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Itaconic acid production by Aspergillus terreus from low-cost carbon and nitrogen sources

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Abstract— **Itaconic acid (IA) is a biobased building block of great interest for polymer of thermoplastic, resin, chelating agents, superabsorbent polymers (SAP), among many others. This study evaluated IA production by Aspergillus terreus with low-cost carbon – granulated sugar and VHP (very high polymerization) sugar – and nitrogen sources – ammonium nitrate and commercial urea. The highest final IA concentration was obtained with the combination of granulated sugar and NH4NO3 (40 g/L IA), while the fermentation of VHP sugar and NH4NO3 produced 25 g/L IA. The use of commercial urea resulted on low IA titers (about 8 g/L IA) with either granulated sugar or VHP sugar. Despite achievement of the highest values of IA production in granulated sugar medium, the lower cost of VHP motivated the evaluation of two different inoculum methods preparation to improve IA (spores and mycelia). The inoculum with mycelia resulted in 45 g/L IA, equivalent to the value obtained with granulated sugar inoculated with spores. Moreover, the productivity was over 3 times higher with mycelia instead of with spores (respectively 0.19 and 0.06 g/L/h). The yield of IA from the fermentation of VHP and NH4NO3 inoculated with mycelium was 53% of the theoretical. This study demonstrates the potential of VHP sugar as carbon source for IA production, which may be applied in a biorefinery concept.**

Index Terms— **Aspergillus terreus, biorefinery, itaconic acid, VHP sugar..**

I. Introduction

The chemical industry gradual shift from petrochemicals to biobased chemicals, whether for economic or environment matter, or both, driving a great boost to develop new products as well as to improve the production or processes of the existing ones. In this scenario, itaconic acid (IA) is one of the building blocks of highest industrial interest [1][2][3]. Expected to have a global market over 216 billion by 2020 [4], the improvement of market competition with other products from renewable sources and from petrochemicals could be achieved with the decrease of feedstock costs with IA production [5].

Commercial IA is obtained by microbial fermentation, mainly by *Aspergillus terreus* with glucose [6], although sucrose is also a possible feedstock [7]. The use of residual materials as feedstocks could be an alternative for decreasing the end-product cost. Nevertheless, the high sensitivity of *A. terreus* to medium impurities impairs the achievement of high IA production yields for different residual feedstocks [8]. For example, the use of molasse [9] or lignocellulosic material [10] requires a pretreatment to remove the impurities, which leads to an increase of production costs [8]. On the other hand, pure carbon sources such as glucose or sucrose may result in greater costs of the end-product, especially because IA production demands high initial concentration of substrate – over 100 g/L for sugars [11]. The use of low cost feedstocks such as granulated sugar or VHP (very high polymerization) sugar, to IA production is proposed on this study.

VHP is a not refined sugar produced on sugarcane industrial plants, which contains more than 99% (wt.) of sucrose [12]. It is the most exported sugar in the world, and it is refined to produce different types of sugar, including the granulated one [13].

The strong recommendation for reducing sugar consumption by humans [14] has decreased the demand of that product in food industry over the years in some countries such as Norway, Canada, India and Brazil [15]. This fact has been one of the subjects to the proposal of European Union governments to redirect part of the sugar production for bioethanol industry and to other fermentative processes [16]. The application of easily assimilated carbon sources, such as those containing sucrose substrate, at a biorefinery concept could be a strategy for the improvement of sugar industry. IA, one of the most promising precursors for bio-based polymers, could be included on the sugarcane industry portfolio.

Besides the requirement of a low-cost feedstock, the improvement of nutritional factors should also be considered for increasing IA market competitivity [11]. The use of a proper nitrogen source is also of great interest as it is used mainly for cell synthesis. In a production medium, the overestimation of the components initial concentration leads to economic loss either by the decrease in the product yield and the cost associated to the extra supplementation of the medium [17].

