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Application of apple cider residue on citric acid bioprocesses using *Aspergillus niger* under solid state fermentation

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Abstract

Apple pomace (AP) is considered among the most damaging agro-industrial wastes to the environment as well as the economy. Therefore, lucrative alternatives have been studied to render this low-value waste into citric acid (CA), which is a high-value product that can be used in several fields such as pharmaceutical, cosmetics, agriculture and biochemical industries. Recently, apple cider residue (ACR) has also become an environmental hazard which, contrary to AP, has not yet been studied for its potential application in industrial bioprocesses. Within this context, the present study was carried out to evaluate the potential of ACR to produce CA through solid state fermentation (SSF) by different *Aspergillus niger* strains. For this, several natural *A. niger* strains and several variations of the fermentation substrates (AP, ACR, mixtures of AP+ACR, supplemented and not supplemented) were tested.

Among the tested strains, MUM 92.13 proved to be the best strain for CA production using AP (41.61 ± 1.14 g/kg), while with ACR the production of CA was lower (13.99 ± 0.09 g/kg). On the other hand, high glycerol production (46 ±0.89 g/kg) and yield (0.92 ±0.08 g/g total sugars) was obtained with ACR when supplements were not added. When both matrices were mixed (75%AP+25%ACR and 50%AP+50%ACR), CA yield of supplemented 75% AP+25%ACR was similar to that obtained with AP, but with lower production (24.81 ±1.24 g/kg). Higher glycerol values of 54.81 ± 0.44 g/kg were achieved with non-supplemented 50%AP+50%ACR. Non supplemented ACR achieved a promising glycerol yield of 2.19 ±0.49 g/g total sugars.

ACR thus proved to be a better substrate for glycerol production than for CA, when compared with AP, and can serve as a natural substrate for SSF, which is an economical alternative technique and does not require sophisticated instruments and techniques.

Keywords: Glycerol, apple pomace, agro-industrial wastes, biotechnology, circular economy.

Resumo

O bagaço de maçã (BM) é um dos resíduos agro-industriais mais prejudiciais para o ambiente, assim como para a economia, uma vez que o seu descarte é geralmente feito por descarga em aterro ou vendido como alimento para animais. Desta forma, alternativas lucrativas têm vindo a ser estudadas para transformar este resíduo em produtos de alto valor, nomeadamente ácido cítrico (AC). Paralelamente ao BM, o resíduo da produção de sidra (RS) tem também vindo a tornar-se um problema ambiental grave, mas ainda não há estudos sobre a sua potencial aplicação em bioprocessos industriais. Neste contexto, o presente estudo foi desenvolvido com o objetivo de avaliar o potencial do RS para a produção de CA através de fermentação por *Aspergillus niger*. Para tal, foram avaliadas várias estirpes naturais de *A. niger* e vários substratos de fermentação (BM, RS, misturas de BM+RS, com e sem adição de suplementos).

Estre as estirpes testadas, a estirpe MUM 92.13 mostrou ser a mais eficiente na produção de AC usando BM (41.61 ± 1.14 g/kg), enquanto a produção de AC a partir de RS foi mais baixa (13.99 ± 0.09 g/kg). Por outro lado, foi observada uma elevada produção de glicerol (46 ±0.89 g/kg), com elevado rendimento (0.92 ±0.08 g/g açúcares totais) com a utilização de RS não suplementado. Quando as duas matrizes foram misturadas (75%BM+25%RS e 50%BM+50%RS), o rendimento de CA a partir de 75%BM+25%RS suplementado foi similar ao obtido a partir de BM, apesar de apresentar produção mais baixa (24.81 ±1.24 g/kg). Os valores de glicerol mais elevados (54.81 ± 0.44 g/kg) foram obtidos usando 50%BM+50%RS não suplementado. A fermentação de RS não suplementado resultou num rendimento de glicerol bastante promissor (2.19 ±0.49 g/g açúcares totais).

Em conclusão, o RS demonstrou ser um substrato mais adequado à produção de glicerol do que de AC, e melhor do que BM. RS pode ser usado como um substrato natural em fermentação sólida usando *A. niger*, que é um bioprocesso bastante económico que não requer equipamentos e técnicas complexos.

Palavras-chave: glicerol, bagaço de maçã, resíduos agro-industriais, biotecnologia, economia circular.

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I. Introduction

I.1. Framework

Fruit processing industries have shown an exceptional growth in the last decade. The constant optimization of their production lines, fruit quality and the variety of the products such as juice, nectars and cider, has positively impacted the economy (Yuan et al., 2017). However, fruit industries produce high amounts of liquid and solid wastes during manufacturing, which must be suitably treated before being discharged to the environment. The solid wastes, generally referred to as "pomace", make up 25-35% of the total production (Kumar et al., 2010), and are considered harmful to the environment, since their biodegradability produces a high amount of greenhouse gases (Gassara et al., 2011). These wastes demand treatments before being buried in soil to minimize the damage, and that becomes a burden on the industries (Gassara et al., 2011).

Apple industries have been struggling the most with discharging apple pomace (AP), which has incurred losses due to the treatment and transportation costs of dumping it in landfill sites. Therefore, alternatives have been studied in the last decade to reintroduce AP into the industrial production lines as a sole raw material that produces high value-added products (Dhillon et al., 2012). By using biotechnology as an interdisciplinary field, microorganisms have been used to convert waste into useful products. This has enabled to consider AP as a potential substrate in the production of citric acid (CA) (Dhillon et al., 2011a; Technavio., 2019).

CA, a natural metabolic intermediate in the Krebs cycle, is generally recognized as safe (GRAS), non-toxic and biodegradable. Due to these properties, it is widely used in food, dairy, pharmaceutical, cosmetics, agriculture and biochemical industries (Dhillon et al., 2013b). The steady increase of worldwide attention and demand of this environmentally friendly molecule have squeezed this profitable market to produce 2 million Tons in 2018 (http://www.prnewswire.com, accessed 09/01/2021). The high production of CA induced an increase in its cost to become almost unprofitable if high energy and first-generation raw materials are used. Therefore, the use of AP or other less expensive agro-industrial by-products have become a trend that meets the increasing needs and exploit the AP discharge (Guardia et al., 2019), promptly responding to one of the main blocks of the European Green

Deal - the Circular Economy Action Plan (https://ec.europa.eu/environment/circular-economy/, accessed 03/02/2021).

On an industrial scale, AP can be used as a substrate for fungi, yeasts and a few bacteria for CA production, as reviewed by Angumeenal et al. (2012). So far, the fungus *Aspergillus niger*, in particular the industrial strain NRRL 567, has produced the highest CA yields on different by-products (cane molasses, banana peel, pineapple waste, corn husk), including AP (Amato et al., 2020). Submerged fermentation (SmF) is the most used method in the production of CA, and more than 80% of the industrial production lines use this as a well trusted process (Dhillon et al., 2011a; Soccol et al., 2017). In recent years, solid state fermentation (SSF) process has attracted worldwide attention as an alternative to SmF since it requires low water content, and the downstream processing is simplified and minimized, resulting in a low waste disposal (Dhillon et al., 2011b; Behera et al., 2020).

The state-of-the-art literature has shown the complexity of *A. niger*'s metabolic conditions for increased CA accumulation, and there is a clear need to optimize important process parameters depending on the substrate, on the type of strain, and on the type of process (Dhillon et al., 2011b, 2012). The literature shows a major gap in the evaluation of the potential use of apple cider (AC) residue as a competitive or complementary substrate for CA production.

I.2. Objectives

This work aimed to develop an optimized bioprocess of CA production using AP and AC residues as substrates, in line with circular economy objectives. The results obtained will be useful to create alternative low-cost production chains for CA production with the simultaneous reduction of the apple industry waste disposal.

For that, several tasks were put in place:

1. Physicochemical and nutritional characterization of the AP and AC wastes obtained from craft cider production;
2. Preliminary evaluation of CA production potential by several local *Aspergillus niger* strains;
3. Evaluation and optimization of the SSF process parameters that maximize the CA production yield by selected *A. niger* strains;
4. Screening for other potential beneficial compound that may appear as fermentation by-products.

II. Literature Review

II.1. Apple industry and agro-industrial wastes

II.1.1. Worldwide economic and environmental impacts

Apple production increased from 82 million tonnes (Mt) in 2016 to 130 Mt in 2019 on a worldwide scale, where 70% were freshly used, 5.8% were processed, 7% were wasted and 0.66% were used as feed (http://www.fao.org/faostat/en/#data/QC/, accessed 04/02/2020). China leads on a worldwide scale with an annual apple production of around 42 Mt, followed by the United States, with a production of 5 Mt, while Portugal produces around 356 000 t per year (http://www.fao.org/faostat/en/#data/QC/, accessed 04/02/2020). The processed apple loses nearly 25-35% of its initial weight as AP, which is considered as agro-industrial waste that cannot be reused to produce an edible, marketable product (Kumar et al., 2010). Studies indicate that apple handling businesses create thousands of tons of AP worldwide each year, as by-products of juice, puree and vinegar production. There is an estimate that 1.4 Mt of fresh AP were generated in 2016 on a worldwide scale (http://www.fao.org/faostat/en/#data/QC/, accessed 15/11/2020). In 2016 AP generation in Portugal was estimated at approximately 25 000 t, and still increasing progressively with the empowerment of the apple processing businesses in the world, that was handled for years through animal feed and fields compost (http://www.fao.org/faostat/en/#data/QC/ accessed 15/11/2020).

In the apple industry, AP from the juice and puree industries is not the only source of waste. Apple cider (AC) is one of the most successful apple-based products, with growing trends, driven by the rising demand for gluten-free and low alcohol beverages. The Global Cider Market was valued at 10 667 million US$ in 2016, and is projected to reach 16 252 million US$ by 2023 (https://www.alliedmarketresearch.com/cider-market, accessed 03/02/2021). In Portugal, ciders have been playing an important role, with great potential growth in the national market. In 2017 it was one of the top 3 FMCG (fast-moving consumer goods) categories in terms of growth (Consultant Nielsen Portugal., 2017), reaching 1.6 million cider consumers in 2018, a value that represents about 19.3% of the population (Marktest., 2019). With this market growth, several Portuguese brands of cider have been

created in the last decade, mostly in the last five years, most of them making use of craft production processes and regional apple varieties.

Figure 1 shows a general workflow of the apple puree, juice, cider and nectar industries, and the resulting waste production: apple pomace (AP), apple cider (AC) lees and apple pomace sludge (APS).

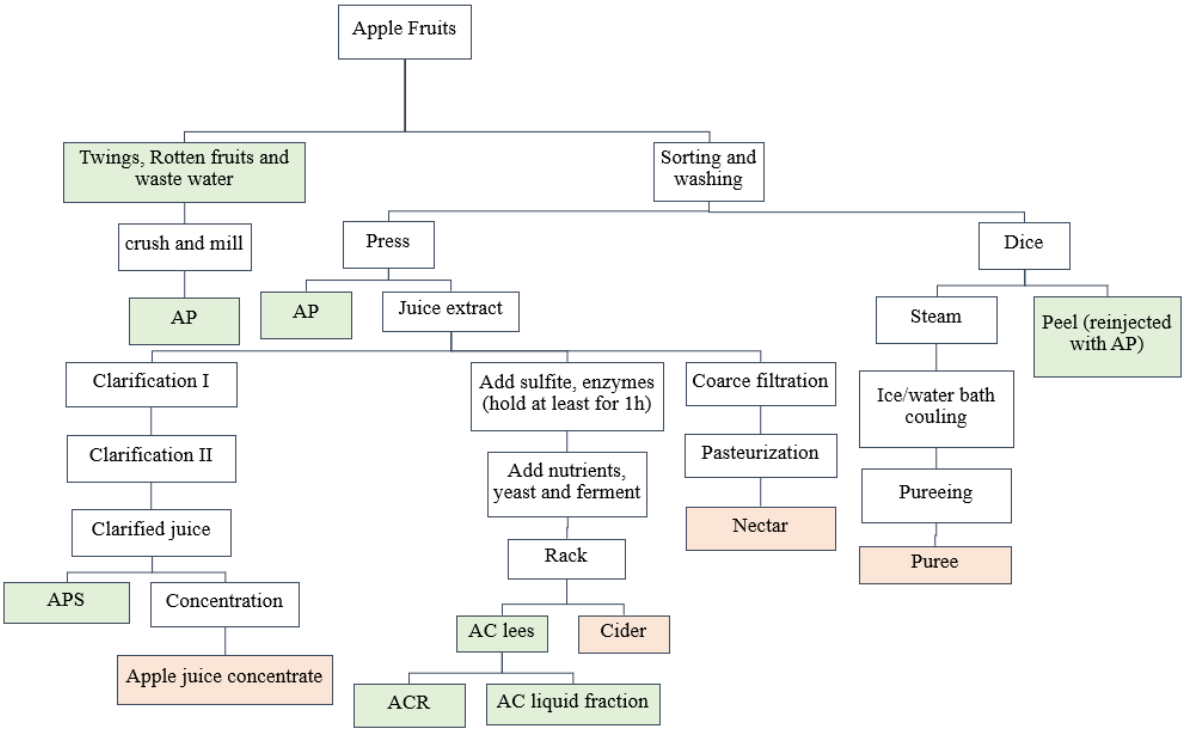

Figure 1. Workflow of the apple puree, juice, cider and nectar industries, and resulting waste production: apple pomace (AP), apple cider (AC) lees and apple pomace sludge (APS) (Source: Bhushanet al., 2008; Merwin et al., 2008; Patriziaet al., 2008)

Throughout the cider-making, several steps can create waste in the form of AP (pre-fermentation solid waste) and lees (post-fermentation residues composed of yeast cells which is the liquid fraction and other precipitate residue (ACR)) (Madrera et al., 2019; Pérez-Bibbins et al., 2015; Seluy et al., 2018). Fresh lees constitute 2–3% of the final cider product, and it is estimated that around 0.35 million hectoliters of lees are produced from this industry in Europe only (Madrera et al., 2019).

