitrogen by a defined factor of 6.25. Ashes were determined by high-temperature treatment at 550 °C for 2 h in a muffle.

Quantification of total lipids by gravimetric method. The mixture (0.4 g of SM and 10 mL of chloroform) was exposed to ultrasound waves in an ultrasonic bath for 2 h to maximize the lipids extraction. Then, the chloroform was recovered by pressing with a syringe and evaporated using nitrogen injection, and total lipids were quantified by weight difference [37].

Determination of minerals by ICP-OES. An inductively coupled plasma with optical emission spectrometry (ICP-OES) (Optima 8000, PerkinElmer) was used to quantify minerals in diluted samples of SM. The samples were previously filtered (pore size of 0.22 μm) and some drops of HNO_3 69 % (v/v) were added to maintain the pKa value. A radio frequency power of 1400 W, 12 $L{\cdot}min^{-1}$ of argon plasma flow, 0.2 $L{\cdot}min^{-1}$ of auxiliary gas flow, and 0.75 $L{\cdot}min^{-1}$ of nebulizer gas flow were the operating conditions used. The plasma view was axial for all analyzed minerals. The wavelengths (nm) evaluated were: K - 766.490, Mg - 280.271, Na - 588.995, and P - 213.617. The calibration curves were constructed with standard elements in ($mg{\cdot}L^{-1}$).

2.7. Statistical analysis

One-way analysis of variance (ANOVA), coupled with Tukey's post hoc test, was carried out for the statistical analysis of the data and to evaluate the statistical differences among means ($p < 0.05$) using the IBM SPSS Statistics 27 software.

3. Results and discussion

In the current study, SM from different suppliers (SM-1 and SM-2) were used as a source of fermentable sugars for GA production by *A. niger* MUM 92.13 in a stirred tank bioreactor. The composition of the two SM samples is significantly different, mainly in the sugar profile (Table 1).

The content of crude protein, fat, and ashes of both molasses is in line with the values described in the literature. The highest ashes (soluble inorganic salts) and fat content were observed in SM-2. Miranda et al.

Table 1
Composition of soybean molasses (SM-1 and SM-2) and data reported by other authors. Experimental data are the average ± standard deviation of three independent analyses. Values followed by the same letter in each row do not present statistically significant differences ($p \geq 0.05$).

	This study		Rakita et al. [5]	Rodrigues et al. [3]	Rodrigues et al. [12]	Cheng et al. [4]	Acosta et al. [38]
Composition (%, w/w)	SM-1	SM-2					
Crude protein	4.8±0.4[a]	4.0±0.3[a]	5–12	5.0	6.4	2	7.6
Fat	4.5±0.7[a]	11.5±1.5[b]	4–20	5	15.6	21.2	6
Ashes	14.5±0.7[a]	18.5±1.2[b]	3–21.9	15	7.9	_	11.2
Total sugars	46.3±2.9[a]	52.2±0.4[b]	58–65	40	60.8	26.8	31
Stachyose	13.8±0.6[a]	17.5±3.5[a]	15.5–34.2	11.9	15.5	10.53	11.4
Raffinose	5.8±0.3[a]	4.9±1.5[a]	4–25.5	5.0	11.7	_	9.6
Sucrose	9.6±0.5[a]	23.1±4.2[b]	18.5–32	19.9	26.0	14.62	7.5
Fructose	4.2±0.4[a]	1.6±0.2[b]	1.2–3.0	2.6	_	<1.19	0.2
Glucose	13.0±1.2[a]	5.13±0.05[b]	0.2–4.7	0.6	_	0.46	1.3
Galactose	n.d.	n.d.	_	4.64	_	_	1.0
Minerals ($mg \cdot L^{-1}$)							
P	0.45±0.04[a]	0.23±0.02[b]	0.58	4.2	_	_	_
Na	0.065±0.35[a]	0.051±0.006[a]	0.26	0.4	_	_	_
Mg	0.15±0.03[a]	0.146±0.003[a]	0.7	1.3	_	_	_
K	3.54±0.41[a]	2.07±0.01[b]	7.2	_	_	_	_
TPC ($gGAE \cdot L^{-1}$)	10.24±0.23[a]	7.42±0.23[b]	_	_	_	_	0.36

n.d.: not detected; TPC: total phenolic concentration

[18] found that SM had 6.25 $g \cdot L^{-1}$ of total phenolics, which is a similar concentration to that observed in SM-2 and lower than SM-1. In general, both SM have lower content of minerals in comparison to the values found by other authors [3,5]. Potassium is the main mineral of SM, and residual amounts of phosphorus, sodium, and magnesium were also detected.

