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Accumulation of malic acid and other industrially important organic acids by *Aspergillus Tubingensis* AN1257

Produção biotecnológica de ácido málico por *Aspergillus Tubingensis* AN1257

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ABSTRACT
Organic acids act as acidifiers which improve sensorial characteristics of beverages, contributing to color, aroma, and taste. Malic acid is one of the industrially important acidifiers, and is also applied in metal cleaning. There is a challenge to establish biotechnological processes for its accumulation. One approach to improve production is searching for better strains, which be adequate for bioconversion and product accumulation from cheaper substrates. The present work aimed to identify a panel of organic acids produced by *Aspergillus tubingensis*, which has shown high biotechnological potential due to the ability of producing a variety of enzymes and of utilizing several substrates. *A. tubingensis* AN1257 was compared to a standard organic acid producer, *Aspergillus niger* 10v10. Secretion of organic acids in dependence of the carbon source concentration, time, and pH was evaluated, and the acids produced were detected and quantified by potentiometric titration and by HPLC. Several industrially important organic acids were accumulated by this strain, including citric and malic acids, with the production being affected by culture variables. Culture pH influenced the quantity and type of acid production by strain AN1257. It was able to convert sucrose to malic acid in submerged bioprocesses, accumulating these industrially important acid in very simple media and culture conditions, posing in perspective the establishment of its biotechnological obtention.

Keywords: Organic acids, filamentous fungi, biotechnology.

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RESUMO
Os ácidos orgânicos atuam como acidificantes que melhoram as características sensoriais das bebidas, contribuindo para a cor, o aroma e o sabor. O ácido málico é um dos acidificantes industrialmente importantes, e também é aplicado na limpeza de metais. Há um desafio para estabelecer processos biotecnológicos para seu acúmulo. Uma abordagem para melhorar a produção é a busca de melhores cepas, que sejam adequadas para a bioconversão e o acúmulo de produtos a partir de substratos mais baratos. O presente trabalho visou identificar um painel de ácidos orgânicos produzidos por *Aspergillus tubingensis*, que tem demonstrado alto potencial biotecnológico devido à capacidade de produzir uma variedade de enzimas e de utilizar diversos substratos. A. *tubingensis* AN1257 foi comparado com um produtor de ácido orgânico padrão, *Aspergillus niger* 10v10. A secreção dos ácidos orgânicos na dependência da concentração da fonte de carbono, tempo e pH foi avaliada, e os ácidos produzidos foram detectados e quantificados por titulação potenciométrica e por HPLC. Vários ácidos orgânicos de importância industrial foram acumulados por esta linhagem, incluindo ácidos cítricos e málicos, com a produção sendo afetada por variáveis de cultura. O pH da cultura influenciou a quantidade e o tipo de produção de ácido pela cepa AN1257. Foi capaz de converter a sacarose em ácido málico em bioprocessos submersos, acumulando estes ácidos industrialmente importantes em condições de meios e cultura muito simples, colocando em perspectiva o estabelecimento de sua obtenção biotecnológica.

Palavras-chave: Ácidos orgânicos, fungos filamentosos, biotecnologia.

1 INTRODUCTION

Organic acids occur naturally in food, being originated from biochemical processes, hydrolysis, and microbial growth. In wines, these acids originate from grapes (tartaric, malic and citric acids) and from the processes of alcoholic and malolactic fermentations (lactic and succinic acids), contributing to color, aroma, and taste, as well as for microbiological and chemical stability (Kallithraka et al.,1997; Rizzon & Sganzerla., 2007).

Malic acid is used as acidifier in foods, in metal cleaning, textile manufacturing, as additive in pharmaceutical products, medical infusions and in paints (Goldberg et al., 2006). The 1,4-dicarboxylic acids, like succinic, malic, and fumaric, are not only produced by all living organisms, being part of the tricarboxylic acid cycle (TCA), but have also been identified among the 12 chemical products of higher value that can be obtained from biomass, according to the Department of Energy of the United States (Werpy & Petersen, 2004).

Among the biotechnological producers, filamentous fungi are of great importance, once they are able to accumulate a variety of economically valuable substances, including enzymes, vitamins, organic acids, antibiotics, and other bioactive compounds and additives for the pharmaceutical, alimentary, chemical, and biotechnological industries (Andersen, 2014; Lange, 2014, Queiroz & Sousa, 2020). Certain genera of filamentous fungi, like Rhizopus and Aspergillus, are known for the production and secretion of large amounts of fumaric and malic acid, when cultivated under

stressing conditions (Abe et al., 1962; Battat et al., 1991; Magnuson & Lasure, 2004). Some of the black Aspergilla are able to secrete higher amounts of organic acids, i.e., Aspergillus niger (Papadaki & Mantzouridou, 2019). A strain of *Aspergillus tubingensis*, a species widely spread in the whole world, was isolated in Diamantina-MG, Brazil, and has shown potential to produce endoglucanases and hemicellulases from plant biomasses (Santos et al., 2015).

Although filamentous fungi are known producers of organic acids, there are no comercial production established for some of these, due to lack of economic viability. Fumaric and malic acids, for instance, are still obtained from petroleum (Ochsenreither et al., 2014). Genetic engineering of *A. niger* allowed the production of malic acid in elevated quantities, attaining almost 70% the theoretical yield of bioconversion from glucose (Brown et al., 2013). Other authors also applied genetic engineering of *A. niger* to produce high amounts of malic acid in fed-batch processes (Xu et al., 2019). However, complex media were used for production, requiring not only glucose in high concentration and regular supplementation, but also an expensive supplement like peptone, and micro-nutrients.

Biochemical and genetical regulatory mechanisms can be obstacles to accumulate intermediate metabolites by natural not engineered strains. Good producers, however, could be searched among more than 12 million fungal species that are estimated to exist (Wu et al., 2019), provided the suitable culture conditions be developed. Molecular improvement of producer strains is a high valuable approach to increase production, but also poses environmental and health-threatening questions, since many of the molecular markers utilized for their preparation are genes conferring resistance to antimicrobials. As metabolism regulation vary among species and strains, another useful approach is the screening for microorganisms able to convert carbon sources to a desirable product, and further investigate culture conditions overcoming feed-back inhibition of the productive pathways.