The high content of organic matter and the muddy nature of the apple waste make this residue especially difficult to handle and treat, and the proper environmental

management creates additional costs (Madrera et al., 2019; Pérez-Bibbins et al., 2015). Their accumulation in soil emanates an uncontrolled fermentation that releases an enormous quantity of greenhouse gases, equivalent to 1841 tons of CO_2 emission per year (reviewed by Gassara et al., 2011), caused by the high biochemical oxygen demand (BOD) and high chemical oxygen demand (300g COD/kg pomace) (Guardia et al., 2019). Not only the high biodegradability and low nutritional value have a negative impact in the environment (Gassara et al., 2011), but also the burdening cost of their treatment and transportation to be discharged in the environment causes economic disruption for apple processing industries (Hang et al., 1984). Therefore, finding suitable ways to recycle apple wastes has been gaining attention to cover these losses (Dhillon et al., 2013b).

II.1.2. Apple residues chemical and nutritional value

AP is the solid residue that remains after the milling and pressing of apples for cider, apple juice or puree production (Figure 2), constituting about 25–35% of the weight of fresh fruit. It contains peel, flesh, stem, core, seeds and juice residues, being thus considered as a heterogeneous mixture (Kumar et al., 2010). AP has been reported to contain in average 54% pulp, 34% peel, 7% seeds, 4% seed core and 2% stem (Kolodziejczyk et al., 2007). AP is rich in carbohydrates and fermentable sugars, but its protein content is low (Kumar et al., 2010). Its mineral content is diverse and is rich in Ca, Cu, Fe, K, Mg, Mn, Na, P, and Zn (Dhillon et al., 2012, 2013a).

Figure 2. Raw apple pomace (Source: Feedipedia; accessed 22/12/2020).

The lees from AC are a mixture a solid and liquid fraction precipitated in the fermentation tanks that can also include pips, fruit skins, grains and seeds. They are rich in microbial biomass (yeasts and bacteria), insoluble carbohydrates (cellulosic or hemi-cellulosic materials), phenolic compounds, lignin, proteins, metals, inorganic salts, organic acids and other materials (Perez-Bibbins et al., 2015). The lees also include a liquid fraction (fermentation broth) rich in ethanol and organic acids such as lactic, acetic, and tartaric acids (Perez-Bibbins et al., 2015). A detailed chemical and nutritional composition of AP and AC lees is shown in Table 1.

Table 1. Proximate composition and nutritional value of AP and AC lees (dry weight basis) (Dhillon et al., 2011b, 2013a; Madrera et al., 2019; Perez-Bibbins et al., 2015; Skinner et al., 2018).

	AP	**AC lees**
General composition		
Moisture (%)	70	90
Alcohol (ethanol) (%)	-	3.8
Protein (%)	4.3	19.4
Fat (%)		4.7
Phenolic compounds (%)	0.7	5.8
Total fiber (%)	70	61.3
Total carbohydrates (%)	55	60
Fermentable sugars (%)		
Glucose	22.7	-
Fructose	23.6	-
Sucrose	1.8	-
Xylose	0.1	-
Organic acids (%)		
Lactic acid	-	1.5
Acetic acid	-	0.27
Tartaric acid	-	0.02
Minerals (%)		
Ca	0.06–0.1	0.08
Na	0.2	0.07
P	0.07–0.076	0.5
Fe	0.0032–0.0038	4.9
K	0.4-1.0	0.72
Mg	0.02-0.36	0.06
Mn	0.00039–0.009	-
Zn	0.0015	Trace

-: not reported

II.3. Citric acid production

II.3.1. Citric acid properties and applications

CA (2-hydroxy-propane-1,2,3-tricarboxylic acid) derives its name from the Latin word citrus. It is a tricarboxylic acid (Figure 3) with a molecular weight of 210.14 g/mol, which contains three carboxylic functional groups with three different values of pKa (3.1, 4.7, and 6.4). It is a primary metabolic product formed in the tricarboxylic acid (or Krebs) cycle (Max et al., 2010). CA crystalizes to its anhydrous form at a 36.6 °C, while at lower temperature its monohydrate form appears. When CA loses a proton in aqueous solutions, citrate ions are produced, which can form salts with many metals (Cavallo et al., 2017).

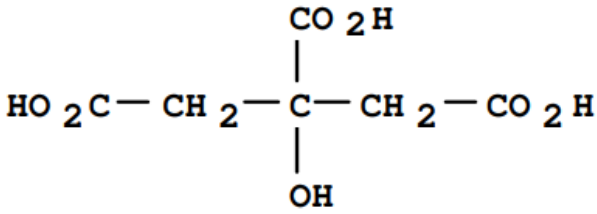

Figure 3. Chemical structure of citric acid.

CA was first introduced to the world in 1784 by Karl Wilhelm Scheele using citrus fruits (lemon, lime, orange) as a natural source (Behera et al., 2020), and was then industrialized until the late nineteenth century as a monopole source (Show et al., 2015). In 1880, CA was chemically synthesized using glycerol as a potential substrate, but this method was not economically efficient compared to the sugar medium fermentation process with *Penicillium* strains (*Penicillium glaucum*) discovered in 1893 by the German botanist Wehmer (Soccol et al., 2006). Later in 1916, the American chemist Currie discovered *A. niger* as a live CA factory cell, having less complicated fermentation process and less bacterial contamination than the *Penicillium* strains (Max et al., 2010; Soccol et al., 2006).

Currently, the extraction of CA is expanding around the globe to reach 2 Mt in 2018 (http://www.prnewswire.com, accessed 12/10/2020). The market is further projected to reach a volume of nearly 3 Mt by 2024, growing at a Compound Annual Growth Rate (CAGR) of

4% during 2019-2024 (http://www.prnewswire.com, accessed 12/10/2020), with a global market estimated at 3.6 billion US$ in 2025 (Mores et al., 2021).

In 2016, the food and beverage industry accounted for 62.5% of the global CA market, used as an acidifier or antioxidant to preserve or enhance the flavors and aromas of fruit juices and ice cream, and to prevent turbidity of wines and ciders. This market was followed by: the pharmaceutical and personal care industry (17.1%), where sodium citrate is used as an anticoagulant in blood transfusions, as an astringent lotion, such as aftershave due to its low pH, as antioxidant to preserve vitamins and effervescent; detergents and cleaners industry (12.3%), for removing metal oxides from surfaces, as a foaming agent for the softening and treatment of textiles or as a phosphate substitute); and others (8.1%), e.g. in photography, in cements, in paper industry and tobacco industry (Behera et al., 2020; Ciriminna et al., 2017; Max et al., 2010; Okafor and Okeke., 2017; Soccol et al., 2006).

II.3.2. *Aspergillus niger* as a citric acid biofactory

Aspergillus niger (Figure 4) is a black mold considered the best cell factory for CA production among fungi, yeasts and bacteria. Studies have been conducted to improve the fermentation processes for several CA-producing microorganisms (Ikram-Ul et al., 2004; Behera et al., 2020; Mores et al., 2021). However, *A. niger* presents numerous strains that differ in CA production rate as well as the fermentation conditions.

Figure 4. *Aspergillus niger* grown on potato dextrose agar (PDA) (Source: Moslem et al., 2010).

To obtain a maximum production rate of CA, *A. niger* needs to grow in an optimum medium containing the precise concentrations and the exact type of the needed nutrients such as carbon, nitrogen, phosphorus, pH, aeration, and various trace elements (Mores et al., 2021). The nature and concentration of sugar in the medium is of extreme importance for CA production. Maltose, sucrose, glucose, mannose, and fructose are the most appropriate, and should be used in a range of 120 g L^{-1} to 180 g L^{-1} (Sun et al., 2018). However, based on several studies, sucrose is classified as the most suitable carbon source for CA production, since it is easily converted (Angumeenal et al., 2013; Ikram-Ul et al., 2004). A 10 to 14% initial sugar concentration is needed at first for *A. niger* growth only, and an increase in sugar content (14-22%) increases CA production, while lower sugar levels (<10%) reduce the size of the mycelium, and affect its morphology, which will directly decline CA yield production (Angumeenal et al., 2013; Behera et al., 2020; Dutta et al., 2019; Sun et al., 2018).

Nitrogen and phosphorus sources can also directly influence CA production (Mores et al., 2021). NH_4NO_3, $(NH_4)_2SO$, $(NH_4)_2SO_4$, KNO_3, $NaNO_3$ and NH_4OH are all potential nitrogen sources that do not affect the fermentation process (Bhattacharjee et al., 2015). Ammonium sulphate [$(NH_4)_2SO_4$] with an optimum concentration of 0.1% is considered the most adequate nitrogen source since it does not produce unwanted oxalic acid, while reducing the pH of the medium. But an increase of its concentration (greater than 0.25%) increases the consumption of sugar and fungal growth, and decreases the amount of CA produced (Chirova et al., 2016; Khattab et al., 2017; Pathania et al., 2018). Phosphate (KH_2PO_4) levels lower than 0.1 gL^{-1} have a positive effect on CA production, while an excess of phosphate concentration (greater than 0.1 gL^{-1}) will lead to a decrease in the fixation of carbon dioxide, which results in the stimulation of fungal growth only, and no CA is produced (Mudyiwa et al., 2013).

The presence of heavy metals such as Zinc (Zn), Manganese (Mn), Iron (Fe), and Copper (Cu) needs to be controlled at minimum concentrations (Mores et al., 2021; Mirminachi et al., 2002). For instance, Zn and Cu must be maintained at 0.3 and 1 mg/L, respectively, for a successful fermentation with CA yields above 80%, while a high Cu concentration of up to 4 mg/L renders yields and productivities mostly unsatisfactory (Mirminachi et al., 2002; Papagianni et al., 2007). The influence of Mn is complex, and it is not yet completely elucidated. Nevertheless, studies indicate that these ions cause undesirable

morphological changes. Therefore, low concentrations (0.5-2 μg/L) must be used based on the amount of sugar provided to *A. niger*, as higher concentrations could completely ruin the production yield and cause organism's morphology to switch from microbial pellets, known as CA productive form, to unproductive filamentous growth (Berovic et al., 2007; Max et al., 2010). Elevated concentrations of the Fe ion (above 1 mg/L) also alters the mycelium morphology, which becomes characterized by long, thin, hyaline hyphae, forming loose flocs appearance instead of compact pellet structures, while at lower concentration (lower than 1 mg/L) *A. niger* switches to the CA producing morphology to become abnormally short, stubby, forked, bulbous mycelium with numerous swollen, oval to spherical-shaped cells distributed throughout the mycelial structure (Mores et al., 2021; Zhang et al., 2002). The mycelial morphology plays an important role in CA production by *A. niger*, but this effect seems to be important mainly in submerged fermentations (Mores et al., 2021).

Usually, calcium (Ca) is used to influence the precipitation and recovery of the CA in crystals, so to recover 1 kg of CA produced, 0.58 kg of $Ca(OH)_2$ is needed (Behera et al., 2020; Mores et al., 2021). Inducers also have a great impact on CA production and excretion. Methanol (MeOH) concentrations out of the 3-4% range (depending on the strain) cause a decrease in CA production activity since MeOH increases the permeability of the microorganism cell membrane, for an easier CA extraction (Dutta et al., 2019).

The pH of culture medium must also be controlled. An initial pH greater than 5 is needed to boost mycelium formation, then will automatically decrease from 5 to 3 due to nitrogen catabolism, activating the CA production. Maximum CA production is obtained at pH lower than 3, which is necessary to hamper oxalic acid and gluconic acid production as well as to prevent contaminations (Karaffa et al., 2019; Maharani et al., 2014).

Temperature is a physical parameter that needs to be taken in consideration for *A. niger* growth and CA production rate, since its production is dependent on glycolysis and TCA cycle enzymes (Maharani et al., 2014), and the sensitivity of these enzymes to temperature fluctuation could disable their function, affecting CA production (Behera et al., 2020; Max et al., 2010; Xie et al., 2009). Incubation temperature ranging from 25 to 30 °C were found to be more suitable for high yields and rapid rates of CA production (Behera et al., 2020). Roukas et al. (2020) have confirmed that, at 25 °C *A. niger* B60 produced

306.8 g/kg dry substrate (DS) of CA, while Dhillon et al. (2011a) retrieved 342–365 g/kg DS of CA at 30 ± 1 °C temperature.

The aeration of the culture medium is also a necessary factor for *A. niger* survival as well as for CA production in liquid and submerged fermentation. Dissolved Oxygen Tension (DOT) is initially regulated to 18-21 mbar for *A. niger* growth, and once the mycelium reaches a stationary phase to start producing CA, the aeration is gradually increased to a maximum of 40-150 mbar (Kubicek et al., 1980; Max et al., 2010).

II.3.3. Fermentation processes used for CA production

The set where the fermentation will occur is as important as the previously mentioned factors (Dhillon et al., 2011a). Fermentation processes using *A. niger* can be performed by using different techniques such as submerged fermentation (SmF) and solid-state fermentation (SSF) (Mores et al., 2021).

SmF is the most used method on an industrial level (Show et al., 2015). It is a process in which microorganisms grow in liquid medium, with high content of free water, having notable advantages regarding instrumentation and control by monitoring of pH, dissolved oxygen, temperature, mixing, aeration, concentration of water-soluble molecules, the easy separation of biomass after the fermentation (Behera., 2020). Usually, beet and sugarcane molasses are used as substrates, but it is possible to use other substrates composed of mono and disaccharides which enable the use of raw by-products such as AP (Soccolet al., 2017; Mores et al., 2021).