This study evaluated IA production with two different carbon sources (granulated sugar or VHP) combined with two different nitrogen sources (NH_4NO_3 or commercial urea sold as fertilizer). The influence of fungal inoculum, mycelial or spores, on IA production was subsequently determined. Also, the inhibition effect of IA on the metabolism of *Aspergillus terreus* NRRL 1960 was evaluated.

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II. Materials and Methods

A. Microorganism

Aspergillus terreus NRRL 1960 was acquired from the ARS (NRRL) Culture Collection. The inoculum, unless indicated otherwise, consisted of fresh spores prepared by propagating stock spores (maintained at 4°C) in PDA medium for 6 days at 33°C. Inoculum with mycelia was done with 5% (v/v) of 20 h old fermented broth prepared by seeding 10^6 spores/mL on production medium.

B. Fermentation

The assays were performed using granulated sugar or VHP as carbon sources, and NH_4NO_3 or commercial urea sold as fertilizer (Dimy, São Paulo-Brazil) as nitrogen sources. The assays with pure sucrose was used as standard, for comparison. The production media composition contained per liter of distilled water: 114 g equivalent sucrose (all carbon sources were considered to contain 100% of sucrose), 3 g NH_4NO_3 or 2.34 g urea, 0.1 g KH_2PO_4, 1 g $MgSO_4$.7 H_2O, 0.008 g $ZnSO_4$.7 H_2O, 0.015 g $CuSO_4$.7 H_2O, 5 g $CaCl_2$. 2 H_2O, 0.00167 g $FeCl_3$.6 H_2O. To evaluate the inhibition effect of IA, 40 g/L of IA (Sigma) was added to medium composed of VHP and NH_4NO_3. The pH of medium was adjusted to 3.0 with H_2SO_4 1M, or NaOH 1M for the experiment with initial addition of IA.

The production media (100 mL) was distributed in 500 mL Erlenmeyer flasks and then sterilized for 20 min. at 111°C (0.5 atm). The $FeCl_3.6H_2O$ solution was separately sterilized by filtration – to avoid precipitation – with 0.1 µm cellulose acetate membrane, which was added to sterile medium before medium inoculation. Fermentation was performed on rotary shaker at 150 rpm and 33°C.

C. Analytical methods

The quantification of ion composition in VHP was done by inductively coupled plasma optical emission spectrometry (ICPOES) with the equipment of Horiba Jobin Yvon, model Ultima 2.

Biomass concentration was determined by filtering the fermented broth with cellulose acetate membrane disks (pores of 0.45 µm, 47 mm) dried at 105°C to constant mass.

IA and sugars concentrations were quantified from the fermented broth, which was filtered with 0.22 µm pore size before the analysis by HPLC (Agilent, model 1260 infinity) with a Bio-Rad HPX-87H column. It was used as mobile phase 5mM H_2SO_4 at a flow rate of 0.7 mL/min and 65°C. Detection was done by refractive index detector (RID).

III. Results and Discussion

A. IA production in pure sucrose medium

IA production by *A. terreus* NRRL 1960 (Fig. 1) showed a 2-days of lag phase followed by an exponential growth phase until the 9th day. Cell growth exhibited a different profile with no lag phase and maximum biomass reached by the 4^{th} day considering the error bars. That behavior is consistent with previous literature, which has shown that high yields of IA are achieved when cell production reaches a stationary growth phase or slow growth rate [18],[19].

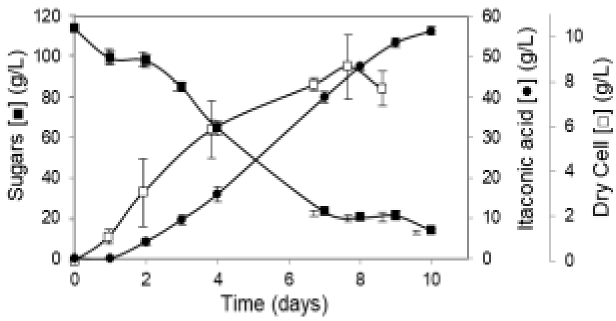

Fig.1 Itaconic acid production in pure sucrose medium by *Aspergillus terreus* NRRL 1960.