SM-2 is composed of 52 % (w/w) of total sugars (with sucrose and stachyose the most abundant), and SM-1 contains approximately 46 % (w/w) of total sugars (mainly stachyose, sucrose, and glucose). The content of total sugars of SM reported by other authors ranged from 27 % (w/w) to 65 % (w/w) (Table 1), which is in line with the composition of molasses used in the current study. The content of oligosaccharides – RFO (stachyose and raffinose) of the two SM are similar to the values reported by Rakita et al. [5], and Rodrigues et al. [12]. SM-1 contains about 3-fold more free glucose and fructose than SM-2, and the amount of these sugars is higher than those reported by Rakita et al. [5] and Rodrigues et al.[3]. In addition, SM-1 contains around 60 % less sucrose than SM-2, which is relatively lower than most studies in Table 1.

In a medium with 100 $g \cdot L^{-1}$ of SM-1 (equivalent to 46 $g \cdot L^{-1}$ of total sugars and 14 $g \cdot L^{-1}$ of free glucose), *A. niger* was able to consume the glucose and sucrose after 12 h and 24 h, respectively (Fig. 2 A). GA titer reached a plateau at 24 h of cultivation. Although the initial glucose was consumed after 12 h, GA continued to be synthesized from units of glucose released from the hydrolysis of sucrose and RFO. This finding is consistent with the fact that *A. niger* has extracellular enzymes which break down RFO [27] and sucrose [39]. A 2.2-fold increase of SM-1 concentration (217 $g \cdot L^{-1}$), equivalent to 100 $g \cdot L^{-1}$ of total sugars and 28 $g \cdot L^{-1}$ of initial glucose, doubled the GA production (Fig. 2 A). Thus, there was no inhibitory effect of SM-1 concentration even containing considerable amounts of phenolic compounds (Table 1), in contrast to what was observed in the GA fermentation using corn stover hydrolysate as raw material [40].

In the experiments with more concentrated SM-1, glucose and sucrose were consumed after 24 h and 32 h, respectively, independently of the agitation rate. As expected, in the first 24 h, during the exponential phase of GA production, dissolved oxygen concentration (DO) decreased due to its consumption for cellular growth and the catalytic reaction of glucose oxidase (GOx) (Fig. 2B). DO decreasing rate was lowest for the less concentrated SM-1 medium, and DO dropped to zero only in the experiment with more concentrated SM-1 at 400 rpm but without significant impact on GA production. In the other experiment with the same SM-1 concentration but at 600 rpm, DO was always above 30 % of saturation (Fig. 2B). For the same conditions of agitation rate, the