SSF, also known as “Koji” process, was first developed in Japan for bread and cheese fermentation, and it has since been used for the production of important biomolecules and products for many industries, including food, pharmaceutical, textile, biochemical and bioenergy, among others (Pandey., 2003; Soccol et al., 2006). SSF is a simple method used as a cheap alternative to invest in agro-industrial waste that consists of the growth of microorganisms in a low-water activity environment on an insoluble material that acts both as physical support and source of nutrients (Soccol et al., 2006). The process can be completed in 4-5 days with an optimum pH range of 4.5–6.0 and a temperature range of 28–30 °C, using a substrate with up to 75% moisture (Behera et al., 2020; Soccol et al., 2017).

SSF can render a higher yield, has lower capital, operating costs, energy and effort for utilizing available agro-industrial substrates without any pre-treatment, the system is less sensitive to the presence of trace elements compared to SmF, and presents a low risk of contamination (Berovic et al., 2007). SSF also presents disadvantages, such as difficulties to scale up to industrial level, difficulty in the process standardization, difficult control of process parameters and problems with heat buildup (Kapilan, 2015).

Dhillon et al. (2011b) compared the CA yield of SSF and SmF using AP as substrate and two *A. niger* strains (NRRL 567 and NRRL2001). SSF rendered a higher yield for both strains (Table 2). This clear advantage in CA yield using the SSF process is mainly due to the fact that SSF mimics the natural habitat of most microorganisms, mainly filamentous fungi. It also enhances enzymatic productivity for many enzymes and it is less susceptible to substrate inhibition, allowing higher concentration of CA (Soccol et al., 2017). Table 3 presents the CA production and yields of several *A. niger* strains under varying fermentation parameters using the SSF process.

Table 2. Comparison between submerged fermentation (SmF) and solid-state fermentation (SSF) based on citric acid production and yield by two industrial strains of *Aspergillus niger* (Source: Dhillon et al., 2011b)

***A. niger* strain**	**SmF**		**SSF**	
	CA (g/kg)	Yield (g/L)	CA (g/kg)	Yield (g/L)
NRRL567	66.0 ±1.9	9.0 ±0.3	127.9 ±4.3	18.2 ±0.4
NRRL2001	61.0 ±1.9	8.9 ±0.3	115.8 ±3.8	13.9 ±0.4

Table 3. Comparison of citric acid production by different *Aspergillus niger* strains using Solid State Fermentation.

Strain of *A. niger*	Carbon source	Culture mode	Time (days)	CA (g/kg)	Reference
B60	Pomegranate peel	F	8	306.8**	Roukas et al. (2019)
NRRL 567	AP	F	6	883*	Vandenberghe et al. (2017)
NRRL 567	AP	F	6	342.41-248.42**	Dhillon et al. (2011a)
NRRL 567	Grape pomace	F	5	600*	Soccol et al. (2006)
NRRL 567	Wheat straw	F	5	231.8**	Kim et al. (2014)
NRRL 567	Corn stover	F	5	213.8**	Kim et al. (2014)
NRRL 567	Peat moss	F	5	240**	Kim et al. (2014)
NRRL 567	AP + rice husk	F	5	364.4 ± 4.5**	Dhillon et al. (2012)
NRRL 2001	Corn husk	F	5	259 ± 10**	Hang et al. (2000)
LPB 21	Cassava bagasse	B	6	269**	Vandenberghe et al. (2004)

*: Based on consumed sugar; **: dry substrate basis; F: Flasks; B: Bioreactor.

Based on all these parameters, Dhillon et al. (2011a) optimized CA production by *A. niger* NRRL567 using AP as substrate. The optimum moisture content of AP was 75-78% supplemented with rise husk. Fermentation was optimal at 30 ± 1 °C with pH 3.43 ± 0.1. 75% moisture content and 3% MeOH were the best conditions to obtain 342.41 g/kg CA in 7 days using SSF (Dhillon et al., 2011a). Roukas et al. (2020) obtained 351.5 g/kg of CA produced by *A. niger* B60 in non-aseptic medium in SSF using non dried pomegranate peel as a substrate. The fermentation was held for 8 days in 25 °C temperature and supplemented with 3% MeOH as an inducer. Sun et al. (2018) used *A. niger* CBS 513.88 in SmF, supplemented with 50 mL of liquefied corn medium and 170 g/L sugar content. The mineral elements consisted of: 0.15 g/L $MgSO_4 \cdot 7H_2O$, 0.25 g/L K_2HPO_4, 1 g/L $(NH_4)_2SO_4$, 0.05 g/L $ZnSO_4 \cdot 7H_2O$, 0.25 g/L $CuSO_4 \cdot 5H_2O$, and 0.06 g/L $MnCl_2 \cdot 4H_2O$. *A. niger* incubation was held for 96 h at 36 °C to produce 174.1 g/L of CA with a sugar conversion rate of 98.36%.

II.4. Potential valorization of cider residue

Although numerous literature reviews indicate high technological maturity of the CA fermentation process, the growing trend in the number of applied documents per year indicates that there is still room for improvements in the development of the fermentation and recovery process, and in discovering new potential, low-value, agro-industrial by-products (Mores et al., 2021; Amato et al., 2020).

Since the discovery of the great potential of AP in *A. niger* CA production rate using SSF, the process has been well developed and the fermentation parameters were well studied. The needed AP concentration in a predefined state (dry matter) was determined, *A. niger* strains were tested, and their nutritional requirements were highlighted in order to obtain a high CA production rate; the physical-chemical conditions of the fermentation process were designed and updated over years to elevate it to an industrial scale (Behera et al., 2020; Dhillon et al., 2011b, 2013a, 2013c; Kumar et al., 2010; Mores et al., 2021).

Conversely, scarce information can be found about the use of ACR as substrate for fermentation processes, while their damaging impact on the environment is as catastrophic as AP. ACR need thus to be integrated into the circular-economy course of action. There are no comprehensive studies reporting the chemical composition and nutritional value of ACR. In-depth analyses in terms of fermentable sugars, nitrogen sources and alcohols are necessary, as they will deliver the needed information of whether ACR could be used as a sole substrate for CA production or incorporated with AP as a mix substrate for *A. niger* fermentation. ACR seem to have an interesting ethanol amount, as well as several minerals (Table 1), which are requirements for optimal CA yields. The result would be promising since CA lees could be integrated as an inducer and as a support substrate to AP. As a result, the process cost would be reduced, with providing a natural inducer and the environmental impact of both AP and ACR would significantly deflate.

III. Materials and Methods

III.1. Base matrices

In the present study, two types of base matrices were used: apple pomace (AP) was used as a control matrix, since it has been previously studied and validated as a good substrate for CA production, and apple cider residue (ACR) which will be a test substrate that has not been previously studied for the production of CA. ACR will be screened and set to fermentation to compare its efficacy to AP in CA production.

III.1.1. Preparation of the matrices

The AP used in this study was retrieved after washing, pressing, crushing, and milling of raw apples (Golden Delicious) that were purchased from Portuguese local market, then dried in hot air oven at 30 °C until constant weight (Figure 5).

Figure 5. Raw apples washed and ready to be pressed (a), dried and crushed apple pomace after pressing and milling (b), sieved apple pomace into 1.7 mm to 2.0 mm particles (c).

The ACR used in this study corresponded to the solid parts of the fermentation product of apple into AC. The cider was produced according to a general flowchart for organic cider production. Firstly, the apples were submitted to a preselection and sanitation, then crushed and the resulting must was left to ferment naturally. Later, the mixture was filtered and racked to collect the juice and clarify it into AC. The solid residue (waste) was collected at the end of the fermentation process and frozen until use. Before use, the ACR was thaw, dried in hot air oven at 30 ºC for 72 hours, grounded and passed through sieves to obtain the desired particle size of 1.7 mm to 2.0 mm (Figure 6). ACR (1,670 g) was dried in hot air oven at 30 °C until constant weight to determine the moisture content. The powdered ACR was used as the basis for the experimental tests described in section III.3.

Figure 6. Dried and sieved apple cider residue.

III.1.2. Determination of moisture content

Moisture was determined by weighting a sample of AP and of ACR before and after hot air drying (fresh weight and dry weight, respectively) at 30 °C until reaching constant weight. The moisture content was calculated using the following equation (Eq. 1):

$$\%\, moisture = \frac{(fw - dw)}{fw} \times 100 \qquad (1)$$

Where fw is the fresh weight (initial mass, before the substrate was dried), and dw is the dry weight (final mass that was held constant after drying), meaning that all moisture was vaporized. For AP drying process took 96 hours and 72h for ACR.

III.1.3. Characterization of the sugars, acids and alcohol content

Sugar and acid concentrations in AP and ACR were determined by High-performance liquid chromatography (HPLC) as presented in section III.7

III.2. Fermentation substrates

Throughout this work, substrates were prepared based on AP and ACR to be used in different steps of the study. The composition of all substrates is described and is summarized in Table 4. Based on Dillon et al (2011a), several nutritional supplements significantly increase the CA production by *A. niger*, and depending on the type of the experiment, multiple media were added with the following supplements: 0.2% potassium dihydrogen phosphate (KH_2PO_4), 0.2% ammonium sulfate ($(NH_4)_2SO_4$), 0.1% magnesium sulfate anhydrous ($MgSO_4$) and 2% methanol (CH_3OH) as an inducer.

Table 4. List of substrates used in this assay.

Substrate	Supplements	Code used in the study
100% AP	Yes	AP_{sup}
100% AP	No	$AP_{n/sup}$
100% ACR	Yes	ACR_{sup}
100%ACR	No	$ACR_{n/sup}$
50% of AP / 50% ACR	Yes	$50\%AP+50\%ACR_{sup}$
50% of AP / 50% ACR	No	$50\%AP+50\%ACR_{n/sup}$
75% of AP / 25% ACR	Yes	$75\%AP+25\%ACR_{sup}$
75% of AP/ 25% ACR	No	$75\%AP+25\%ACR_{n/sup}$

Moisture was set through adding 75 g of sterile distilled to 25 g of dry substrate. The pH was adjusted to 3 by adding hydrochloric acid (HCl) 37% or sodium hydroxide (NaOH) 1N.

III.3. *Aspergillus niger* strains

III.3.1. Strains used in this study

Several strains of *A. niger* previously isolated from different sources were studied in order to identify the best CA producer in terms of production and yield (table 5). The strains were propagated in Potato Dextrose Agar (PDA, Liofilchem, Italy) at 25 °C, for 7 days, and preserved in 30% glycerol with 0.1% tween-80 solution, at -20 °C.

Table 5. *Aspergillus niger* strains used in the study and their origin.

***A. niger* strains**	**Host**	**Reference**
S09-6	Palm dates (*Phoenix dactylifera*)	Nikolchina and Rodrigues, 2021
S11-2	Palm dates (*Phoenix dactylifera*)	Nikolchina and Rodrigues, 2021
A. niger A	Palm dates (*Phoenix dactylifera*)	Nikolchina and Rodrigues, 2021
LBTsp-60HR	Almonds (*Prunus dulcis*)	Jelassi, 2020
LGsp-T9	Almonds (*Prunus dulcis*)	Jelassi, 2020
LBL4%T9	Almonds (*Prunus dulcis*)	Jelassi, 2020
MUM 92.13	Collection culture (Micoteca da Universidade do Minho)	http://www.micoteca.deb.uminho.pt/

III.3.2. Mycotoxigenic profile of the strains

Even though *A. niger* is generally recognized as safe and labeled with the GRAS by the US Food and Drug Administration (FDA), this epithet is not applicable to all strains, as numerous studies revealed the ability of several strains to produce mycotoxins depending on the environmental conditions and nutrient source (reviewed by Nielsen et al., 2009). Therefore, as a way to guarantee the safety of the tested strains and of the obtained products, the strains used in the study were characterized for their mycotoxigenic profile in both mycotoxin inducing conditions (synthetic medium) and in the fermentation substrates.

III.3.2.1. Culture conditions

For the determination of the mycotoxigenic profile of the tested strains, strains were cultivated in Yeast Extract Sucrose (YES; composed of 20 g/L of yeast extract, 150 g/L of saccharose and 15 g/L of agar) agar at 25 °C for 9 days, to induce the production of mycotoxins. After the incubation, three portions of the culture (6 mm diameter each portion) where cut with a cork borer and deposited in 1.5 mL of methanol (CH_3OH; CAS n. 67-56-1, Honeywell) for 60 minutes, with regular agitation (vortex), and filtered with 0.2 µm PTFE disposable syringe filter. The methanol was evaporated with a nitrogen flux. To test the presence of mycotoxins in the CA aqueous extract, 0.5 mL of the extract resulting from fermentation (prepared as described in section III.7.1) were filtered and lyophilized. The

dried samples were sent for multi-mycotoxin analysis using multi-mycotoxin method of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

III.3.2.2. LC-MS/MS analysis

The LC-MS/MS analyses were done at the Department of Agrobiotechnology, IFA-Tulln, Institute of Bioanalytics and Agro-Metabolomics, University of Natural Resources and Life Sciences, Vienna (BOKU).

Detection and quantification were performed with a QTrap 5500 MS/MS system (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray electrospray ionization (ESI) source and a 1290 series UHPLC system (Agilent Technologies, Waldbronn, Germany). The LC-MS/MS protocol was applied as previously described (Sulyok et al., 2020), and has in the meantime been extended to cover 710 metabolites overall. Confirmation of positive analyte identification was obtained by the acquisition of two MS/MS transitions per analyte (with the exception of moniliformin and 3-nitropropionic acid, that each exhibit only one fragment ion), which yields 4.0 identification points according to commission decision 2002/657/EC.

The accuracy of the method is verified for major mycotoxins on a routine basis by participation in interlaboratory testing schemes organized by BIPEA (Bureau Interprofessionnel des Etudes Analytiques, France) and by CODA-CERVA (National Reference Laboratory for mycotoxins, Belgium).