The theoretical yield of IA production from glucose is 1 mol of glucose for 1 mol of IA [5],[20], thus, the maximum theoretical yield of IA synthesized by *A. terreus* with sucrose may be 1 mol of sucrose to produce 2 mols, i.e., 0.76 g IA/ g sucrose. Therefore, the result of 0.56 g IA/g sucrose obtained in this study is 74% of the maximum theoretical yield, indicating that sucrose is also a possible substrate for high yield IA production. Moreover, the result with sucrose in this study was greater than the results in 180 g/L initial glucose medium in 1.5 L stirred tank reactor with the same strain (72% of the maximum yield) [18].

B. IA production in different carbon and nitrogen sources

This experiment combined two different carbon, cheaper than sucrose, and two nitrogen sources to evaluate IA production. The fermentations were performed with (a) granulated sugar and NH_4NO_3, (b) VHP and NH_4NO_3, (c) granulated sugar and commercial urea and (d) VHP and commercial urea (Fig 2).

The four conditions showed likewise a lag phase for IA production (Fig. 2), also similar to the fermentation of pure sucrose medium (Fig. 1). Nonetheless, their IA profiles were rather distinct, as well as the pH variation along the fermentation.

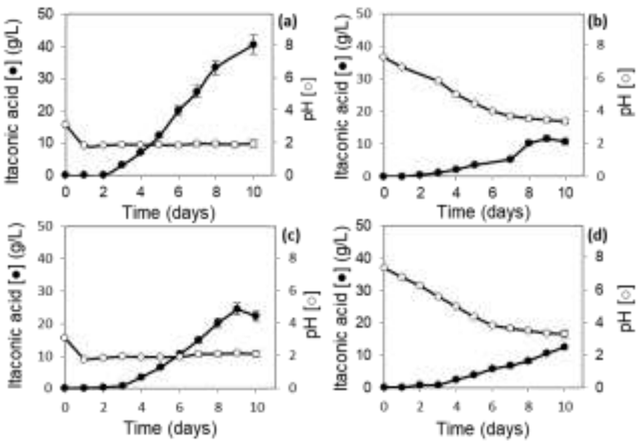

Fig. 2 Itaconic acid production by *Aspergillus terreus* with different carbon and nitrogen sources: granulated sugar and NH_4NO_3 (a), granulated sugar and commercial urea (b), VHP

and NH_4NO_3 (c), VHP and commercial urea (d).

The combination of granulated sugar and NH_4NO_3 resulted in the highest IA production (Fig. 2a) among all four combinations, which was 30% less IA than the fermentation with pure sucrose (Fig. 1). The pH value had a significant drop from 3 to 2 at the first day of fermentation, remaining unchanged until the end of fermentation.

The use of commercial urea instead of NH_4NO_3 with granulated sugar (Fig. 2b) resulted in a final 70% less IA concentration. Those results indicated that the use of commercial urea, under tested conditions, was less appropriated for IA production than NH_4NO_3, but it did not impair it. References [21] and [22] also reported low IA yields in fermentation with urea, although the authors did not present the IA production values obtained.

The combination of VHP and NH_4NO_3 (Fig. 2c) resulted in the second highest IA production considering the combinations evaluated. IA production reached 25 g/L before it was consumed by the microorganism on the 10^{th} day. The pH profile was almost identical to the one presented on Fig 2 (a), which contained the same nitrogen source.

The final IA titer in VHP and urea fermented medium (Fig. 2d) was equivalent to that obtained with the same nitrogen source and granulated sugar, i.e., 12 g/L. Also, the pH profile was similar to that observed in medium formulated with the same nitrogen source, but the other carbon source (Fig. 2 b).

The IA productivity values from the different media (Table 1) reinforced that granulated sugar and NH_4NO_3 were the best combination of carbon and nitrogen sources. The use of urea impaired IA production in both media containing this nitrogen source.