In this work, a strain of *A. tubingensis* AN1257, previously studied by its ability of producing enzymes and of converting alternative substrates, was investigated to determine a gama of organic acids produced. Further, variables interfering in the production, i.e. concentration of carbon source, time of cultivation, and pH were investigated and varied to increase acid accumulation in batch processes.

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2 MATERIALS AND METHODS

Strain cultivation and maintenance

A. tubingensis AN1257 and *A. niger* 10v10 were replicated in Potato Dextrose Agar (PDA) and incubated for 7 days at 30 °C, before harvesting conidia to be used as inocula. For maintenance, small blocs of agar covered by mycelium were submerged in sterile distilled water contained in 2 mL micro tubes, and stored under refrigeration (4 ± 1 ºC). Stocks were replaced at every 6 months (Castelani, 1963).

Screening the production of organic acids

The potential of *A. tubingensis* AN1257 for organic acid production was screened in solid media and compared to a known acid producer, *A. niger* 10v10. For semi-quantitative determination of total acid production, solid media (120 g . L^{-1} sucrose; 3 g . L^{-1} $(NH_4)_2SO_4$; 1 g . L^{-1} KH_2PO_4; 1 g . L^{-1} K_2HPO_4; 15 g . L^{-1} agar) was prepared and supplemented with bromocresol green at 0.4 g . L^{-1} as indicator; pH was adjusted at 4.0 and 6.0. Conidia from 7 days colonies cultivated in PDA at 30 °C were collected in sterile distilled water and diluted to a concentration of 50 conidia in 10 µL, which were deposited in the centre of a Petri dish containing the screening medium. Cultures were incubated at 30 ºC for 7 days. Colony diameter for radial growth determination, and the acidic halo for estimation of total acid production were measured in two directions for 7 days at 24 h intervals. Acid formation index (IA) was calculated by the ratio among the diameters of the acidic halo and the colony diameter. IA was determined as media of three independent cultures.

Central composite design for organic acid production by submerged cultivation

Concentration of carbon source (sucrose) and pH were investigated as independent variables of Central Composite Design, (CCD) in order to determine the conditions influencing organic acid production by A. tubingensis AN1257. Four bioprocesses combining the lowest and highest levels of each variable were designed, in which the concentration of sucrose varied from 60 to 120 g . L^{-1}, and pH varied from 4 to 7. Another four processes were designed by combining the higher (132.4 g . L^{-1} for sucrose; 7.62 for pH) and lower (47.7 g . L^{-1} for sucrose and 3.37 for pH) axial levels of each variable with the central level of the other. The central point condition combined the medium level of both variables (90 g . L^{-1} sucrose; pH 5.5). A total of 9 productive conditions were generated and used to investigate organic acid production by A. tubingensis AN1257 by submerged process, as shown in Table 1: SA to SD (high and low levels of sucrose and pH); SE to SH (axial conditions); SI to SL (replicates of the central point condition) (Table 1).

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Table 1: Bioprocesses generated by Central Composite Design for organic acid production by *A.tubingensis* AN1257

Bioprocess	Sucrose (g . L^{-1})	pH
SA	60.0	4.0
SB	60.0	7.0
SC	120	4.0
SD	120	7.0
SE	47.57	5.5
SF	132.4	5.5
SG	90.0	3.37
SH	90.0	7.62
SI-SL	90.0	5.5

Other medium constituents were kept as simple as possible, including an inorganic primary nitrogen source [3 g . L^{-1} $(NH_4)_2SO_4$], phosphate and potassium sources [1 g . L^{-1} KH_2PO_4 and 1 g . L^{-1} K_2HPO_4]. Processes were conduced to contain three replicates of each condition, which were stopped after 3, 6, and 9 days. The central point processes were prepared in 3 replicates. A sample of each medium was collected before inoculation (zero time) for control. Cultures were inoculated with conidia to a final concentration of 10^6 . mL^{-1} and incubated at 30 °C under 150 rpm.

Production of organic acids was determined by potentiometric titration and by high performance liquid chromatography (HPLC).

Potentiometric Titration

Samples of 1 mL were collected from each bioprocess and diluted with 19 mL of distilled water. Potentiometric titration was performed against NaOH [0.05 mol . L^{-1}] previously standardized with potassium acid biftalate, to determine medium acidity and the potential of A. tubingensis AN1257 for secretion of total acid under submerged cultivation. Analysis was performed by automatic titration in Titroline 7000 (SI Analytics). Titration equivalent volumes were determined by the derivative method (Harris, 2005).

Determination of organic acids by HPLC

Analyses were performed in chromatograph Shimadu, in a flow of 0.6 mL . min^{-1}, at 60 °C, injection of 5 µL, and detection at 220 nm. It was utilized a column Rezex ROA 30 x 9 for organic acids. Samples of 1.5 mL were removed from bioprocesses after 0, 3, 6, and 9 days of cultivation,