III.3.3. Preparation of spore suspensions

For each experiment, strains were freshly cultivated in 6 cm diameter Petri dishes containing 10 mL of PDA and incubated for 7 days at 30 °C. Spores were harvested by flooding the plates with 1 mL of a sterile 0.1% Tween-80 solution and collected with a pipette into 1.5 mL sterile Eppendorf tubes, then counted using a hemocytometer (Neubauer chamber) to adjust the count to approximately 10^7 spores/mL. Spores were simultaneously stored in a 30% glycerol plus 0.1% (w⁄v) Tween-80 solution in the freezer (-20 ºC).

III.4. Preliminary assay – Screening for CA producing strains

A preselection step was established in order to identify the best CA producer among the prementioned *A. niger* strains (*cf.* section III.3) using AP_{sup} as a substrate (*cf.* Table 4, section III.2) in SSF, as described by Dillon et al (2011a). The prepared amount of substrate (160 g) was divided equally into 16 sterilized glass jars (10 g each). A spore suspension (10^7 spores/mL) was prepared for each strain and each fermentation unit was inoculated with 1 mL of the suspension. The fermentation process was held for 4 days at 30 °C, in an incubator (Figure 7).

Figure 7. Jars of 10 g of apple pomace inoculated with *Aspergillus niger* strains.

III.5. Assessment of ACR as substrate for CA production

A second set of experiments was conducted to assess the ACR adequateness as a potential substrate for CA production using the three *A. niger* strains that produced the highest amounts of CA under optimal conditions, as determined in the preliminary assay (section III.4): LBTsp-60HR, LGsp-T9, and MUM 92.13, and to determine the impact of the supplements on CA yield. Three substrates were prepared in triplicates (110 g of each): AP_{sup} as a control, ACR_{sup} and $ACR_{n/sup}$ (cf. Table 4, section III.2), then divided equally into 33 sterilized glass jars (10 g each). A spore suspension (10^7 spores/mL) was prepared for each strain as previously described and each fermentation unit was inoculated with 1 mL of the suspension. The substrates were fermented for 4 days at 30 °C and the fermentation products were extracted and analyzed as described in section III.7.

III.6. Combination of ACR and AP for CA production

As a result of the screening of strains for CA production, MUM 92.13 was selected as the best CA producer among all three strains to be used in this assay. A combination of different substrate mix was prepared: 100%AP, 75%AP+25%ACR, 50%AP+50%ACR, and 100%ACR, as described in Table 4. One hundred and ten grams of each mixture were prepared and divided into 11 sterilized jars. Fifty grams were supplemented as described in section III.2. In this experiment, instead of adding methanol as a fermentation inducer, 0.3% of ethanol were added. This was done in order to test the efficacy of the ethanol naturally present in the ACR (0.3%, previously measured by HPLC), to substitute the need for methanol addition. The prepared glass jars for inoculation were inoculated with a 10^7 spores/mL spore suspension previously prepared. The substrates were fermented for 4 days at 30 °C and the fermentation products were extracted and analyzed as described in section III.7.

III.7. Sample Analyses

The samples from all the experiments were prepared and analyzed by HPLC as follows.

III.7.1. Sample preparation

At the end of the incubation period, 3 g of each fermented medium was mixed with 15 mL of distilled water in a 100 mL Erlenmeyer at 120 rpm for 60 mins at 25 °C, in an orbital shaker (Figure 8.a), to extract the water-soluble compounds, specifically the sugars and the CA, following the protocol described by Dillon et al (2011a). Samples were then filtered through filter paper Whatman #3 (Figure 8.b), to remove the solids. The flowthrough was filtered again with 0.22 μm syringe filter and analyzed by HPLC.

Figure 8. Orbital shaker set for 120 rpm (a), sample filtration with Whatman #3 paper in glass wholes to retrieve the filtrate (b).

III.7.2. HPLC

The system used was equipped with a binary pump (Varian Prostar 220), an injector (Rheodyne 7725i; Loop 20 µL), an infrared detector (Varian RI-4) for the detection and quantification of sugars, alcohol and organic acids. Data were analyzed using the Varian Chromatography Workstation software (version 4.5). The chromatographic separation was achieved using isocratic elution with a prepacked HPLC analysis column Aminex HPX-87H (Bio Rad; 300×7.8 mm, hydrogen form, 9 µm particle size, 8% cross linkage, pH range 1–3) operating at 30 °C (JonesChromatography 7981 oven). The mobile phase consisted of an isocratic program of sulfuric acid 0.004 M, pumped at 0.6 mL/min for a total run time of 30 min. The injection volume was 20 µL. Calibration curves, limit of detection (LOD), and limit of quantification (LOQ) were determined with seven concentration levels of HPLC-grade standards of glucose, fructose, sucrose, glycerol (0.10 to 8.0 g/L), CA, lactic acid, malic acid, acetic acid (0.019 to 1.5 g/L), and ethanol and methanol (0.044 to 3.5 %).

Methanol was included in the analyses even if it is not naturally present in the fermentation products but because it is added during the substrate preparation.

Standards injections were used to build regression lines for each compound using the areas for each concentration level, to obtain the following linear equation (Eq. 2):

$$y = bx + a \qquad (2)$$

where *y* is the retrieved signal, *b* is the slope, *a* is the intercept, and *x* is the concentration.

LOD and LOQ were calculated according to the following Equations (Eq. 3 and Eq. 4, respectively):

$$\mathrm{LOD} = 3 \times \left(\frac{\mathrm{sa}}{\mathrm{b}}\right) \qquad (3)$$

$$\mathrm{LOQ} = 10 \times \left(\frac{\mathrm{sa}}{\mathrm{b}}\right) \qquad (4)$$

where *sa* is the standard deviation of the intercept of the regression line obtained from the calibration curve and *b* is the slope of the line. LOD and LOQ values were multiplied by the dilution factors resulting from the sample preparation method to obtain sample LOD and LOQ.

III.7.3. Quantification of fermentation products and product yield

The areas used to calculate glucose, fructose, sucrose, glycerol, methanol, and ethanol were retrieved from the refracted index (RI) chromatograms, as for CA, lactic acid, malic acid, and acetic acid the areas were retrieved from UV chromatograms.

The concentration of the compounds was calculated from the HPLC results as g/kg of substrate for glucose, fructose, sucrose, glycerol, CA, lactic acid, malic acid, and acetic acid, and in % for methanol and ethanol, taking in consideration the dilution factors.

The product yield (i.e, the rate of conversion of a given substrate into a given product) was calculated with the following formula (Eq. 5):

$$\mathrm{Y}\left(\frac{\mathrm{P}}{\mathrm{S}}\right) = \frac{(P_{final} - P_{initial})}{|(S_{final} - S_{initial})|} \qquad (5)$$

where $Y_{(P/S)}$ is the yield of product (CA or glycerol) to substrate (sugar), P_{final} is the amount of product (CA or glycerol) produced at the end of fermentation (in g/kg), $P_{initial}$ is the initial amount of CA or glycerol contained in the medium (in g/kg), $S_{initial}$ is the amount of substrate

(total sugars or one type of sugar - glucose, fructose or sucrose) preexistent in the medium, and S_{final} is the amount of substrate (sugar) remaining in the medium after fermentation (in g/kg).

III.8. Statistical analysis

The results are expressed as mean values ± standard deviation (SD), for the preliminary assay where the *A. niger* strains were evaluated for their ability to produce CA from AP_{sup} were performed in one replicate.

The results were analyzed with ANOVA two factors with replication, followed by a Post Hoc test (Scheffe test) for multiple comparison in mean significance within groups. In case where there are less than three groups of results, an independent t-student test was conducted, to determine the significance of the difference between the treatments and the strains to produce CA. A 5% significance level ($p < 0.05$) was considered for all tests. The analyses were performed using IBM SPSS statistics 28.0.0.0.

IV. Results and Discussion

IV.1. HPLC method performance parameters

The performance parameters of the HPLC method for each of the compounds are presented in Table 6. The LOD and LOQ values presented in g/L are relative to the equipment, and those presented in g/kg are relative to the sample, as a result of the extraction method dilutions.

Table 6. Performance parameters of the HPLC method for the analyzed compounds (in g/L for the equipment; in g/kg for the sample extraction method).

			Equipment		Method	
Compound	**Equation**	**R^2**	**LOD (g/L)**	**LOQ (g/L)**	**LOD (g/kg)**	**LOQ (g/kg)**
Glucose	y = 1.31E+06x - 5.20E+03	1.0000	0.02	0.06	0.004	0.012
Fructose	y = 1.63E+06x - 3.23E+03	1.0000	0.01	0.03	0.002	0.006
Sucrose	y = 9.92E+05x + 3.19E+03	1.0000	0.07	0.22	0.014	0.044
Glycerol	y = 3.05E+05x - 3.74E+03	0.9998	0.16	0.52	0.032	0.104
Ethanol	y = 6.45E+06x - 2.57E+04	1.0000	0.01	0.04	0.002	0.008
Methanol	y = 2.04E+06x + 7.79E+02	0.9999	0.03	0.11	0.006	0.022
Citric acid	y = 8.49E+05x - 4.05E+03	1.0000	0.01	0.04	0.002	0.008
Lactic acid	y = 5.41E+05x - 1.34E+02	0.9999	0.02	0.07	0.004	0.014
Malic acid	y = 7.32E+05x - 1.33E+03	1.0000	0.00	0.09	0.001	0.018
Acetic acid	y = 1.13E+06x - 4.22E+03	0.9999	0.02	0.08	0.004	0.016

IV.2. Sugars and acids composition and moisture content of the matrices

ACR is the result of cider fermentation, which explains the presence of glycerol, organic acids (malic and citric acids) and alcohol (ethanol) that was determined by using a fresh ACR matrix.

AP presented a moisture content of 89.12% of the initial weight. The difference in moisture content of AP to other studies such as 70.5% moisture determined by Dhillon et al (2011a, 2011b), can be explained by the fact that the apple pressing in our study was manual and not mechanical, which is not as strong on juice removal. As for ACR (also homemade), the moisture content was about 75.37% which is adequate since it is the solid residue that forms at the bottom of the recipients containing cider, after fermentation.

The difference in moisture content to other studies is due to the fact that the used AP and ACR in this study are homemade and the pressing, crushing and fermentation techniques (cf. section III.1.1) are different from the industrial processes. Overall, ACR presents an interesting nutritional appraisal compared to AP, which primely can be screened for CA production as a solo substrate or as a mixture substrate with AP.

As for the composition of AP and ACR in terms of sugars (glucose, fructose, sucrose), glycerol, organic acids (lactic, malic and citric acids), and alcohol content is presented in

Table 7. Acetic acid was not detected in any of the analyzed samples. Analyses showed that, on a dry weight (dw) basis, the sugars glucose, fructose and sucrose summed up 52.7% and 31.4% of AP and ACR, respectively.

In AP, the sugar contents were similar to those detected in previous studies. Antonic et al. (2020), Bhushan et al. (2008), and Kołodziejczyk et al. (2007) reported that fructose content in AP can reach up to 498 g/kg (dw), glucose content can range from 25 to 227 g/kg (dw), and sucrose can range from 18–67 g/kg (dw) depending on the type of processed apples and the pressing techniques. Dhillon et al. (2011b, 2012) also reported that 227, 236, and 18 g/kg (dw) were glucose, fructose and sucrose content respectively in AP.

Table 7. Sugars, acids and glycerol content of apple pomace (AP), and apple cider residue (ACR); values presented in g/kg dry weight basis (dw)

Constituents	Composition (g/kg)	
	AP	ACR
Glucose	135	185.4
Fructose	273.5	186.3
Sucrose	21.8	17.5
Glycerol	5	25
Lactic acid	13	-
Malic acid	10.45	25.3
Citric acid	-	1.6
Ethanol	-	2.34*

-: not detected
* Ethanol content determined on a fresh wet basis and converted to dry basis.

However, there are no sufficient studies about the sugars content of ACR to relate to. Therefore, when compared to other substrates that were studied to produce CA besides AP, ACR is presenting significant quantity of sugars. Dhillon et al. (2011b) used brewery spent grain containing 215 g/kg of glucose and were able to produce 14 ± 1.2 g/kg (dw) of CA by *A. niger* NRRL567. Citrus waste also contained 183 g/kg carbohydrates and was set as a substrate for *A. niger* NRRL567, and *A. niger* NRRL 2001 to produce 59.3 ± 1.8 and 63.6 ± 2.9 g/kg of CA at 72h.

IV.3. Preselection of *Aspergillus niger* strains for maximum CA production

IV.3.1. CA production and yield

The screening of *A. niger* strains for their CA production ability showed distinct abilities to produce CA with AP_{sup} as a substrate (Table 8).

Table 8. Citric acid (CA) production (g/kg) and Yield (g/g total sugars, CA/total sugars) obtained from *Aspergillus niger* strains.

A. niger strain	CA production (g/kg)	Yield (CA/total sugars)
A. niger A	1.99	0.06
LBL4°/T9	5.24	0.18
LBTsp-60HR	36.46	0.59
LGsp-T9	36.58	0.58
MUM 92.13	41.63	0.57
S09-6	7.89	0.12
S11-2	0.32	0.00

The strain with the lowest CA production was S11-2, with as low as 0.32 g/kg, while the highest CA production (41.63 g/kg (dw)) and yield (0.57 ± 0.003 g/g total sugars) were achieved by the strain MUM 92.13. The CA production by this strain is comparable with *A. niger* Tieghem MTCC 281 that produced 46 g/kg of CA (Kumar et al., 2010), *A. niger* NRRL 567 with 65.6 ± 1.9 g/kg, and *A. niger* NRRL 2001 with 61.1 ± 1.9 g/kg. However, these two strains where industrially enhanced and the fermentation conditions were optimized to

achieved higher CA production of 127 ± 4.3 and 115.81 ± 3.8 g/kg (dw) respectively (Dhillon et al., 2011a, 2011b).