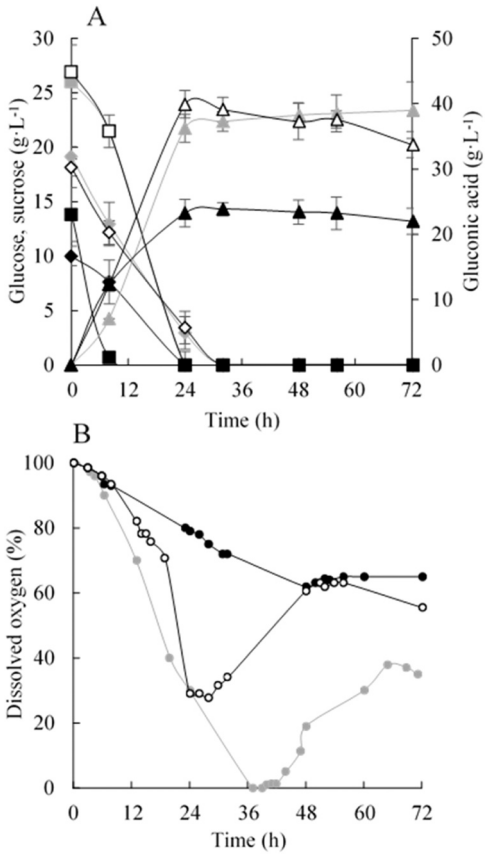

Fig. 2. Time course of glucose (■,■,□), sucrose (◆,◆,◇), and GA(▲,▲,Δ) concentration (A), and dissolved oxygen (% of saturation) (●,●,○) (B) obtained in batch cultures of *A. niger* MUM 92.13 using 100 $g \cdot L^{-1}$ SM-1 (black) and 217 $g \cdot L^{-1}$ SM-1 (gray: 400 rpm; white: 600 rpm). The error bars represent the standard deviation of two independent experiments.

increase of molasses concentration decreases the value of k_La at 400 rpm (Table 2), probably due to the high viscosity of molasses (9000–14000 cP), as reported by Rodrigues et al. [3]. Thus, to compensate for this effect, the increase in agitation rate is of most importance. Nevertheless, for the range of k_La values used in this work, only a slight increase of 10 % in GA productivity was observed by

Table 2
Experimental values of k_La, GA productivity (P_{GA}), and GA yield ($Y_{GA/s}$) obtained in batch cultures of *A. niger* MUM 92.13 performed in STR bioreactor under 400 rpm and 600 rpm of agitation rate with SM-1 and SM-2 media. Data are average ± standard deviation of two independent experiments. For each SM sample, values followed by the same letter in each row do not present statistically significant differences ($p \geq 0.05$).

Medium	SM-1			SM-2		
Molasses ($g{\cdot}L^{-1}$)	100	217	217	290	560	560
Total sugars ($g{\cdot}L^{-1}$)	46	100	100	150	290	290
Initial glucose ($g{\cdot}L^{-1}$)	14	28	28	14	28	28
Stirring rate (rpm)	400	400	600	400	400	600
k_La (h^{-1})	35±1[a]	22±1[b]	30±5[a,b]	18±1[a]	14±5[a]	23±2[a]
P_{GA} ($g{\cdot}L^{-1}{\cdot}h^{-1}$)	1.01±0.06[a]	1.51±0.09[b]	1.66±0.09[b]	0.55±0.02[a]	0.85±0.05[b]	1.41±0.02[c]
$Y_{GA/s}$ ($g{\cdot}g^{-1}$)	1.04±0.08[a]	0.90±0.03[a]	0.87±0.08[a]	0.53±0.01[a]	0.54±0.04[a]	0.71±0.005[b]

P_{GA} was calculated at 24 h and 56 h in the SM-1 and SM-2 experiments, respectively; $Y_{GA/s}$ was calculated considering the initial free glucose and glucose released from sucrose, raffinose, and stachyose.

increasing the agitation rate from 400 rpm to 600 rpm, which is not statistically significant ($p > 0.05$). The experiments with SM-1 demonstrated that this molasses could be used for GA production, being GA productivity improved by 50 % - 70 % by increasing molasses concentration without significant change of GA yields (GA produced considering the consumption of initial free glucose and glucose provided by hydrolysis of sucrose and RFO) (Table 2). Regardless of the agitation rate used, GA yields achieved from SM-1 were as promising as those obtained from sugar beet molasses [39], sugarcane molasses [33], banana must [41], corn stover [42], grape must [33], breadfruit hydrolysate [43], and pure glucose [33,44].