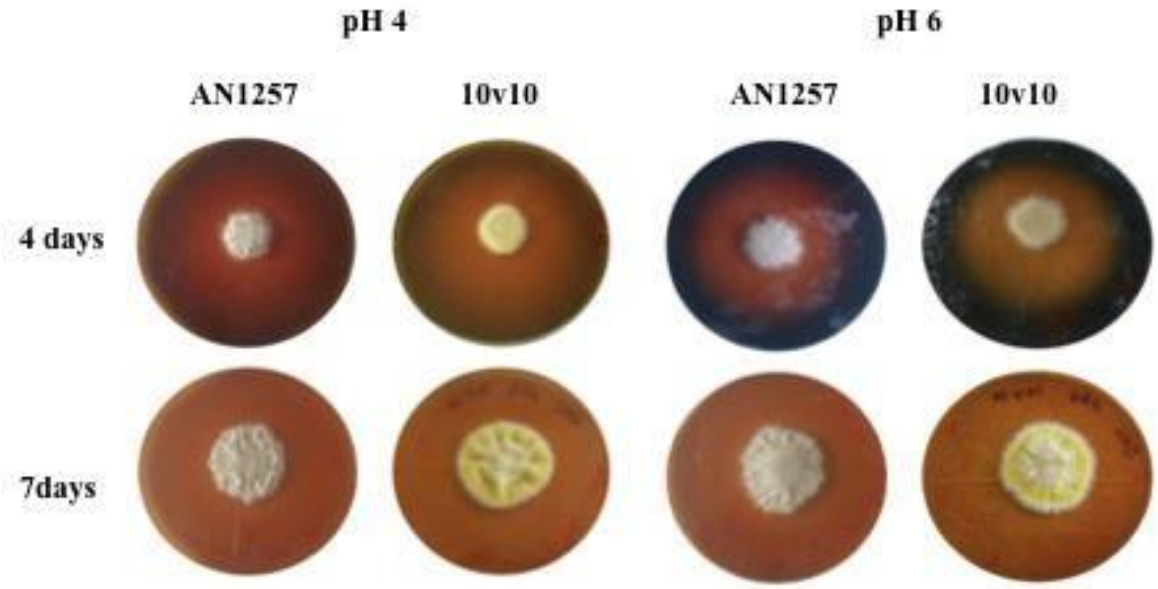
and centrifuged at 10.000g for 5 minutes. An aliquot of 150 µL were collected from the supernatant and diluted (10x) in the eluent H_2SO_4 [0,005N]. After treatment, samples were placed in vails and injected.

3 RESULTS AND DISCUSSION

Screening the production of organic acids

Both A. tubingensis AN1257 and A. niger 10v10 showed good acid secretion in solid media. As shown in Figure 1, both strains secreted high amounts of acid, as indicated by the acidic yellow halo in each culture against the green color of the media. After 4 days of cultivation, A. tubingensis AN1257 showed good potential for acid secretion, when compared to the standard strain A. niger 10v10. After 7 days of cultivation acid secretion was so intense that the whole media were acidified in cultures of both strains, either in pH 4 or 6. Starting cultures at pH 6 decreased the index of acidification, yet the strains showed good ability to secrete acids enough to form large halos covering most of the area of the dish after 4 days (Figure 1).

Figure 1: Cultivation of A. tubingensis AN1257 and A. niger 10v10 in media to evaluate acid secretion.



Media were supplemented with sucrose as the sole carbon source, and pH was adjusted for pH 4 and pH 6. Cultures were incubated at 30 ˚C and observed daily to measure the radial growth and the acid halo diameter. Cultures of 4 and 7 days are shown.

Thus, *A. tubingensis* AN1257 presented very high potential for acid secretion, so does A. niger 10v10. After determining colony diameter and acidity halo, the index of acid formation (IA) was calculated for each time of cultivation. Curves for radial growth and IA are shown in Figure 2.

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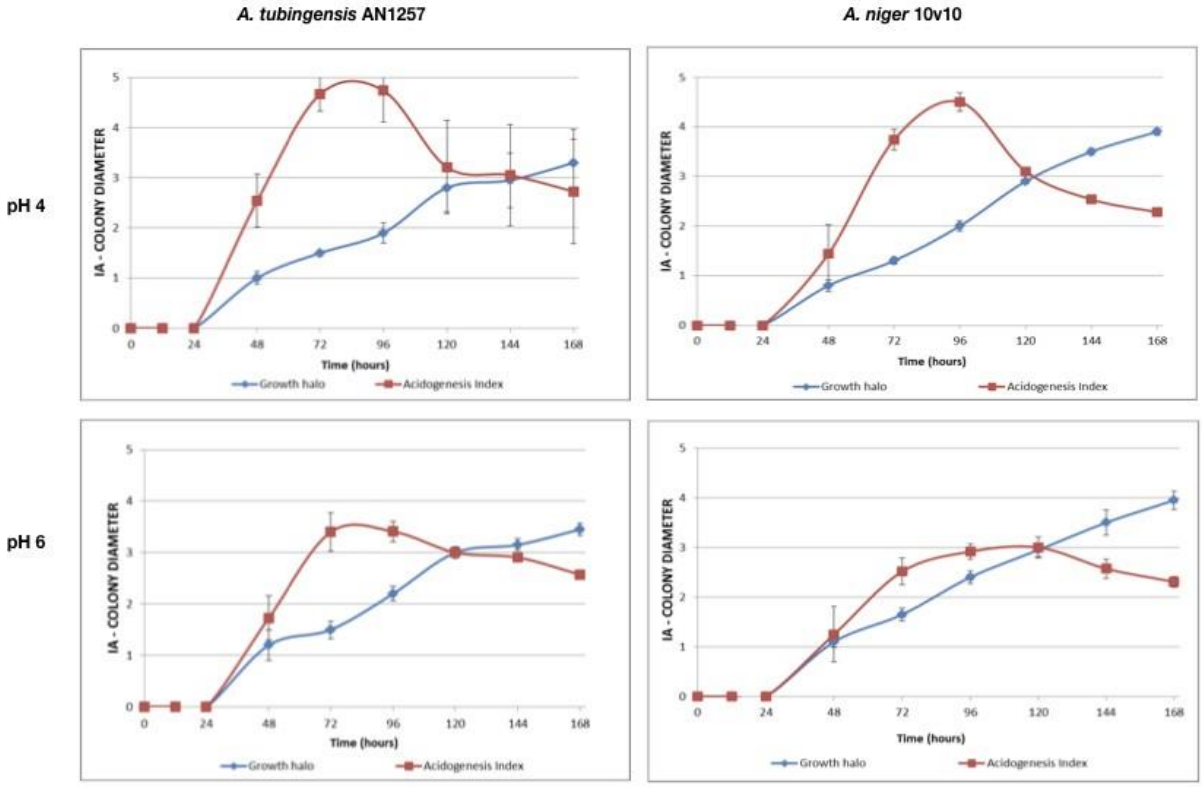
Figura 2: Growth and índex of acid secretion (IA) of A. tubingensis AN1257 and A. niger 10v10 cultivated in pH 4 and 6.



Cultures were prepared in media supplemented with sucrose as the sole carbon source, inoculated with 50 conidia at the center, and incubated at 30 ºC. Radial growth and acidity halo were measured daily. IA is expressed as the ratio among the diameters of the acidity halo and the colony.