The strains used in this experiment are local and there is no genetic or molecular modification in their nature. Therefore, the amount of CA produced by MUM 92.13, LBTsp-60HR and LGsp-T9 was considered very promising, and they were thus selected for further CA fermentation process optimization using an ACR-based substrate. Since this was a preliminary assay for the screening of CA producers, only one replica was used.

IV.3.2. Mycotoxigenic profile of *Aspergillus niger* strains

Considering that the strains LGsp-T9, LBTsp-60HR and MUM 92.13 were selected for further CA production assays, they were screened for mycotoxins production in both inducive medium (YES) and the tested substrates AP_{sup}, ACR_{sup}, and $ACR_{n/sup}$. The obtained results are presented in Table 9.

Previous studies reported that *A. niger* mainly produces ochratoxin A (OTA) and fumonisin B_2 (Perrone et al., 2011; Samson et al., 2007). The FAO/ WHO expert committee on food additives (JECFA) limited ochratoxin A (OTA) to 0.1 ng/kg as a tolerable weekly intake, with a regulation of 10 ng/g tolerated in daily food and feed products, 2 ng/g in grapes, wine and must, and 0.5 ng/g in baby and infant food, since it is a nephrotoxin, considered as potential carcinogen, and an immunosuppressive compound (FAO/WHO, 2016). Fumonisin B2 is reported also to be a problematic mycotoxin in food, especially products based on fumonisin producers since it has a severe toxic effect on the human's kidneys and liver. Yet, fumonisin B2 is considered less toxic than OTA, and a daily intake of 165 ng/g is tolerated (FAO/WHO, 2016).

Table 9. Fungal secondary metabolites (including mycotoxins) found after fermentation of LGsp-T9, LBTsp-60HR and MUM 92.13 on supplemented apple pomace (APsup), supplemented apple cider residue (ACR_{sup}), and non-supplemented apple cider residue ($ACR_{n/sup}$), in ng/g of agar or substrate.

Detected compound	LGsp-T9				LBTsp-60HR				MUM 92.13			
	YES	AP_{sup}	ACR_{sup}	$ACR_{n/sup}$	YES	AP_{sup}	ACR_{sup}	$ACR_{n/sup}$	YES	AP_{sup}	ACR_{sup}	$ACR_{n/sup}$
Fumonisin B_2	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	22.4	7586.0	15.4	< LOD	< LOD
Fumonisin B4	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	3285.8	< LOD	< LOD	< LOD
Fumonisin B6	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	329.1	< LOD	< LOD	< LOD
Malformin A	35.34	699.0	19872.0	54.5	41000.0	18.6	66.0	15900.0	< LOD	29.3	< LOD	< LOD
Pyranonigrin	1694.2	628.0	15708.0	2444.0	8200.0	< LOD	< LOD	16372.0	81220.0	31480.0	83100.0	102100.0
Fonsecin	4.4	< LOD	< LOD	1.8	4.9	< LOD	< LOD	0.2	24..2	4.5	1243.2	1411.6
Kotanin A	1.4	< LOD	< LOD	< LOD	0.6	< LOD	< LOD	23.5	7330.0	102.0	2.9	5.1
Pyrophen	13.3	< LOD	< LOD	< LOD	7.0	< LOD	< LOD	10.9	7610.0	102.0	3310.0	1863.2
Kojic acid	106.3	5802.0	9290.0	57560.0	70.8	4810.0	4146.0	5866.0	399.6	9440.0	9890.0	2652.0
Tryptophol	< LOD	279.0	118.8	16.2	< LOD	276.6	135.7	10.0	< LOD	< LOD	119.2	83.2

Primarily, all 3 strains did not produce detectable amounts of OTA, either in the inducive medium (YES) or in the substrates. Fumonisin B2 was produced in high amounts by MUM 92.13 in YES, but the toxin was detected in very small amounts in the AP_{sup} substrate, indicating that this substrate in not adequate for the production of this toxin or that the aqueous extraction was not adequate for the extraction of the toxin. Either way, the aqueous extract used for CA extraction seems to be safe from fumonisin B2. This toxin was also detected in small amounts in the substrate $ACR_{n/sup}$ by LBTsp-60HR. Malformin A was detected in all samples from LBTsp-60HR and LGsp-T9 in various amounts, while MUM 92.13 produced low or undetectable amounts.

Malformins are not considered mycotoxins *sensu stricto* since they are not toxic by oral administration, only if they are injected into the peritoneum (mostly performed on animals only) (Nielsen et al., 2009). Pyranonigrin was detected in very high concentrations in all samples from the $ACR_{n/sup}$ substrate, in particular from MUM 92.13. This compound, along with fonsecin, is not considered a mycotoxin, and its presence in significant amounts is interesting for having a characteristic radical scavenging reagent rarely found in nature. They are in fact a family of antioxidant compounds generally isolated from *A. niger* (Carboué et al., 2019; Tang et al., 2018; Riko et al., 2014).

Kojic acid, a by-product of several fermentation processes is conjointly found in all fermentation media. It is a mild inhibitor of pigment formation in plants and animal tissues, and an on-demand compound for its wide use in cosmetics and food where it is a precursor of flavors enhancers and in seafood to prevent color change (Ezekiel et al., 2020; Saeedi et al., 2019). Moreover, the presence of pyrophen in $ACR_{n/sup}$ and ACR_{sup} gives another reason for integrating it in the circular economy, since pyrophen have antimicrobial activities against *Escherichia coli, Staphylococcus aureus,* and *Candida albicans* (Zhang et al., 2010), while other studies confirmed that 400 ng/mL of pyrophen have cytotoxic effect on Brest cancer cells. To Conclude ACR can be considered as safe to be used as a solid-state substrate for CA production and extraction.

IV.4. Production of CA using AP and ACR substrates

IV.4.1. Total sugars consumption and CA production

An analysis was made to determine the production of CA and glycerol and the consumption of sugars using AP_{sup}, ACR_{sup} and $ACR_{n/sup}$ as solid substrates with 75% moisture, by the three selected *A. niger* strains MUM 92.13, LGsp-T9 and LBTsp-60HR. Table 111 summarizes the results obtained for the various compounds, as determined by HPLC. Figure 9 shows the results in terms of consumption of sugars and production of CA and glycerol, and Figure 10 shows the yield of CA and glycerol, in g of compound produced by g of sugars consumed.

Table 10. Analysis of variance between subjects; the interaction effect of the nature of substrate and the presence of supplements in the medium on citric acid (CA) and glycerol production.

	CA				glycerol			
	df	MS	F	p	df	MS	F	p
Substrate	2	3200.697	16766.03	<.001	3	2561.512	1071.562	<.001
strains	2	47.866	250.735	<.001	2	2.367	0.990	0.391
Interaction (substrate× supplements)	4	3.426	17.949	<.001	4	316.571	132.432	<.001
Error	18	0.191			17	2.390		

df: degree of freedom, MS: mean square, F: fisher ratio = (between groups)/(within groups),
p: significance level

Table 11. Composition of supplemented apple pomace (AP_{sup}), supplemented apple cider residue (ACR_{sup}), and non-supplemented apple cider residue ($ACR_{n/sup}$), before (Zero) and after four days of fermentation for LBTsp-60HR, LGsp-T9, and MUM 92.13. Glucose, fructose, sucrose, glycerol, citric acid (CA), and malic acid are quantified in g/kg in dry weight basis, methanol is quantified in %. The values represent average (n=3) ± standard deviation

	Sample ID	Glucose (g/kg)	Fructose (g/kg)	Sucrose (g/kg)	Glycerol (g/kg)	Methanol (%)	Citric acid (g/kg)	Malic acid (g/kg)	pH
AP_{sup}	Zero	46.79±2.34	52.90±1.03	9.15±3.19	6.17±2.17	1.99	0.03±0.00	8.07	3.29
	LBTsp-60HR	11.21±5.25	33.10±5.60	0.60±0.03	75.54±1.05	0.32±0.02	35.72±0.45	3.9±0.441	2.3±0.06
	LGsp-T9	6.61±2.58	31.15±6.29	0.52±0.09	67.69±1.46	0.24±0.032	37.48±0.40	1.46±0.234	2.327±0.05
	MUM 92.13	11.39±2.16	22.45±0.26	0.48±0.06	51.53±3.39	0.4±0.0057	41.63±1.14	1.31±0.171	2.27±0.042
ACR_{sup}	Zero	74.08±0.18	68.55±0.22	1.34±0.00	7.99±0.39	2.01	0.02±0.00	19.07	3.28
	LBTsp-60HR	52.88±0.73	48.77±0.81	0.77±0.18	24.90±0.43	0.05±0.0378	8.98±0.11	2.77±0.205	2.664±0.0115
	LGsp-T9	50.87±1.37	45.43±0.67	0.39±0.08	30.74±0.59	0.08±0.0115	9.50±0.21	3.0±0.123	2.60±0.0264
	MUM 92.13	58.60±2.16	40.97±0.67	0.91±0.06	36.84±1.06	0.07±0.01	14.01±0.09	2.97±0.185	2.60±0.04
$ACR_{n/sup}$	Zero	74.08±0.18	68.55±0.22	1.34±0.00	7.89±0.39	0.04	0.02±0.00	19.31	3.29
	LBTsp-60HR	48.37±0.20	45.50±0.23	0.17±0.26	44.61±1.24	0.12±0.0057	1.52±0.01	14.92±0.08	3.3±0.046
	LGsp-T9	48.17±2.38	41.25±0.01	0.72±0.00	44.88±1.66	0.08±0.01	1.42±0.02	14.17±0.05	3.23±0.082
	MUM 92.13	44.65±0.68	48.65±0.39	0.42±0.03	53.61±0.90	0.06±0.00	3.51±.025	12.30±0.01	2.811±0.315

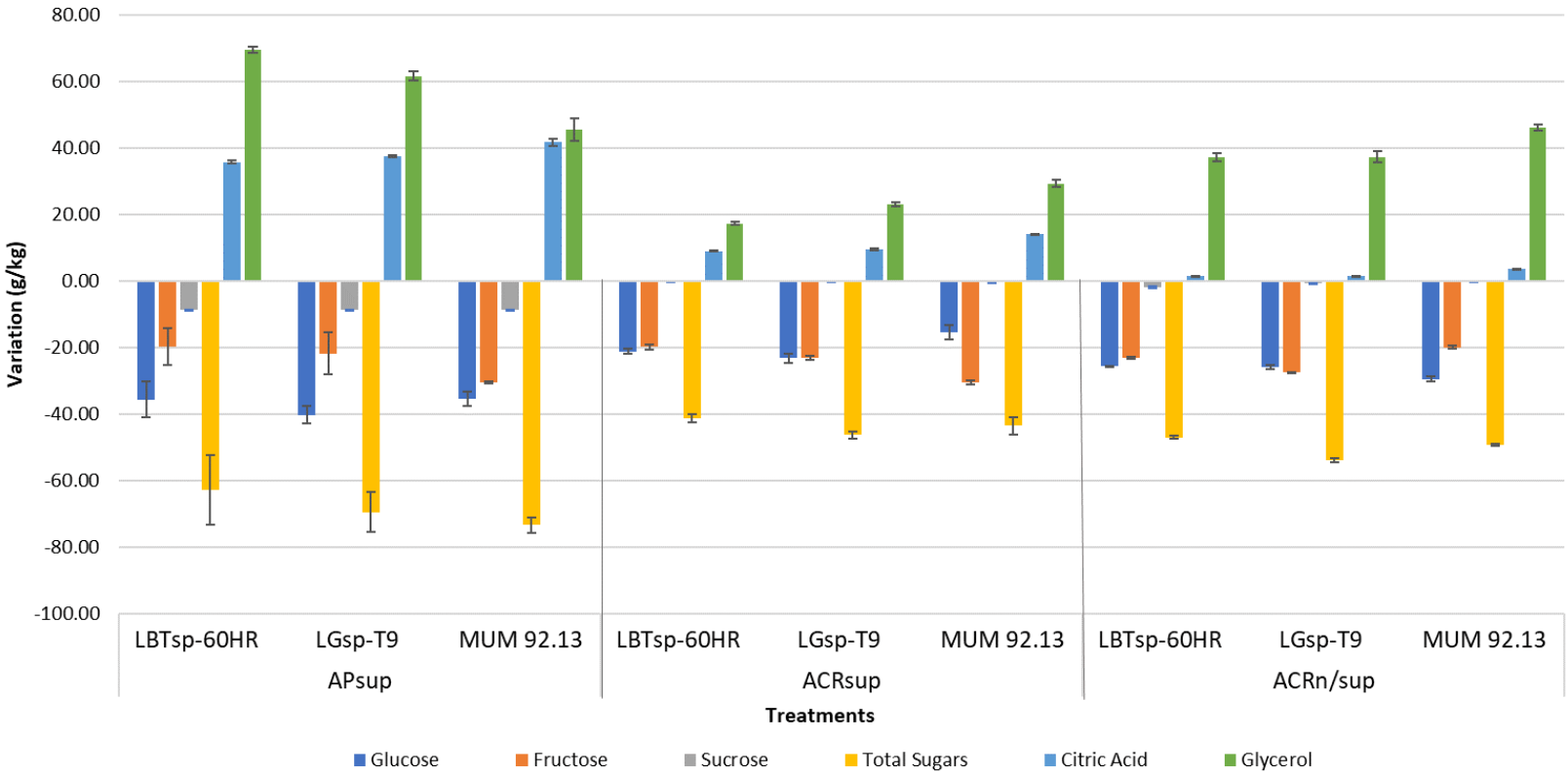

Figure 9. Total sugars consumed and citric acid (CA) and glycerol produced (g/kg) by MUM 92.13, LGsp-T9, and LBTsp-60HR fermentation using supplemented apple pomace (AP_{sup}), supplemented apple cider residue ($ACR_{sup)}$, and non-supplemented apple cider residue ($ACR_{n/sup}$).