In addition to glucose, from hydrolysis of RFO are obtained monomers of galactose and fructose and from sucrose are also released units of fructose. A previous study reported that *A. niger* MUM 92.13 (the same strain of *A. niger* used in this work) started to consume fructose when glucose was scarce or no longer available, synthesizing oxalic acid [33]. This organic acid can also be obtained from galactose, as observed by other authors [45] via glycolysis and the TCA cycle. In the current study, the fungus also produced oxalic acid, reaching concentrations between 11 $g{\cdot}L^{-1}$ and 26 $g{\cdot}L^{-1}$ at the end of the batch experiments with SM-1 (Table 3). Only around 5 $g{\cdot}L^{-1}$ of fructose was not consumed in the SM-1 medium performed with 217 $g{\cdot}L^{-1}$ of SM-1 at 400 rpm (Table 3). No RFO and galactose were found at the end of the batch cultures performed with SM-1. It should be noted that from 48 h, *A. niger* started to use GA as a carbon source (Fig. 2 A) via the pentose phosphate pathway [46] since all sugars were consumed.

Table 3
Values of oxalic acid, stachyose, raffinose, fructose, and galactose obtained at the end of the bioreactor experiments for each condition and SM tested. Data are average ± standard deviation of two independent experiments. For each SM sample, values followed by the same letter in each row do not present statistically significant differences ($p \geq 0.05$).

Medium	SM-1				SM-2	
Molasses ($g{\cdot}L^{-1}$)	100	217	217	290	560	560
Total sugars ($g{\cdot}L^{-1}$)	46	100	100	150	290	290
Initial glucose ($g{\cdot}L^{-1}$)	14	28	28	14	28	28
Stirring rate (rpm)	400	400	600	400	400	600
Stachyose ($g{\cdot}L^{-1}$)	n.d.	n.d.	n.d.	n.d.	14±3[a]	14±3[a]
Raffinose ($g{\cdot}L^{-1}$)	n.d.	n.d.	n.d.	n.d.	5±1[a]	7.3 ±0.4[a]
Fructose ($g{\cdot}L^{-1}$)	n.d.	4.8 ±0.3	n.d.	47 ±12[a]	97±1[b]	68±1[a]
Galactose ($g{\cdot}L^{-1}$)	n.d.	n.d.	n.d.	13±1[a]	19±1[b]	n.d.
Oxalic acid ($g{\cdot}L^{-1}$)	10.8 ±0.5[a]	18.2 ±0.5[b]	26.3 ±0.4[c]	10.1 ±0.9[a]	7.3 ±0.6[a]	21.3 ±0.3[b]

n.d.: not detected

As previously noted, SM-2 has a reduced concentration of free glucose compared to SM-1. Thus, to achieve similar values of initial free glucose as those attained in experiments with SM-1 (around 14 $g{\cdot}L^{-1}$ and 28 $g{\cdot}L^{-1}$), a higher SM-2 concentration was used than with SM-1 (Table 2 and Fig. 3 A). As a result, SM-2 medium contained about 6 times and 3 times more sucrose and RFO, respectively, than SM-1 medium.

Contrariwise to SM-1, GA production from SM-2 at 400 rpm continuously increased through operation time since there was glucose in the medium. Probably, the rate of enzymatic hydrolysis of sucrose and RFO into monomers of glucose was higher than the bioconversion of glucose into GA, which led to the accumulation of this sugar in the medium in the first 24 h (Fig. 3 A).

At 400 rpm, glucose slightly decreased from 24 h, and 8 $g{\cdot}L^{-1}$ and 14 $g{\cdot}L^{-1}$ of glucose were not consumed in the media with 290 $g{\cdot}L^{-1}$