According to Max et al. (2010) and Yang et al. (2017) different species of *Aspergillus* like *A. wenti, A foetidus, A. aculeatus, A. awamori, A. fonsecaeus, A. phoenicis,* and *A. carbonarius* have been shown to be good producers of citric and other acids. Here we show that *A. tubingensis* is also a good acid producer, secreting acidity in a magnitude that encouraged evaluation of the panel of acids which could be produced in a liquid process. Elucidation of the variety of acids produced by a fungus is not only important for biotechnological applications, but is also useful as a tool for species identification, which is particularly difficult among black aspergilla.

Dezam *et al.* (2017) also screened 6 fungal general for organic acid production by Solid State Fermentation (SSF), utilizing sucrose as carbon source and bromocresol green as pH indicator. Acidification of the media and formation of a yellow halo around inocula was used as indicative of organic acid secretion. Their screening showed that only the genera *Aspergillus* and *Fusarium* were suitable for acid secretion.

Either *A. tubingensis* AN1257 and *A. niger* 10v10 started measurable radial growth after 24 h. Both strains grew until 168 h, showing good adaptation to the conditions utilized for cultivation and acid secretion, including pH 4 or 6, sucrose as carbon source, and ammonium sulphate as

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nitrogen source (Figure 2). Maximum IA produced by *A. tubingensis* AN1257 was determined after 72 h of cultivation either in medium adjusted for pH 4 or 6. *A. tubingensis* AN1257 showed more potential for acid secretion than strain 10v10, once the maximum IA of the later was obtained after 96 h, and was lower than the IA of strain AN1257 during the whole period studied. Analysis of Figure 2 allowed to conclude that *A. tubingensis* AN1257 could be investigated as an acid producer in submerged processes, once it yielded a high IA of near 5 when cultivated in the conditions similar to that utilized for citric acid fermentation. As shown, strain AN1257 overcame the standard citric acid producer *A. niger* 10v10.

Increasing pH from 4 to 6 impaired the index of acidification of both strains, decreasing IA in about 1.5 unit after 72 and 96 h of cultivation. As pH and pOH are logarithm scales, increasing the initial pH in 2 units (pH 4 to 6) increases the concentration of OH^- in media from 10^{-10} M to 10^{-8} M, meaning that the fungi had do secrete 100x more acid to acidify completely the media, in order to turn to the yellow color. Yet, both strains kept a high index of acidification, especially strain AN1257, whose IA was above 3 after 72 and 96 h (Figure 2).

Organic acid production by *A. tubingensis* AN1257 in submerged processes

After designing conditions for organic acid production by submerged fermentation, as shown in Table 1, all processes were analyzed for the amount of acid produced after 0 (control), 3, 6, and 9 days of cultivation. Curves of potentiometric titration for processes SA, SB (6% sucrose), SG, SH (9% sucrose), and SC, SD (12% sucrose) are shown in Figure 3.

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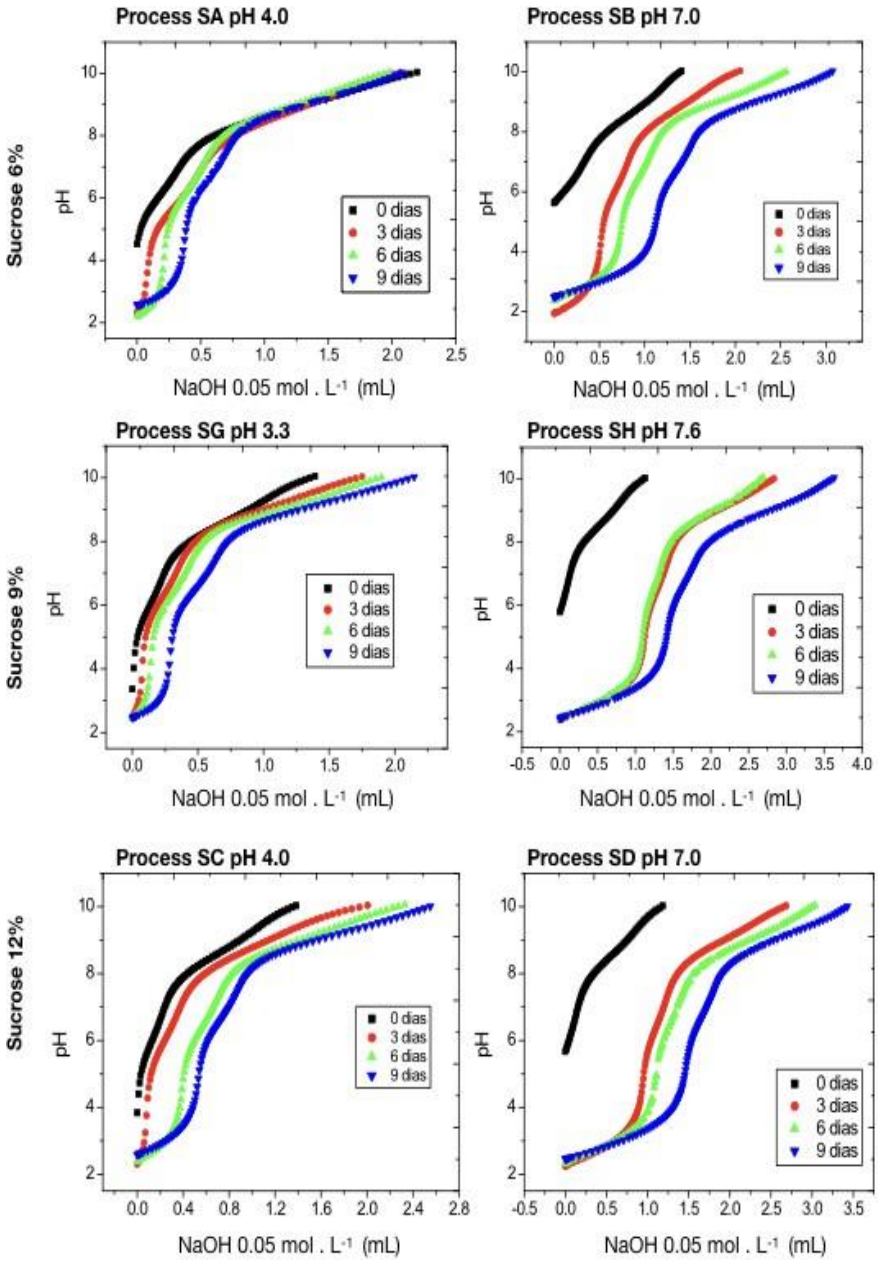
Figura 3: Total acid production by *Aspergillus tubingensis* AN1257 in submerged bioprocesses supplemented with sucrose at 6%, 9%, and 12% at acid pH and at pH near neutrality.