Table 1 IA productivity values for *Aspergillus terreus* NRRL 1960 cultivated in granulated sugar or VHP sugar as carbon sources, and NH_4NO_3 or urea after 9 (b and c) or 10 days (a and d).

Carbon source / Nitrogen source	Granulated sugar (g/L/h)	VHP sugar (g/L/h)
NH_4NO_3	0.10	0.06
Urea	0.04	0.05

As mentioned before, medium impurities may prevent IA production by *A. terreus* [8]. Granulated sugar and VHP have different content of impurities, as the first is the refined form of the second. Considering the kinetic profiles and productivity values for the two carbon sources with NH_4NO_3 (Fig. 2a and 2b, and Table 1), the best results were obtained from granulated sugar fermentation, i.e., the source with less contaminants, but whose content of impurities is greater than the pure sucrose (Fig. 1). On the other hand, the fermentation of VHP (Fig. 2c) produced almost 40% less IA than that with granulated sugar (Fig. 2a). Comparatively, both results of IA production with commercial urea (Fig. 2b and 2d) were similar and lower than the values obtained with NH_4NO_3, suggesting impurities in the carbon sources were negligible in relation to the effect of the used nitrogen source. The effect of low IA when urea was applied may concern to one or more of the following reasons: impurities inherent to the feedstock, as mentioned previously; the nitrogen source itself; and the initial high pH.

Regardless of the adjustment of the medium to pH 3, the high temperature during autoclaving and the acid environment must have promoted the dissociation of urea to ammonium and carbon dioxide [23]. Therefore, the issue would not be nitrogen source as ammonium was formed on the medium and that nitrogen source is preferably assimilated by filamentous fungus [24]. Instead, the increase of pH concerning the formation of ammonium and the consequent increase of pH to neutral is a strong possible cause of lower IA production compared with the use of NH_4NO_3.

Acid environment (pH 2-3) has been reported as an essential condition for high yield IA production [19], [25], [26]. However, this IA production with urea in medium with pH medium close to neutral obtained in this study contradicts that state. Reference [26] suggested that the synthesis of essential enzymes for IA production only occur in acid environment (about pH 2), which was the opposite of the results presented by a previous study that described IA production at a wide pH range. Other study reported that IA production occurs at different initial pH ranging from 1.9 to 4.9 without pH control during the cultivation, and that IA production was not different at pH ranging from 3.1 to 4.9 [25]. This study showed that IA is produced in pH close to neutral, however, lower pH results in higher IA production.

C. IA production in VHP and NH_4NO_3 medium with different inoculum

Despite significantly higher IA production in granulated sugar medium, the lower cost of VHP feedstock motivated further investigation to promote greater IA yields. Besides the lower cost of VHP, its production occurs at sugarcane mill, while granulated sugar is processed from VHP in food industry plants. The possibility to produce IA where sugarcane is processed rather than where it is refined is economically more feasible, and the bioprocess may be applied as a biorefinery concept. Therefore, it must be investigated fermentations parameters to improve IA production from VHP source. It was evaluated the use of mycelia instead of spores as a strategy.

IA production with mycelia was undoubtedly more efficient than the fermentation with spores (Fig. 3). Moreover, IA production had a longer lag phase when the inoculum was done with spores. The productivity was over 3 times higher with mycelia than with spores (respectively 0.19 and 0.06 g/L/h), which indicates that the use of mycelia as inoculation method is a simple alternative to achieve greater IA production in VHP medium.

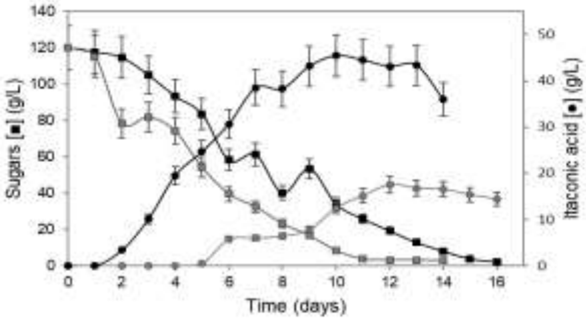

Fig. 3 Itaconic acid produced by *Aspergillus terreus* NRRL 1960 in VHP medium. Inoculum done with spores (*gray line*) or mycelia (*black line*).