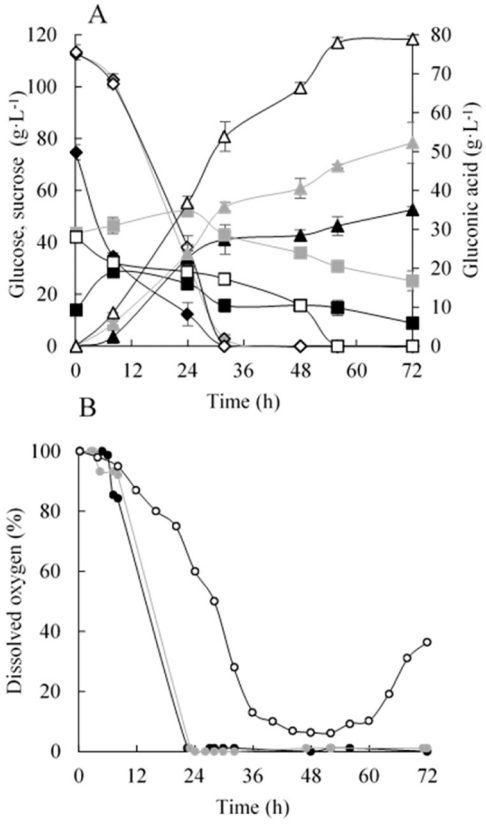

Fig. 3. Time course of the concentration of glucose (■,■,□), sucrose (◆,◆,◇), and GA (▲,▲,△) concentration (A) and dissolved oxygen (% of saturation) (●,●,○) (B) obtained in batch cultures of *A. niger* MUM 92.13 using 290 $g{\cdot}L^{-1}$ SM-2 (black) and 560 $g{\cdot}L^{-1}$ SM-2 (gray: 400 rpm; white: 600 rpm). The error bars represent the standard deviation of two independent experiments.

(equivalent to 150 g·L^{-1} of total sugars) and 560 g·L^{-1} (equivalent to 290 g·L^{-1} of total sugars) of SM-2, respectively (Fig. 3 A). Sucrose was completely hydrolyzed after 32 h in all experiments (Fig. 3 A). At 400 rpm, a 2-fold increase in SM-2 concentration improved GA final titer by 50 % (Fig. 3 A) instead of duplicating as observed with SM-1 (Fig. 2 A), even though similar GA yields were attained (Table 2). The highest GA titer of 79 g·L^{-1} was reached with the highest SM-2 concentration (2.3-fold higher than that obtained with less concentrated SM-2) at an agitation rate of 600 rpm. In this case, glucose decreased in the first 8 h, remained practically constant until 32 h, and was completely consumed after 56 h (Fig. 3 A). Similar to what was found with SM-1, increasing SM-2 concentration led to an improvement in GA productivity. However, contrarily to SM-1, a 1.6-fold enhancement in GA productivity was obtained by increasing the agitation rate from 400 rpm to 600 rpm. This agitation rise prevented total oxygen depletion from the medium (Fig. 3B), which was observed in the other conditions. These observations may be explained by the k_La values in the SM-2 medium that were slightly lower than those observed with SM-1, probably owing to SM-2's high viscosity, high concentration, and high level of fat (Table 1) that affects the oxygen transfer from air to the liquid phase [47]. In a previous study, the use of sugarcane molasses in the production of 2,3-butanediol led to a significant decrease in oxygen mass transfer rate compared with that achieved in the glucose medium [48]. In addition, in the work of Fernandes et al. [32], 42 h^{-1} of k_La value was obtained using 400 rpm of agitation rate and 1 vvm of aeration rate in the same bioreactor to produce GA from pure glucose by *A. pullulans* NCYC 4012. Thus, using molasses as a raw material in bioprocesses led to a decrease in OTR, which may explain the low values of k_La obtained in this work (Table 2).