Production of total acid was determined by potentiometric titration of culture supernatants after 3 (red), 6 (green), 9 (blue) days of cultivation. Zero time (black curve) is shown for each bioprocess as control (media not inoculated).

Potentiometric determination of pH in samples collected at zero time of each culture (control) showed that the initial pH adjusted in these media was decreased in samples collected after 3, 6, and 9 days of cultivation, as can be seen from titration curves before addition of NaOH (Figure 3). Control samples (zero time) show curves for potentiometric titration with two buffering regions, one that is near pH 6.0, which may be due to carbonate formation, and other near pH 9.0, due to the presence of phosphate and ammonium salts (Figure 3).

Analysis of samples collected from bioprocess SG (9% sucrose, initial pH 3.37) and SH (9% sucrose, initial pH 7.6) showed that after 3 days of cultivation, medium pH was decreased to approximately 2.5. In all bioprocesses, the culture media were acidified, showing pH values below 3 after 3 days of cultivation and on, as can be seen by comparing control samples (zero time) and samples collected after 3, 6, and 9 days of cultivation before starting titration with NaOH (Figure 3).

This comparison evidences acid secretion by strain AN1257 after 3 days in all conditions shown, similarly of results obtained in solid media (Figure 2). Independently of the amount of sucrose in the media, pH of samples collected after 3, 6, and 9 days of cultivation decreased in comparison to the initial pH (zero time), indicating the presence of acid products.

As expected from analysis of acid secretion in solid media, after fungal growth and acid secretion in submerged bioprocesses, medium pH was decreased, and the equivalence volume increased from 0.03 mL (3 days), 0.3 mL (6 days), to 0.5 mL (9 days) as seen in the titration curve of bioprocess SC (12% sucrose, pH 4.0) (Figure 3). When sucrose concentration is 12% and starting pH is 7, in bioprocess SD, acid production increased after 6 and 9 days of cultivation, once these samples presented higher resistance to pH elevation, with a weak buffering tendency observed in the beginning of titration (Figure 3). As shown, buffering was a little stronger in the sample collected from bioprocess SD after 9 days of cultivation. This indicates that at the higher sucrose concentration and at neutral initial pH, acid production was increased in the conditions of the present study.

It is important to consider that conidial germination in Aspergillus is influenced by pH, with better values raging from pH 4.0 to 7.0. Thus, while acid secretion and medium acidification was observed in all cultures, the amount of acid produced may vary due to metabolic reasons and to germination and growth.

By means of the derivate method (supplementary material) it was possible to determine the volume of NaOH (0.05 mol . L^{-1}) needed to neutralize the acids produced, as well as to quantify the total acid amount (Figure 4).

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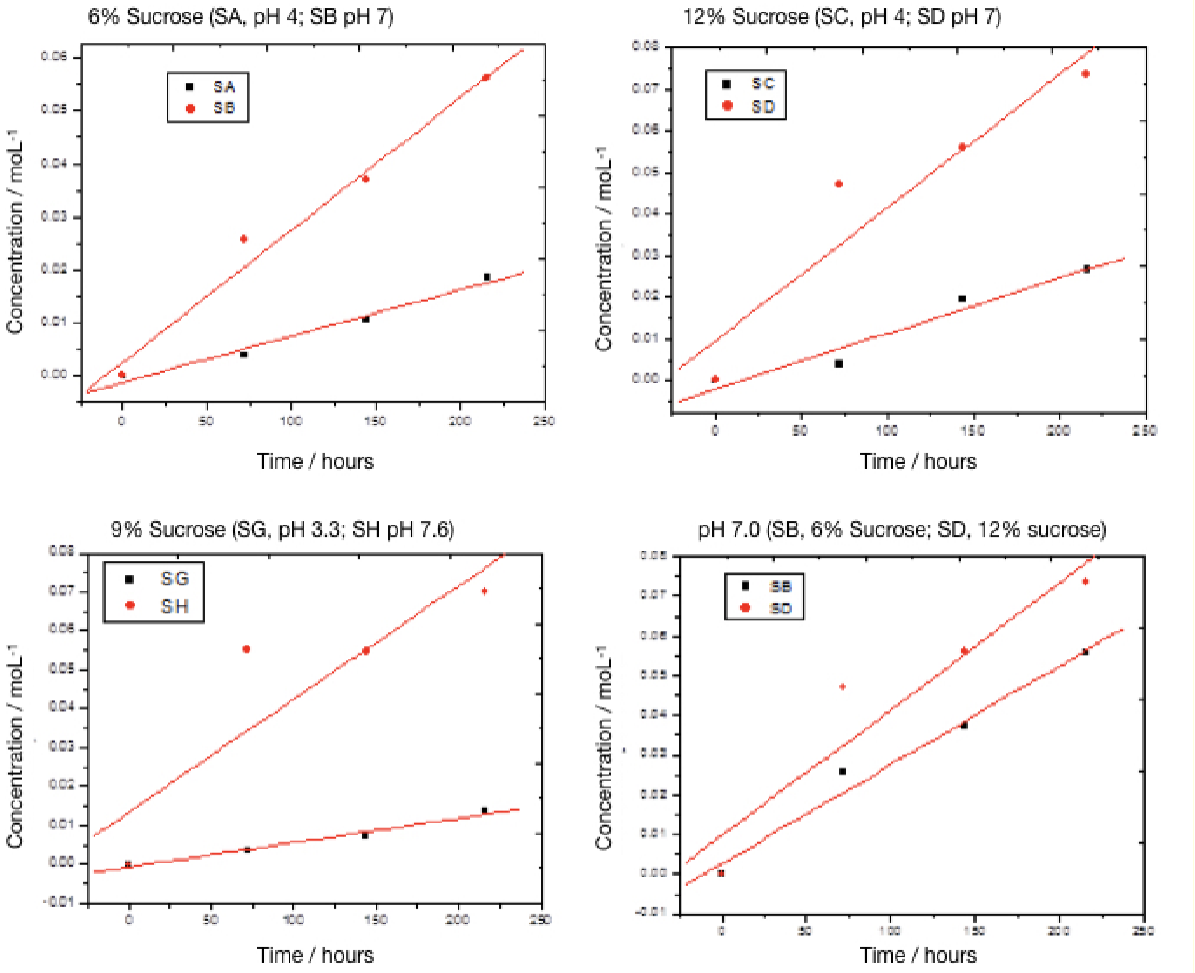
Figure 4: Effects of pH and sucrose concentration on acid production by *Aspergillus tubingensis* AN1257