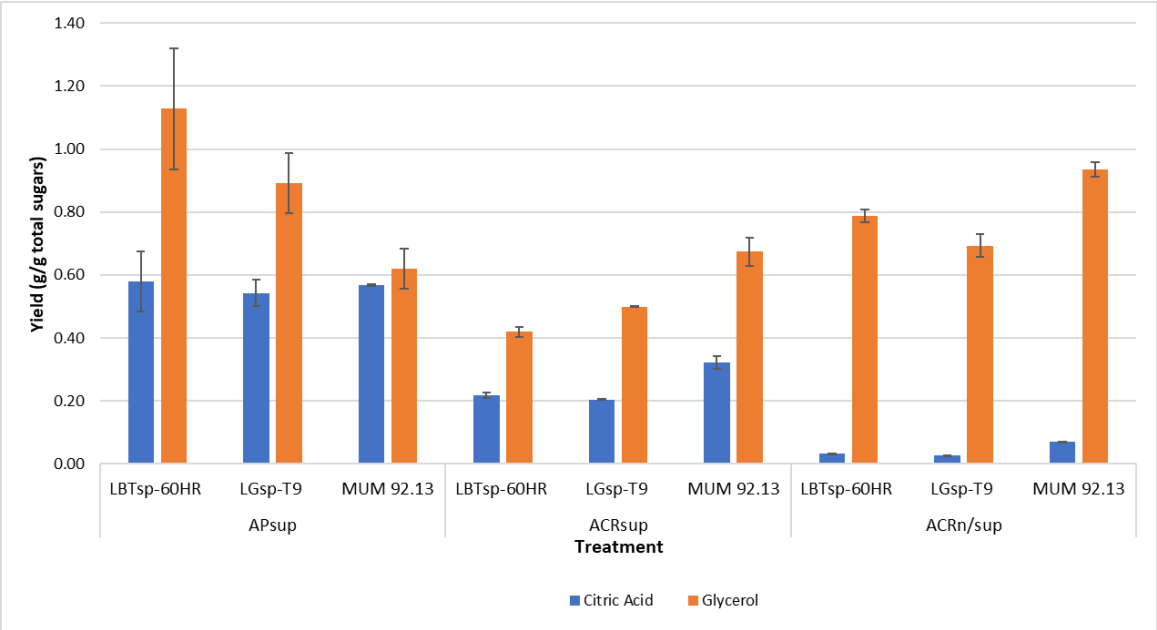

Figure 10. Citric acid (CA) and glycerol yield (g/g total sugars) using supplemented apple pomace (AP_{sup}), supplemented and non-supplemented apple cider residue (ACR_{sup}, $ACR_{n/sup}$) by LBTsp-60HR, LGsp-T9, and MUM 92.13.

AP and ACR contained 108.84 and 143.64 g/kg respectively of total sugars (glucose, fructose, and sucrose). Total sugar consumption from $ACR_{n/sup}$ was lower than AP_{sup} and $ACR_{n/sup}$ (Figure 9). After 4 days of solid-state fermentation, glucose was the most consumed sugar (Figure 9, Table 111), fructose consumption rate was at its highest from ACR_{sup}, and sucrose was totally consumed. In fact, glucose, fructose and sucrose are suitable carbohydrate sources for CA production. For *A. niger* strains, sucrose consumption is preferred over glucose consumption, and only then fructose, in order of decreasing CA yield (Show et al., 2015). However, the total sugar consumption kinetics was not performed in this study to confirm that sucrose was firstly consumed.

Studies developed over several decades have shown that the carbohydrates content affects the CA yield directly (reviewed in Amato et al., 2020 and in Legisa and Mattey, 2007). MUM 92.13 consumed higher amounts of total sugars than LBTsp-60HR and LGsp-T9, and its CA production was the highest among all three strains with all prepared substrates, with a maximum production of 41.61±1.14 g/kg (dw) and a yield of 0.57 ± 0.003 g/g total sugars with AP_{sup} (Figure 9). The highest amount of CA produced with ACR_{sup} did not

exceed 14 g/kg which is a very low amount compared to AP_{sup} and to other mentioned studies. The lowest CA production was observed with $ACR_{n/sup}$ by LGsp-T9 with 1.42 ± 0.02 g/g (dw), which can be explained by the absence of supplements, causing stressful conditions to CA metabolism (Behera et al., 2020).

Meanwhile, a high glycerol production was determined with AP_{sup} by LBTsp-60HR with 69.37 ± 1.05 g/kg and a maximum yield of 1.13 ± 0.19 g/g total sugars. For ACR, the highest glycerol production of 46 ± 0.89 g/kg and a yield of 0.92 ± 0.02g/g total sugars were determined when the supplements were not added ($ACR_{n/sup}$). In a study of CA and glycerol production by *A. niger* using Amberlite as substrate, Gutiérrez-Rojas et al. (1995) reported a maximum glycerol production of 12.5 g/L when using 400 g/L of glucose. Also, Semkiv et al. (2020) and Wang et al. (2001) mentioned that *Saccharomyces cerevisiae* was able to produce 55 g/kg from molasses in batch sulfite with a yield of 0.25 g/g total sugars, 35 g/kg from glucose in batch mode with a yield of 0.23 g/g total sugars, and 80 g/kg of glycerol from molasses in fed batch sulfite/vacuum mode with a yield of 0.25 g/g total sugars. Glycerol production in this study is promising with not only high amounts being produced per kg of substrate but also high yield by sugars consumed. The high concentration of glycerol in the medium is due to its role as an osmoregulatory metabolite (Legisa and Kidric., 1989) and its production by the fungus stimulates CA production. It is responsible for the inhibition of mitochondrial $NADP^+$ dependent isocitrate dehydrogenase, which leads to CA accumulation (Gutiérrez-Rojas et al. (1995). However, when the substrate is not supplemented with an inducer and other trace elements, CA secretion is inhibited and glycerol production is stimulated (Wang et al., 2001).

Malic acid was consumed in high amounts during the fermentation process with AP_{sup} and ACR_{sup} compared to $ACR_{n/sup}$. No studies were found that explain the consumption rate of malic acid in relation with the production of CA. However, Xu et al. (2020) mentioned that the accumulation of malic acid in the medium can be done through the consumption of CA by enhancing the glycolytic flux, which means that malic acid consumption or the inhibition of its production was due to the accumulation of CA. This could explain the low consumption of malic acid in $ACR_{n/sup}$ since there is a low production of CA.

The pH of the substrate after fermentation (Table 11) with AP_{sup} was the lowest, around 2.3, while producing high CA production, which indicates a high fermentation rate, compared to the use of ACR_{sup} where pH levels were near 2.6. As for $ACR_{n/sup}$, pH levels (around 3.11) were almost the same as the initial pH (3.28), in line with the low CA production and indicating a low fermentation rate (Behera et al., 2020). The decrease in pH is due to the consumption of ammonia and the release of protons during the spore germination phase, thereby increasing the medium acidity to favor CA production (Show et al., 2015), which explains the lower pH measures of AP_{sup} than ACR_{sup}. The deviation of pH decrease is dependent on the nature of the engaged microorganism as well as its metabolic functions for accumulating CA in the medium. Also, the nature of the substrate and the production technique influence pH kinetics (Show et al., 2015; Dashen et al., 2013).

The addition of supplements had an impact on fungal growth and morphology as shown in Figure 11(supplemented medium in Figure 11.a and non-supplemented medium in Figure 11.b) This interpretation was confirmed by Behera et al. (2020) and Show et al. (2015), as with optimum concentrations of trace elements fungal growth is inhibited, favoring CA production. While the absence or the presence of non-suitable trace elements concentrations can boost fungal growth rather than the anabolic pathway of CA.

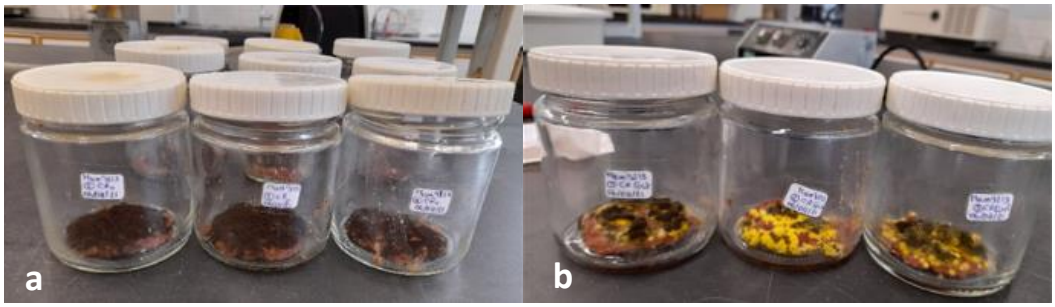

Figure 11. Difference in MUM 92.13 morphology after 4 days of fermentation in supplemented apple cider residue (ACR_{sup}) as solid substrate (a), and non-supplemented apple cider residue ($ACR_{n/sup}$) (b).

IV.4.2. Effect of the substrate and the *A. niger* strain on CA and glycerol production

To understand the effect of both substrate (AP and ACR) and *A. niger* strains involvement in CA and glycerol production, a 3 (substrate: AP_{sup}, $ACR_{sup,}$ and $ACR_{n/sup}$) by

3 (*A. niger* strain: LBTsp-60HR, LGsp-T9 and MUM 92.13) levels factorial ANOVA was conducted. This analysis allows to study the reaction of the *A. niger* strains as a function of the consumed substrate to produce both CA and glycerol, to study their effect and interactions towards each other. The result of the analysis is presented in Table 100. A Scheffe test was conducted to evaluation the significant difference in between AP_{sup}, ACR_{sup} and $ACR_{n/sup}$ means and LBTsp-60HR, LGsp-T9, and MUM 92.13 means, as shown in Table 12 and Table 13.

Table 12. Scheffe test; multiple comparison between substrates based on observed means with citric acid (CA), and glycerol as dependent variables.

		CA		glycerol	
Substrate i	Substrate j	Md	p	Md	p
AP_{sup}	ACR_{sup}	27.447*	<.001	33.7351*	<.001
	$ACR_{n/sup}$	36.127*	<.001	17.4118*	<.001
ACR_{sup}	AP_{sup}	-27.447*	<.001	-33.7351*	<.001
	$ACR_{n/sup}$	8.680*	<.001	-16.3233*	<.001
$ACR_{n/sup}$	AP_{sup}	-36.127*	<.001	-17.4118*	<.001
	ACR_{sup}	-8.680*	<.001	16.3233*	<.001

Md: Mean difference, p: significance level, *: The mean difference is significant at the 0.05 level.

Table 13. Scheffe test; multiple comparison between *A. niger* strains based on observed means with citric acid (CA), and glycerol as dependent variables.

		CA		glycerol	
Strain i	Strain j	Md	p	Md	p
MUM92.13	LGsp-T9	3.5833*	<.001	-1.0226	0.832
	LBTsp-60HR	4.3067*	<.001	-0.4439	0.393
LGsp-T9	MUM92.13	-3.5833*	<.001	0.4439	0.832
	LBTsp-60HR	0.7233*	<.001	-0.5787	0.734
LBTsp-60HR	MUM92.13	-4.3067*	<.001	1.0226	0.393
	LGsp-T9	-0.7233*	<.001	0.5787	0.734

Md: Mean difference, p: significance level, *: The mean difference is significant at the 0.05 level.

Based on the ANOVA analysis for CA production (Table 10), there is a significant main effect of the substrate composition (

Table 12), such that $ACR_{n/sup}$ (M=2.151, SD=1.02) and ACR_{sup} (M=10.83, SD=2.39) have a significantly lower effect on CA production compared with the AP_{sup} (M=38.27, SD=2.71), which is considered as a control substrate, having the highest main effect on CA production. The effect of *A. niger* strains is also significant (Table 13), F (2.18) =250.73, p<0.001, where MUM 92.13 (M=19.72, SD=17.06) has a significant higher CA production ability than LGsp-T9 (M=16.13, SD=16.38) and LBTsp-60HR (M=15.41, SD=15.57) (figure12). In addition, the interaction effect (substrate*strain) is significant (Table 10) indicating that the ability of producing CA using ACR_{sup} and $ACR_{n/sup}$ as substrates are not as promising as using AP_{sup} for all strains. Furthermore, the use of supplements has a significant effect on CA production for MUM 92.13 more than for LBTsp-60HR and LGsp-T9 (Figure 12). Thus, the production efficiency of CA by *A. niger* does not only depend on the substrate nature but also on the capacity of the strain to produce and accumulate CA.

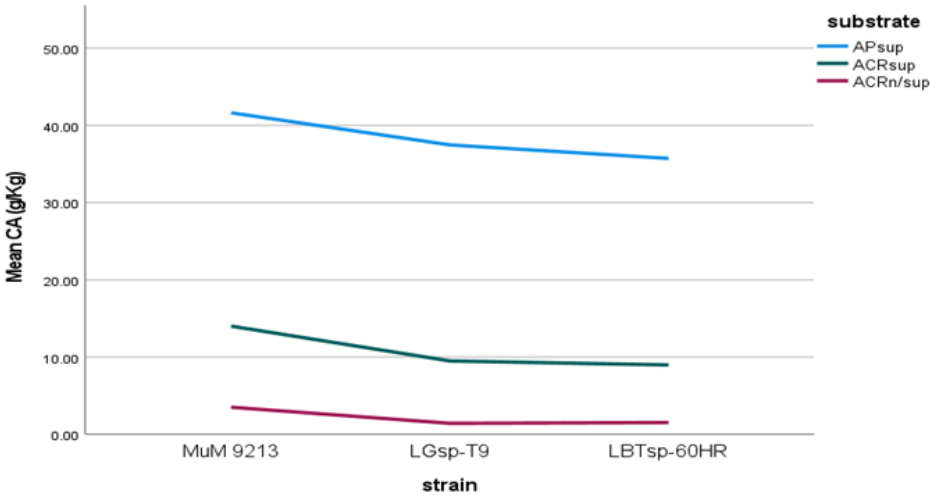

Figure 12. Multiple line Means of citric acid (CA) production by strain by substrate, showing the effect of each substrate on the strain to produce citric acid.