Previous studies have demonstrated that several strategies to enhance the oxygen availability in the medium, namely increasing agitation and/or aeration rates [32,39,43], or using pure oxygen [44,49,50] or increased air pressure [33] have a positive impact on submerged fermentation of GA production. SM-2 can deliver about 3 times more glucose than SM-1 (taking into account the monomers of glucose obtained from the hydrolysis of sucrose and RFO), which resulted in higher oxygen requirements for cell growth, as well as, biosynthesis of GA, leading to a subsequent lower DO concentration in the medium, as described by Qian et al. [51]. As can be seen in Fig. 3 B, at 400 rpm of agitation rate, DO dropped to zero at 24 h and remained in this value until the end of the process, whereas at 600 rpm, DO decreased from 36 h to 10 % and increased from 56 h to 36 %. The DO limitation in the medium may explain the lower yields obtained from SM-2 compared with those achieved from SM-1 (Table 2). Furthermore, the highest concentration of SM-2 could significantly increase osmotic pressure in the cells, as reported by Liu et al. [52]. The high osmolality decreases GA yield because cells first need to maintain the intercellular and external osmotic equilibrium, encouraging cell growth or ensuring the membrane integrity with compatible solutes and osmoprotectants, instead of GA biosynthesis [52]. Besides the highest sugar concentration, SM-2 has a double ashes content than SM-1 (Table 1), which may affect the biosynthesis of GA and other organic acids by *A. niger* [53,54]. Cheng et al. [4] observed that the use of untreated SM resulted in low polymalic acid production. The pretreatment of SM with H_2SO_4 and $Ca(OH)_2$ was advantageous to remove 20 % of its viscous substances (e.g., soybean oils, proteins, and cell debris), which can hinder oxygen transfer to the cells and consequently decrease polymalic acid yield. Sophorolipid production by *Candida bombicola* from high-sugar concentrated SM was lower than that from pure glucose due to the high SM viscosity, which hindered the OTR gaseous to the culture medium [55]. Therefore, the absence of pretreatment in SM-2 and the highest concentration of SM-2 could also explain the lower GA yields achieved when compared to those attained from SM-1. Moreover, the highest fat content in SM-2 could lead to a metabolism deviation for the production of other organic acids, namely citric acid by *Aspergillus niger* [56]. Pretreatments and clarification have been used in other cheaper glucose sources to produce GA. Purification of grape and banana must [57] and several pretreatments applied to sugarcane molasses to reduce trace metals led to improved GA production by *A. niger* [53,57]. In a previous study, the use of HCF-treated sugar-cane molasses led to a 3.43-fold increase in GOx levels (2.62 U·mL^{-1}) when compared to that obtained with crude form [58]. Nevertheless, using cheaper raw materials without any purification and pretreatments is cost-effective since these procedures increase the overall costs of GA production. It should be highlighted that the highest titer of GA obtained from SM-2 was similar to the results reported by other authors using pretreated corn stover (76.67 g·L^{-1}) [40] and sugarcane molasses (76.3 g·L^{-1}) [59], and higher compared to GA reached from purified grape must (54.6 g·L^{-1}), banana must (61.8 g·L^{-1}), and clarified sugarcane molasses (69.8 g·L^{-1}) [57]. Moreover, in most of these studies, engineering strains of *A. niger* were used.

In 560 g·L^{-1} SM-2 experiments, approximately 6 g·L^{-1} raffinose and 14 g·L^{-1} stachyose remained in the medium at the end of time cultivation, regardless of agitation rate (Table 3). Though there was RFO in the medium, GA production at 600 rpm stabilized from 56 h, coinciding with the depletion of free glucose (Fig. 3 A). At 600 rpm, 68 g·L^{-1} of fructose was quantified in the medium, whereas at 400 rpm, galactose (19 g·L^{-1}) and a considerable amount of fructose (97 g·L^{-1}) remained unconsumed at the end of cultivation (Table 3). In the experiments carried out with more diluted SM-2, 47 g·L^{-1} of fructose and 13 g·L^{-1} of galactose lasted in the medium after 72 h. No raffinose and stachyose were detected in this condition. Previous studies have reported that the isolated galactose is a poor carbon source for *A. niger* cultures and its catabolism is affected by the presence of other sugars [60–64]. The Leloir pathway is the main route of fungi to assimilate galactose [30] and some authors reported that the biosynthesis of Leloir proteins requires high energy [65] and dissolved oxygen [66] to enable efficient utilization of galactose by the cells. This fact could explain the highest consumption of galactose in SM-2 medium at 600 rpm. Considering the concentration of fructose and galactose that remained in the SM-2 medium, galactose was consumed faster than fructose by *A. niger*. Thus, fructose was the less preferred sugar by *A. niger* MUM 92.13. It should be noted that the assimilation of different types of sugars (RFO, sucrose, and monosaccharides glucose, galactose, and fructose) from SM-2 by *A. niger* is in line with previous studies. Lameiras et al. [67] observed that *A. niger* assimilated sugars sequentially rather than simultaneously in a medium composed of a mixture of sugars. The authors explained that the carbon catabolite repressor protein CreA ensures that the presence of a preferred carbon source (e.g., glucose) prevents the expression of genes involved in the utilization of less preferred sugars, being this mechanism dependent on medium pH. In another study, *A. niger* CreA mutant also consumed part of the sugars sequentially during cultivation, similar to *A. niger* wild-type [68]. Thus, sugar catabolism and its regulatory mechanisms in *A. niger* are not consensual in the literature.