Cultures were incubated at 30 ºC for 3 (72 h), 6 (144 h), and 9 days (216 h). Samples were collected, titrated against NaOH 0.05 mol . L^{-1} and the amount of total acid was estimated by the derivative method.

Graphical comparison of bioprocesses SA and SB, which were both supplemented with 6% sucrose, but at pH 4 and 7, respectively, allow to verify that acid production was higher in pH 7 (2.5 . 10^{-4} mol . L^{-1}) than in pH 4 (8.7 x 10^{-5} mol . L^{-1}), as shown in Figure 4. The same occurs when sucrose concentration is increased to 9% or 12%. Analysis of bioprocesses SC and SD, which were supplemented with 12% sucrose, show 2x more acid production at pH 7 (Bioprocess SD, 3.2 . 10^{-4} mol . L^{-1}) than at pH 4 (Bioprocess SC, 1.3 . 10^{-4} mol . L^{-1}). Theses analyses indicated that A. tubingensis AN1257 is more suitable for acid production at pH 7.0 or near neutrality (Figure 4). Investigating acid production at axial levels of pH (Bioprocess SG, pH 3.3 and Bioprocess SH, pH 7.6) and supplementing the media with 9% sucrose confirmed this tendency. The lowest amount of acid production was obtained in Bioprocess SG, as shown in Figure 4.

Considering neutral pH as the best condition for acid production by strain AN1257, sucrose concentration (Bioprocess SB, 6% sucrose; Bioprocess SD, 12% sucrose) was compared to

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determine its effect for acid production (Figure 4). As shown, increasing the sucrose concentration elevates acid production, which reaches 3.2. 10^{-4} mol . L^{-1} in bioprocess SD.

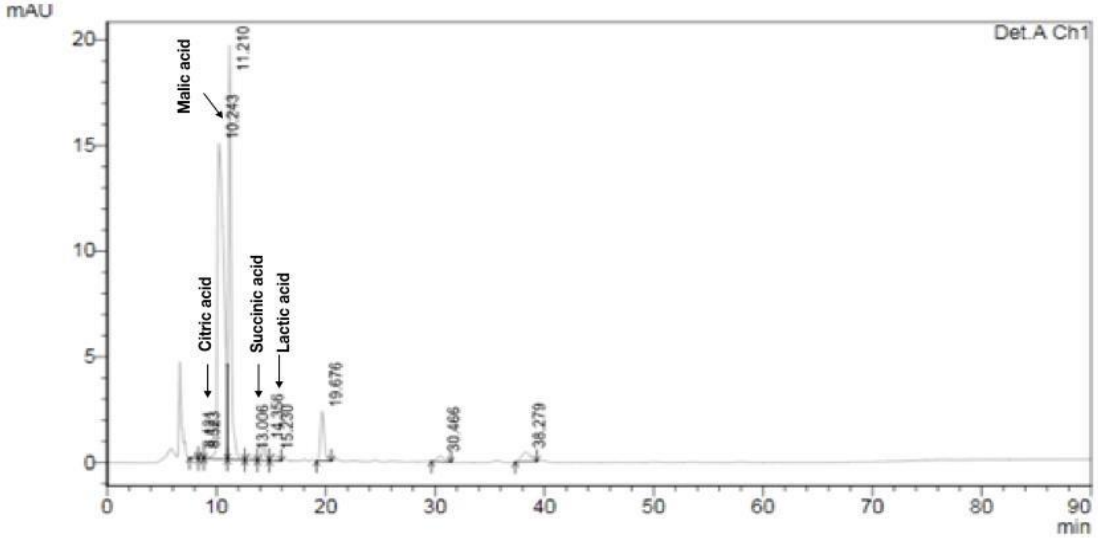
After determining the best conditions for acid production, samples were analyzed by HPLC for identification and quantification of each acid produced.

Determination of organic acids by HPLC

HPLC analysis of supernatants collected from submerged bioprocesses evidenced the presence of organic acids consistent to the standards citric, succinic, malic, lactic, and tartaric acids. Acetic and butyric acid were not detected.

Supernatant from Bioprocess SD, after 9 days of cultivation, was the best condition for total acid production, as shown in Figures 3 and 4. Analysis of this sample by HPLC is shown in Figure 5. Some pikes of organic acids were detected, with their retention times (RT) being compatible to citric acid [0.2 g . $L^{-1,}$] (TR 8.5 min.), malic acid [13.2 g . L^{-1}] (TR 10.2 min.), succinic acid [0.35 g . L^{-1}] (TR 13.0 min.), and lactic acid [0.93 g . L^{-1}] (TR 14.3 min.). The final concentrations were calculated by multiplying by 10, once this was the dilution used for sample preparation.

Figura 5: Analysis of Bioprocess SD (12% sucrose, pH 7) to detect the profile of organic acids produced by *A. tubingensis* AN1257.



Sample was collected from supernatant after 9 days of cultivation at 30 ºC and diluted 10x prior to analysis.

Other bioprocesses planned by CCD were also analyzed by HPLC, whose results are summarized in Table 2. There was considerable production of malic acid in some bioprocesses which were adjusted with initial pH 7.0, after 6 days of cultivation (Table 2).