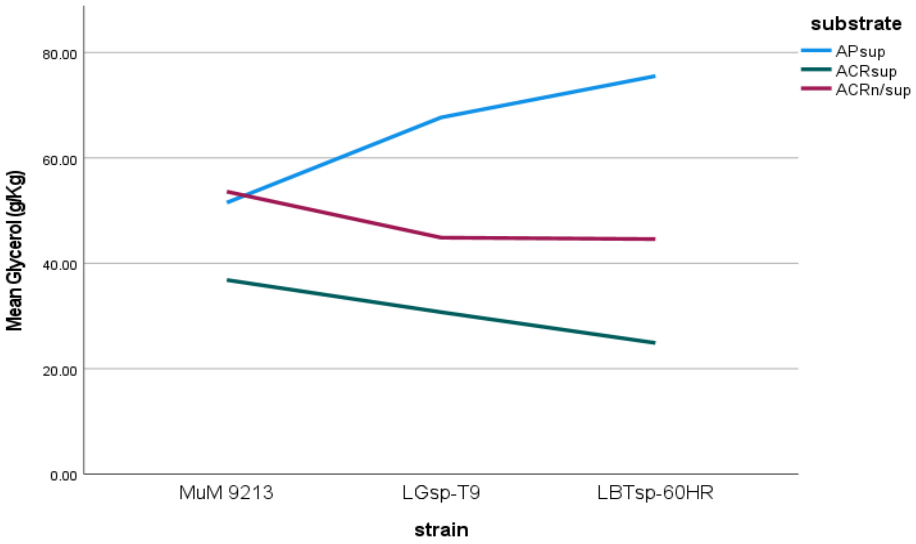

Figure 13. Multiple line Means of glycerol production by strain by substrate, proving the effect of each substrate on the strain to produce glycerol.

As previously mentioned, glycerol is also a very important molecule that is produced in distinct amounts in the different treatments, therefore a second 3 (substrate: AP_{sup}, $ACR_{sup,}$ and $ACR_{n/sup}$) by 3 (*A. niger* strain: LBTsp-60HR, LGsp-T9 and MUM 92.13) levels factorial ANOVA was conducted to study the reaction of *A. niger* strains as a function of the consumed substrate to produce glycerol. Based on the ANOVA results (Table 10), there is a significant main effect of the substrate composition, such that AP_{sup} (M=57.2215, SD=10.77) has overall the highest significant effect on glycerol production followed by $ACR_{n/sup}$ (M=39.8098, SD=4.57) compared to ACR_{sup} (M=23.4865, SD=5.21), which means that supplements inclusion in the ACR inhibits the production of glycerol (Figure 13).

The effect of the *A. niger* strains is not significant, as shown in Table 10, which is confirmed by the Scheffe (Table 13): there is no significant difference when using MUM 92.13 (M=39.68, SD=7.89), LGsp-T9 (M=40.13, SD=16.06) or LBTsp-60HR (M=40.71, SD=21.99) as glycerol producers. However, the interaction effect (substrate*strain) on glycerol production is significant (Table 10) meaning that using $ACR_{n/sup}$ has a significantly greater impact on glycerol production by MUM 92.13 than ACR_{sup} and AP_{sup} (Figure 13). In that order, MUM 92.13 has proven a higher performance

in CA and glycerol production in all 3 prepared substrates. For this reason, further experiment development will be conducted using MUM 92.13.

IV.5. Analysis of AP and ACR mixtures as substrates for CA and glycerol production by MUM 92.13

IV.5.1. Fermentation outcome and total sugars consumption

An analysis was made to determine the production of CA and glycerol and the consumption of sugars using AP_{sup}, $AP_{n/sup}$, 75%AP+25%ACR_{sup}, 75%AP+25%$ACR_{n/sup}$, 50%AP+50%AP_{sup} ,50%AP+50%$ACR_{n/sup}$, ACR_{sup} and $ACR_{n/sup}$ as solid substrates with 75% moisture, by MUM 92.13. Table 155 summarizes the results obtained for the various compounds, as determined by HPLC. Figure 14 shows the results in terms of consumption of sugars and production of CA and glycerol, and Figure 15 shows the yield of CA and glycerol, in g of compound produced by g of sugars consumed.

Table 14. Analysis of variance in between subjects (substrate mixture, and presence of supplements); nature of substrate and the presence of supplements in the medium effect.

	CA				glycerol			
	df	MS	F	p	df	MS	F	p
Substrate	3	142.132	123.346	<.001	1	1330.232	1811.580	<.001
Supplements	1	811.253	704.025	<.001	3	77.572	105.642	<.001
Interaction (substrate×supplements)	3	11.328	9.830	0.005	8	299.827	408.321	<.001
Error	8	1.528			8	0.734		
Total	16				16			
Corrected Total	15				15			

df: degree of freedom, MS: mean square, F: fisher ratio = (between groups)/ (within groups), p: significance level.

Table 15. Composition of supplemented and non-supplemented substrates fermented by MUM 92.13: 100% apple pomace (100% AP), 75% apple pomace+25% apple cider residue (75%AP+25%ACR), 50% apple pomace+50% apple cider residue (50% AP+50% ACR), and 100% apple cider residue (100% ACR). Values represent average (n=2) ± standard deviation in dry weight basis.

Substrate	supplements	Glucose	Fructose	Sucrose	Glycerol	Ethanol	CA	Malic acid	pH
100% AP	Zero	43.90±0.00	53.63±0.00	7.54±0.30	2.93±2.41	0.33*	0.06±0.04	5.58±0.28	3.39±0.016
	sup	1.06±0.06	16.49±1.18	0.65±0.23	24.09±0.46	0.12±0.007	32.39±0.23	1.10±0.283	2.52±0.20
	n/sup	4.11±0.18	13.68±0.72	1.19±0.11	7.43±0.26	0.03±0	14.39±0.68	0.43±0.042	2.79±0.034
75% AP+25% ACR	Zero	45.41±3.13	62.79±1.73	1.36±0.00	3.77±0.19	0.32*	0.05±0.04	4.97±0.22	3.69±0.084
	sup	1.40±0.56	19.52±0.09	0.83±0.02	30.12±0.71	0.02±0.007	24.87±1.24	0.33±0.134	2.52±0.088
	n/sup	18.95±0.95	49.59±0.90	0.77±0.32	33.45±1.44	0.03±0	13.94±0.10	3.53±0.102	2.75±0.101
50% AP+50% ACR	Zero	53.09±1.47	58.54±0.95	1.17±0.02	6.70±0.09	0.31*	0.02±0.00	4.13±0.00	3.61±0.064
	sup	2.37±0.09	15.47±0.65	0.74±0.01	56.28±0.38	0.03±0	21.09±2.67	0.35±0.007	2.38±0.055
	n/sup	35.85±2.61	49.03±2.78	1.15±0.19	61.51±0.44	0.03±0.007	4.97±0.05	3.20±0.537	2.59±0.113
100% ACR	Zero	31.73±0.47	30.88±0.00	1.54±0.02	3.23±0.03	0.30*	1.66±0.09	3.60±1.40	3.49±0.023
	sup	1.94±0.71	5.22±0.77	0.48±0.20	12.66±0.65	0.02±0.01	16.25±0.25	5.43±0.084	2.48±0.024
	n/sup	28.82±1.02	19.05±0.18	0.63±0.01	38.37±1.50	0.03±0.01	4.32±0.04	12.05±0.26	2.45±0.002

*: Ethanol concentration present only in the supplemented substrates before fermentation

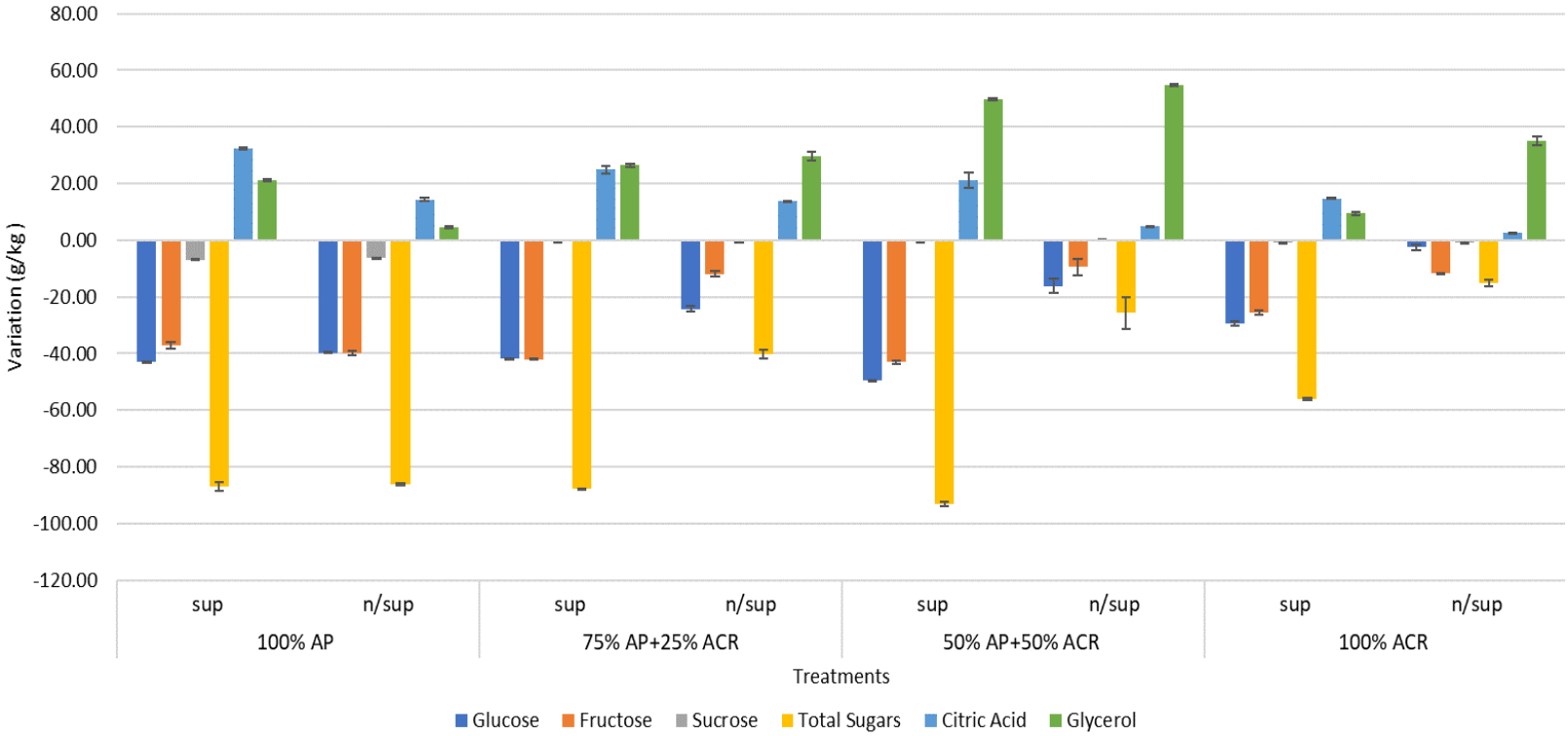

Figure 14. Total sugars consumed and citric acid (CA) and glycerol produced (g/kg) by MUM 92.13 fermentation using supplemented and non-supplemented substrates: 100% apple pomace (100% AP), 75% apple pomace+25% apple cider residue (75%AP+25%ACR), 50% apple pomace+50% apple cider residue (50% AP+50% ACR), and 100% apple cider residue (100% ACR).

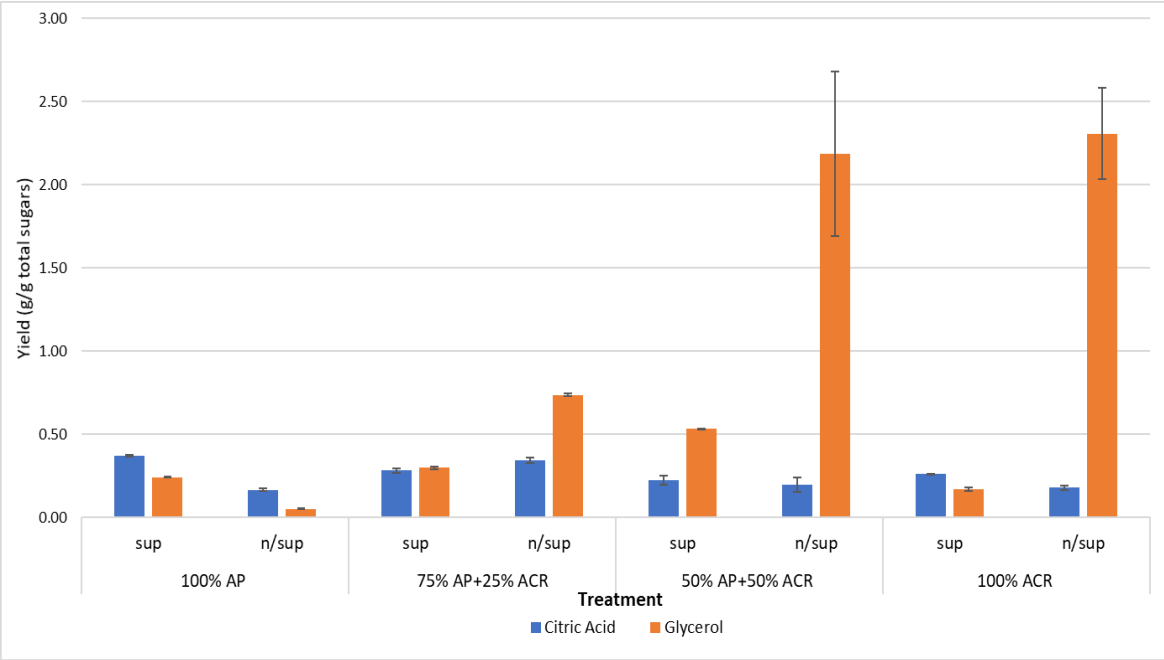

Figure 15. Citric acid (CA) and glycerol yield (g/g total sugars) obtained by MUM 92.13 fermentation using supplemented and non-supplemented substrates: 100% apple pomace (100% AP), 75% apple pomace+25% apple cider residue (75%AP+25%ACR), 50% apple pomace+50% apple cider residue (50% AP+50% ACR), and 100% apple cider residue (100% ACR).