The rates of substrate consumption were rather similar, but total consumption of sugars was reached faster with mycelia inoculum. Probably due to the times required for spore germination and the formation of essential enzymes related to IA synthesis.

Itaconate, the ion of IA, is synthesized at the cytosol, where *cis*-aconitate is converted to that ion by *cis*-aconitate decarboxylase [27],[28]. *Cis*-aconitate is produced inside the mitochondria at the TCA cycle and is rapidly transported to the cytosol thought mitochondrial tricarboxylic transporter(s) (Mtt) [28]. The deviation of the TCA cycle to high yield IA production results in decreased or null cell production [18],[19]. In the present study, the initiation of the fermentation with mycelia reduced the lag phase for spore germination. Moreover, the previous cultivation in production medium to form mycelia may have induced the formation of CAD at the cytosol for the conversion to itaconate. Fermentation with mycelia induced a faster production rate, which resulted in 0.41 g IA/g sucrose, 53% of the theoretical yield with VHP, a feedstock that was not previously treated for impurities removal.

D. IA uptake by A. terreus in VHP and NH_4NO_3 medium

Researches regarding degrading pathway of IA are in the early stages [29]. Reference [29] showed that fermentation by *A. terreus* LYT10 with glucose at an initial concentration of 5 to 40 g/L IA as sole carbon source did not promote cell growth. On the other hand, the combination of 40 g/L of IA with an initial 130 g/L of glucose resulted in a partial consumption of IA during the first 36 h of fermentation (inoculum with mycelium), when IA was, then, produced. The present study investigated whether the same effect may occur with *A. terreus* NRRL 1960 in VPH medium with NH_4NO_3 inoculated with mycelia.

The final IA concentration in medium with 40 g/L of IA added in the beginning of the fermentation (Fig. 4) was significantly lower than that naturally produced by the filamentous fungus – close to 50% less IA (Fig. 3). It was not observed a consumption of IA in the beginning of the fermentation, which contradicts the results presented by [29].

An inhibition effect was observed when IA is already present in the medium. That effect was not related to substrate consumption, as sugar uptake profile was similar to that presented on Fig. 3 for inoculum with mycelia. IA was consumed when substrate source was no longer available in the medium.

IV. Conclusion

This study showed that VHP sugar is a potential feedstock for IA production since it does not require previous impurities removal for achieving high IA production yields when inoculum is done with mycelia. The application of NH_4NO_3 as nitrogen source showed to be more advantageous than urea from commercial origin. The possibility of applying that carbon source in a biorefinery concept would decrease the end-product cost of IA. Moreover, the use of VHP could be an alternative for the eventual issues regarding the decrease of sugar demand on the food market.

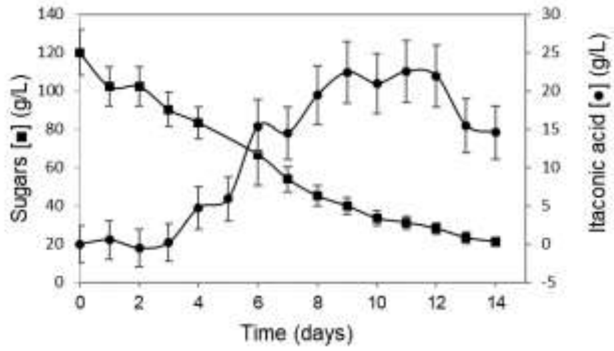

Fig. 4 Itaconic acid production by *Aspergillus terreus* NRRL 1960 in VHP medium with addition of 40 g/L of itaconic acid in the beginning of the fermentation. To present the consumption effect of itaconic acid, the secondary vertical axis represents the product value withdrawn the initial 40 g/L added to the medium.

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