However, when sucrose concentration is increased to 132.42 g . L^{-1} (13.4%) (Bioprocess SF), malic acid production decreases (Table 2). This decrease in production could be due to the

excess of carbon source, whose concentration would be reaching limiting values, or more probably may be due to the low initial pH, which was 5.5. It is widely known that citric acid production by filamentous fungi, especially by *A. niger*, is strongly influenced by pH, which must be near pH 2 during the active phase of production. Other acids, on the opposite, are produced near neutrality. Results in Table 2 show that malic acid production by *A. tubingensis* AN1257 occurs preferentially near neutral pH. It is possible to conclude that this species produces a panel of organic acids and presents potential to accumulate malic acid.

In bioprocess SB (6% sucrose, pH 7) the concentration of malic acid reached 8.30 g . L^{-1} after 9 days of cultivation. The augment of sucrose to 9% increased malic acid production to 12 g . L^{-1} after 9 days of cultivation, as determined in the supernatant of Bioprocess SH (pH 7.62). As seen in Table 2, further increase in sucrose concentration (12%) in Bioprocess SD (pH 7) correlated with the maximum concentration of malic acid: 13.6 g . L^{-1} after 9 days of cultivation, what is in accordance to the titration and derivative analysis, which showed this condition to be the best for total organic acid accumulation (Figures 3 and 4).

Table 2: Production of organic acids by Aspergillus tubingensis AN1257 in bioprocesses planned by CCD.

Bioprocess	**Time (days)**	**Sucrose (g . L^{-1})**	**pH**	**Citric acid (g . L^{-1})**	**Succinic acid (g . L^{-1})**	**Malic acid (g . L^{-1})**	**Lactic acid (g . L^{-1})**	**Tartaric acid (g . L^{-1})**
SA	3	60.0	4.0	-	-	-	-	0.57
SA	6	60.0	4.0	0.15	0.37	0.32	0.70	0.55
SA	9	60.0	4.0	0.15	0.32	1.09	0.77	0.49
SB	3	60.0	7.0	-	-	2.40	0.67	0.56
SB	6	60.0	7.0	-	0.29	0.56	0.72	0.57
SB	9	60.0	7.0	0.28	0.32	8.30	0.97	0.53
SC	3	120.0	4.0	-	0.24	0.27	-	0.60
SC	6	120.0	4.0	0.14	0.32	2.30	0.71	0.51
SC	9	120.0	4.0	-	0.30	0.32	0.75	0.52
SD	3	120.0	7.0	-	-	5.40	-	0.50
SD	6	120.0	7.0	-	0.26	11.90	0.65	0.57
SD	9	120.0	7.0	0.20	0.35	13.60	0.93	-
SE	3	47.57	5.5	-	0.22	0.40	-	0.50
SE	6	47.57	5.5	-	0.21	1.50	0.70	0.54
SE	9	47.57	5.5	0.17	0.28	0.39	0.76	0.52
SF	3	132.4	5.5	-	0.28	0.27	-	0.50

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SF	6	132.4	5.5	-	0.32	0.94	0.70	0.54
SF	9	132.4	5.5	-	0.28	2.70	0.71	0.53
SG	3	90.0	3.37	-	0.23	0.20	-	0.50
SG	6	90.0	3.37	-	0.27	0.28	0.66	0.54
SG	9	90.0	3.37	0.16	0.29	0.25	0.82	0.51
SH	3	90.0	7.62	-	-	6.00	-	0.54
SH	6	90.0	7.62	-	0.28	11.00	0.74	0.53
SH	9	90.0	7.62	0.21	0.49	12.00	0.96	0.52
SI	3	90.0	5.5	-	0.23	0.26	-	0.56
SI	6	90.0	5.5	-	0.34	1.39	0.65	0.56
SI	9	90.0	5.5	0.15	0.59	0.76	0.84	0.54
SJ	3	90.0	5.5	0.27	-	0.40	0.63	0.63
SJ	6	90.0	5.5	0.14	0.28	0.31	0.68	-
SJ	9	90.0	5.5	0.15	0.33	0.69	0.74	0.54
SL	3	90.0	5.5	-	0.23	0.37	0.95	-
SL	6	90.0	5.5	-	0.30	1.30	0.79	0.57
SL	9	90.0	5.5	0.19	0.40	1.14	0.71	0.51

West (2011) utilized *A. niger* and Aspergillus flavus as producers of malic acid by converting vinasse composed of glucose, glycerol and lactic acid. This author obtained a production of 17 g . L^{-1} of malic acid by *A. niger* ATCC9142 after 192 hours of cultivation. The production described in the present work by *A. tubingensis* AN1257 was 13.6 g . L^{-1} after 216 h (9 days). This production encourages further studies for a variety of reasons. First, this strain is not well studied yet, and showed ability to accumulate malic acid even without genetic engineering. Second, sucrose concentration may be further adjusted to increase production, and finally, it is important to establish a biotechnological route for ecological sustainability, whereas malic acid is still obtained from petroleum. Adjustment of the biotechnological process for malic acid production can pave the pathway for its obtention in larger scales at a reduced cost, once the media and conditions applied in the present work were simple and utilized cheap and common substrates.