The production of oxalic acid was also observed from SM-2, ranging from 10 g·L^{-1} to 21 g·L^{-1} at the end of the experiments (Table 3). Though there were no significant differences in k_La between the two agitation rates, considering the media with 560 g·L^{-1} of SM-2, high agitation led to approximately 4-fold and 2-fold enhancement of fructose and galactose consumption and, consequently, a high titer of oxalic acid was obtained. The non-limiting oxygen conditions at 600 rpm (Fig. 3B) may also contribute to enhancing oxalic acid synthesis. As was already been mentioned, in the experiment with SM-1 under a high agitation rate all sugars were consumed and more 8 g·L^{-1} of oxalic acid was obtained when compared to the concentration found in the experiments performed at 400 rpm. A previous study observed that in highly aerated cultures of *A. niger*, carried out at 4 bar of total air pressure, fructose consumption (after depletion of glucose) from grape must was faster, and a higher accumulation of oxalic acid was attained compared to the results obtained at atmospheric pressure [33]. By contrast, Lu et al. [69] verified that *A. niger* under oxygen limitation increases the

biosynthesis of oxalic acid. According to these authors, the intracellular metabolic response of *A. niger* to oxygen limitation is still unclear.

From batch cultures with SM-2, unconsumed fructose can be recovered as a high-fructose syrup, which can be used as a sweetener in food and medicine formulations [70]. Fructose and galactose have the potential as substrates for microbial lipids accumulation [17]. Hence, the use of SM-2 as feedstock can provide three added-value compounds (GA, oxalic acid, and high-fructose syrup). The purification and concentration of GA obtained from two SM media can be performed using a nanofiltration membrane system, as demonstrated by Pal et al. [71] in the GA production from sugarcane juice by *Glucanobacter oxydans*. This system allows for the removal of cells and unconsumed sugars from the culture broth. In the SM media, the unconsumed fructose can be separated from other sugars using functionalized polyamide nanofiltration membranes [72] or membrane modification techniques, such as LbL and plasma treatment [73]. Though in a biorefinery it is desirable a selective production of added-value chemicals, the recovery and purification of oxalic acid and GA from broth can be performed by electrodialysis with bipolar membranes processes combined with monovalent anion-selective membranes as described by Wang et al. [74].

This work proved, for the first time, efficient utilization of two SM samples without prior enzymatic or acid pretreatments for GA production by *A. niger*. Moreover, high-fructose syrup and oxalic acid were also added-value co-products obtained from SM.

4. Conclusion

Soybean molasses (SM) is an alternative source of glucose for gluconic acid (GA) production by *Aspergillus niger*. However, the GA production depends on SM composition, as was shown in this work where the highest yields of mass GA obtained per glucose consumed were attained from SM with the highest ratio of free glucose to sucrose concentration. However, the highest GA titer (79 $g{\cdot}L^{-1}$) was reached in the SM medium with less free glucose but the highest total sugar concentration, despite the lowest yield. High sucrose and oligosaccharides present in SM are sources of glucose that the fungus can use to convert to GA. From the SM hydrolysis by the fungus and glucose conversion, a high-fructose syrup can be obtained. Oxygen and SM composition were important factors in controlling the metabolism shift from GA to other organic acids, such as oxalic acid. GA at interesting titers for industrial production was obtained, creating a new route of SM valorization under a biorefinery and circular bioeconomy perspective.

CRediT authorship contribution statement

Isabel Belo: Writing – review & editing, Validation, Supervision, Conceptualization. **Bruna Dias:** Methodology, Investigation. **Sílvia Fernandes:** Writing – original draft, Methodology, Investigation, Formal analysis. **Marlene Lopes:** Writing – review & editing, Validation, Supervision, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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