The association of different sugar concentrations in the medium (75%AP+25%ACR and 50%AP+50%ACR) has shown lower sucrose consumption than when using AP and ACR by themselves. Using supplements in all different substrate mixture resulted in higher glucose consumption (around 50 g/kg) compared to the non-supplemented medium (less than 3 g/kg) (Figure 14). And fructose consumption from 50%AP+50%ACR_{sup} was the highest among all substrate mixtures (around 43 g/kg), while 50%AP+50%$ACR_{n/sup}$ presented the least consumed fructose among all studied substrates in this experiment (Figure 14). The total amount of sugars present in AP (Table 155) is clearly lower than in the AP used in the previous experiment even though they are the same prepared matrix (cf. section III.1.1). The decrease is explained by the chemical reaction that can occur with time, resulting in partial degradation of sugars even in the dry material. This decrease in the sugar content, especially sucrose, has ultimately affected CA production using AP (Table 155).

After the fermentation of the various substrate mixtures by MUM 92.13, none of the substrates with mixed AP and ACR delivered a higher CA production than AP_{sup} (control)

(Table 15) with a maximum production of 24 ± 1.24 g/kg with 75%AP+25%ACR_{sup}. The CA yield of 75%AP+25%$ACR_{n/sup}$ is similar to the CA yield of AP_{sup}.

$ACR_{n/sup}$ presented the highest glycerol yield (Figure 15), which is also higher than the yield achieved when using 2% of methanol as an inducer (*cf.* section IV.5; Figure 10). The highest glycerol production was with 50%AP+50$ACR_{n/sup}$ with 61.51± 0.44 g/kg and a yield of 2.19 ± 0.49 g/g total sugars, while $AP_{n/sup}$ produced low amount of 7.43 ± 0.26 g/kg.

Even though MUM 92.13 did not produce CA when mixing substrate and when using 0.3% ethanol (natural inducer), as high as the previous experiment (using 2% methanol cf. section III.2), it did present promising glycerol production (Table 155) with 50%AP+50% $ACR_{n/sup}$ use. A promising glycerol yield was achieved with $ACR_{n/sup}$ which is considered to be the highest obtained yield in this study (Figure 15). With that, it can be concluded that supplements can be dispensed and that the inducer will be provided naturally for further optimization of the glycerol production process.

IV.5.2. Effect of the type of substrate and supplementation on CA and glycerol production by *Aspergillus niger* MUM 92.13

To understand the effect of both supplements in the substrate and nature of the substrate (100%AP, 75%AP+25%ACR, 50%AP+50%ACR, 100%ACR) in CA and glycerol production, a 4 (substrate: 100%AP, 75%AP+25%ACR, 50%AP+50%ACR, 100%ACR) by 2 (treatment: supplemented, non-supplemented) levels factorial ANOVA was conducted. This analysis allows to study the reaction of the supplements as a function of the consumed substrate mixture to produce CA and glycerol, and to study their effect and interactions towards each other. The results of the analysis are presented in Table 14, Table 16 and Table 17.

Table 16. Scheffe test; multiple comparison in between substrate mixture based on observed means with citric acid (CA), and glycerol as dependent variables.

		CA		Glycerol	
Substrate I	Substrate J	Md	p	MD	p
100% AP	75%AP+25%ACR	6.6050*	0.000	-16.7600*	0.0000
	50%AP+50%AP	-3.7325*	0.009	-43.0075*	0.0000
	100%ACR	9.3425*	0.000	-11.1400*	0.0000
75%AP+25%ACR	100% AP	-6.6050*	0.000	16.7600*	0.0000
	50%AP+50%AP	-10.3375*	0.000	-26.2475*	0.0000
	100%ACR	2.7375*	0.045	5.6200*	0.0001
50%AP+50%AP	100% AP	3.7325*	0.009	43.0075*	0.0000
	75%AP+25%ACR	10.3375*	0.000	26.2475*	0.0000
	100%ACR	13.0750*	0.000	31.8675*	0.0000
100%ACR	100% AP	-9.3425*	0.000	11.1400*	0.0000
	75%AP+25%ACR	-2.7375*	0.045	-5.6200*	0.0001
	50%AP+50%AP	-13.0750*	0.000	-31.8675*	0.0000

Md: Mean difference=I-J, p: significance level, *: the mean difference is significant at the 0.05 level.

Based on Table 14 and Table 16 we can confirm that both factors have a significant effect on CA and glycerol production. In fact, AP (M=23.39, SD=0.39) had the highest significant effect on CA production but the lowest significant effect on glycerol production (M=15.75, SD=9.62), while mixing 75%AP+25%ACR (M=19.40, SD=6.34) had a higher significant effect on CA than when mixing 50%AP+ 50% ACR (M=13.02, SD=9.43) (figure16). However, glycerol production was significantly affected by a 50%AP+50% ACR mixture (M=58.89, SD=3.03) more than a 75%AP+25%ACR mixture (M=31.78, SD=2.13) and ACR (M=25.51, SD=14.8) (figure 17).

Conjointly supplements added with 0.3% ethanol to the medium had a significant main effect on glycerol production. In fact, supplements have a significantly higher effect on

CA production (M=23.65, SD=6.41) than when the media are not supplemented (M=9.40, SD=5.1) (Figure 16), which is confirmed by an independent t-test (

Table ***17***), that showed that the magnitude of the differences in the means were significant. A Scheffe test was not performed for supplements because there were fewer than three groups. Therefore, a t-test was performed.

Table 17. Independent samples t-test performed to identify the mean difference in CA and glycerol production when supplements are present or absent in the medium.

		glycerol	CA
		Equal variances assumed	Equal variances assumed
F		0.176	0.030
p		0.681	0.865
t		-0.471	4.918
df		14	14
p (2-tailed)		0.645	0.000
Mean Difference		-4.40375	14.24125
Standard Error Difference		9.35037	2.89580
95% Confidence Interval of the Difference	Lower	-24.45831	8.03037
	Upper	15.65081	20.45213

F: fisher ratio, df: degree of freedom, t: student test

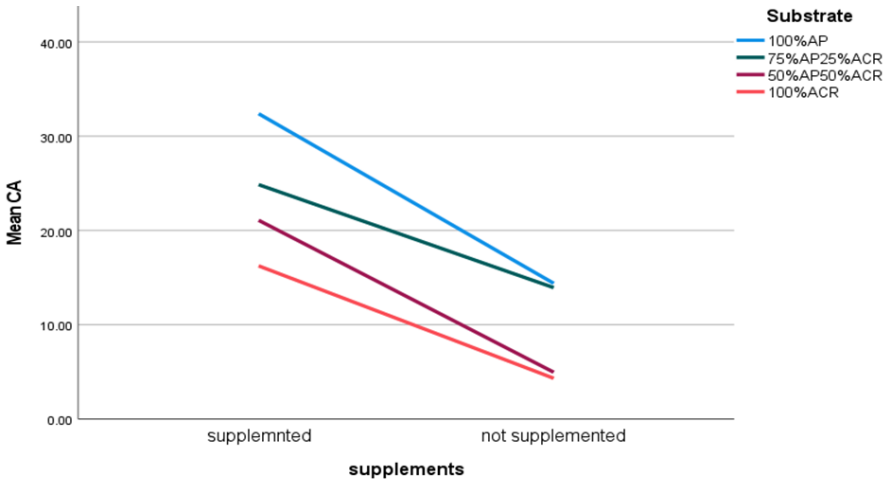

Figure 16. Multiple line Mean of citric acid by supplements by substrate. Demonstrating the significant impact of the supplements on all substrates to induce citric acid (CA) production (g/kg).

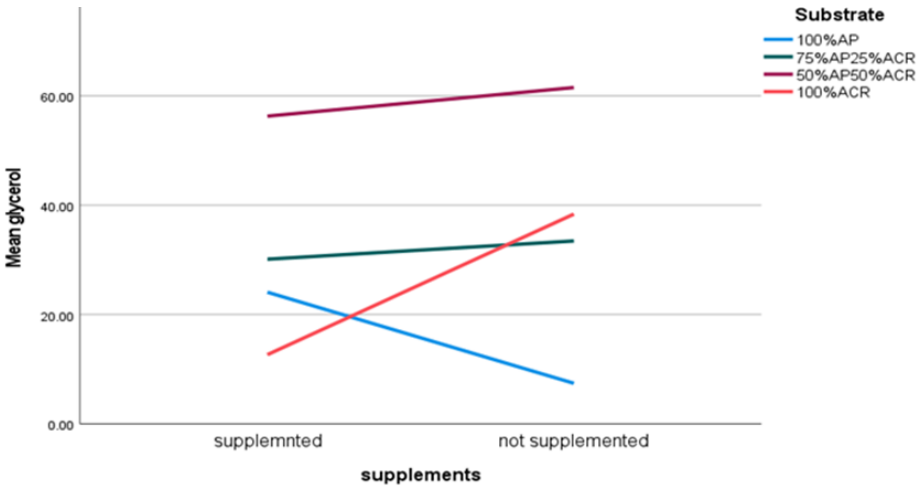

Figure 17. Multiple line alignment of glycerol by supplements presentencing negative and positive effect on the substrate to produce glycerol.

Oppositely, for glycerol production, the presence of supplements (M= 26.65, SD=16.63) had a lower effect than its absence (M= 35.19, SD=20.55) in the medium (Figure 17). However, the difference was not considered significant based on the conducted independent t-test, where the magnitude of the differences in the means was very small. With that, it can be observed that a higher CA production requires the presence of supplements, while for a higher glycerol production supplements should not be used.

V. Conclusions and future work

During recent years, apple cider industries have been witnessing an increase demand, reaching a global market value of 10,667 million US$ in 2016. However, such production has annually generated around 0.35 million hectoliters of apple cider residue in Europe only. Their accumulation in soil results in an uncontrolled fermentation that releases around 1,841 tons of CO_2 emission per year. Therefore, ACR needs to be integrated into the circular-economy course of action.

In-depth AP and ACR analyses in terms of fermentable sugars, acids, and alcohol content were carried out in the present study. Primarily, AP was used as a control substrate in solid state fermentation to test CA production using multiple local *Aspergillus niger* strains; S09-6, LBL4°/T9, LBTsp-60HR, *A. niger*, LGsp-T9, S11-2, and MUM 92.13, in the same fermenting conditions as Dhillon et al.(2011b) set, since AP is a well-studied by-product for its potential for providing optimal nutritional conditions for a maximum CA production yield.

The results revealed that among all strains MUM 92.13 presented the highest CA production of 41.61±1.14 g/kg with a yield of 0.57±0.0003 g/g total sugars with AP_{sup}. During the second set of experiments, glycerol production was found to be prominent with a production of 69.37±1.052 g/kg using AP_{sup}, while ACR did not show high CA production, with a maximum production of 14.01±0.09 g/kg and a yield of 0.32±0.02 g/g total sugars. However, interesting, glycerol production was detected using $ACR_{n/sup}$ (46.61±0.90 g/kg).

Other beneficial by-products, such as pyranonigrin, fonsecin, kojic acid, and pyrophen were also determined, and could be the target of a future study. Hence, the next step of the study included glycerol production since it is a very used compound in multiple industries such as cosmetics, paint, automotive, food, and textile industries (Wang et al., 2001).

Furthermore, in mixtures of both by-products – AP and ACR –, the inducer ethanol was provided naturally by ACR and used to study the impact of both variety of carbohydrates amount and the natural inducer effect in the fermentation medium using MUM 92.13. Results proved that using both prepared mixtures did not induce high CA production, which did not

exceed 24.81±1.24 g/kg compared to when using AP_{sup} as a solo substrate that reached 32.33±0.23 g/kg and a yield of 0.37±0.004 g/g total sugars. It is also lower than the predetermined CA value when the inducer is 2% of industrial methanol. The highest glycerol production was obtained with 50%AP+50%$ACR_{n/sup}$ (54.81±0.44 g/kg) with a high production yield of 2.19±0.49 g/g total sugars, and with $ACR_{n/sup}$ with a yield of 2.31±0.27 g/g total sugars.

It can be concluded that the use of ACR as a solo substrate, with no use of supplements, seems to be more promising in glycerol production than in CA production, while bringing economic benefits resulting from the unnecessary addition of supplements. Further optimization of the process can be done taking in consideration the produced beneficial by-products pyranonigrin, fonsecin, kojic acid, and pyrophen, through studying the corresponding production kinetics.

Other potential studies can be focused on MUM 92.13 because it presents an interesting aptitude to produce both CA and glycerol. A genetic modification similar to *A. niger* NRRL567 can potentially upgrade it to an industrial level. Consequently, the environmental impact of both AP and ACR would significantly deflate while serving the economy through the generation of high-added-value and eco-friendly products.

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