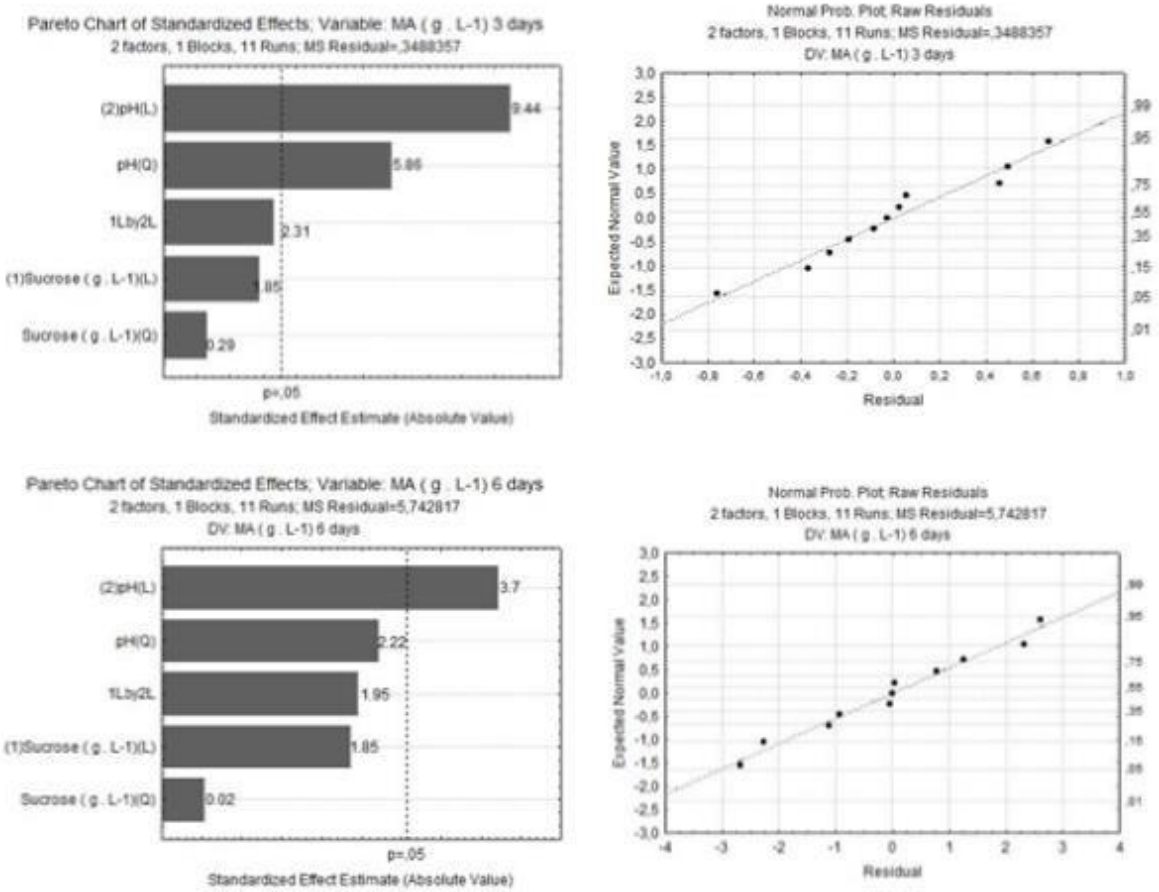
Stastistical analysis of pH and sucrose effects on malic acid production by *A. tubingensis* AN1257

Results of bioprocesses planned by CCD were analyzed with the software Statistics, in order to determine the significance of the effects of pH and sucrose concentration on malic acid production

by strain AN1257. The analysis did not reveal a significant effect of sucrose concentration on product accumulation in the present study (Figure 6). Pareto graphs in Figure 6 show that sucrose effect on malic acid production was not significant at each time of cultivation (3, 6, and 9 days). Thus, while increasing sucrose concentration coincides with increases in malic acid production, this effect was not statistically significant, meaning that the variation from 60-120 g. L-1 did not cause significant differences in malic acid accumulation. In other words, similar production can be reached with lower concentrations.

Production of malic acid was significantly influenced by culture pH, which presented both linear and quadratic positive effects. The linear effects of pH were 9.44; 3.70; and 9.25 for the production of malic acid after 3, 6, and 9 days, respectively (Figure 6). This positive effects confirms statistically that higher pH is better for malic acid accumulation by *A. tubingensis* AN1257. As shown in Figure 6, the positive quadratic effect of pH (5.4) on malic acid accumulation after 9 days - the best time for production studied in the present work - was also positive. The statistics of experiments show correspondence to the normal probability.

Figura 6: Effect of independent variables pH and sucrose concentration on malic acid production by A. tubingensis AN1257.



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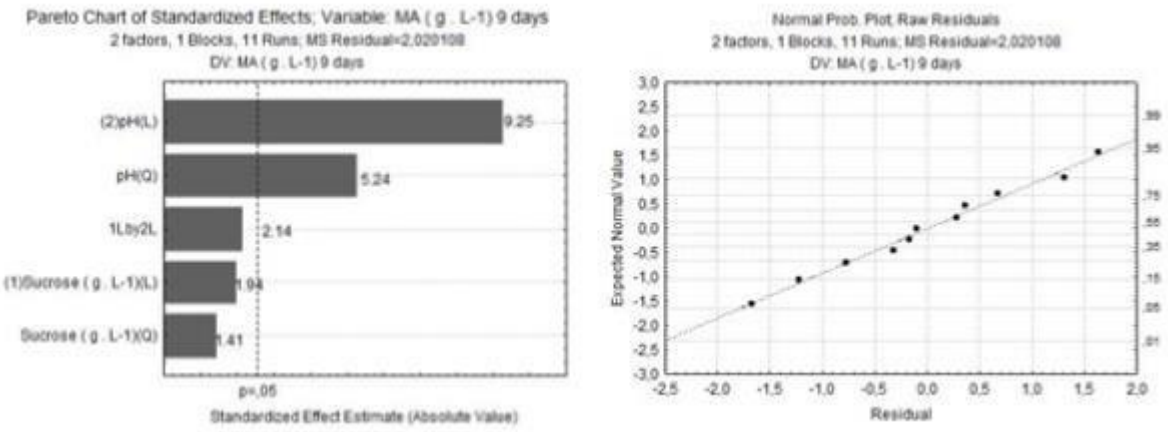

Pareto Graphs show the intensity of the linear and quadratic effects of pH and sucrose on the concentration of malic act after 3 (Graph A), 6 (Graph C), and 9 days (Graph E). Graphs B, D, and F show the normal probability plots for each analysis.

The possible pathways for 4 of the acids determined in the supernatants of cultures of *A. tubingensis* AN1257 - citric, succinic, malic, and lactic acids - are summarized in Figure 7, except for tartaric acid, whose pathway of accumulation in fungi remains unclear.

The metabolic pathway for citric acid production by *A. niger* is well established. It is obtained from the pyruvate produced in carbohydrate catabolism, in an excess of glucose or other fast assimilating saccharide. For citric acid production, a combination of factors is necessary, including high rates of carbon catabolism through glycolysis, low pH, and limitations of manganese and other metals.

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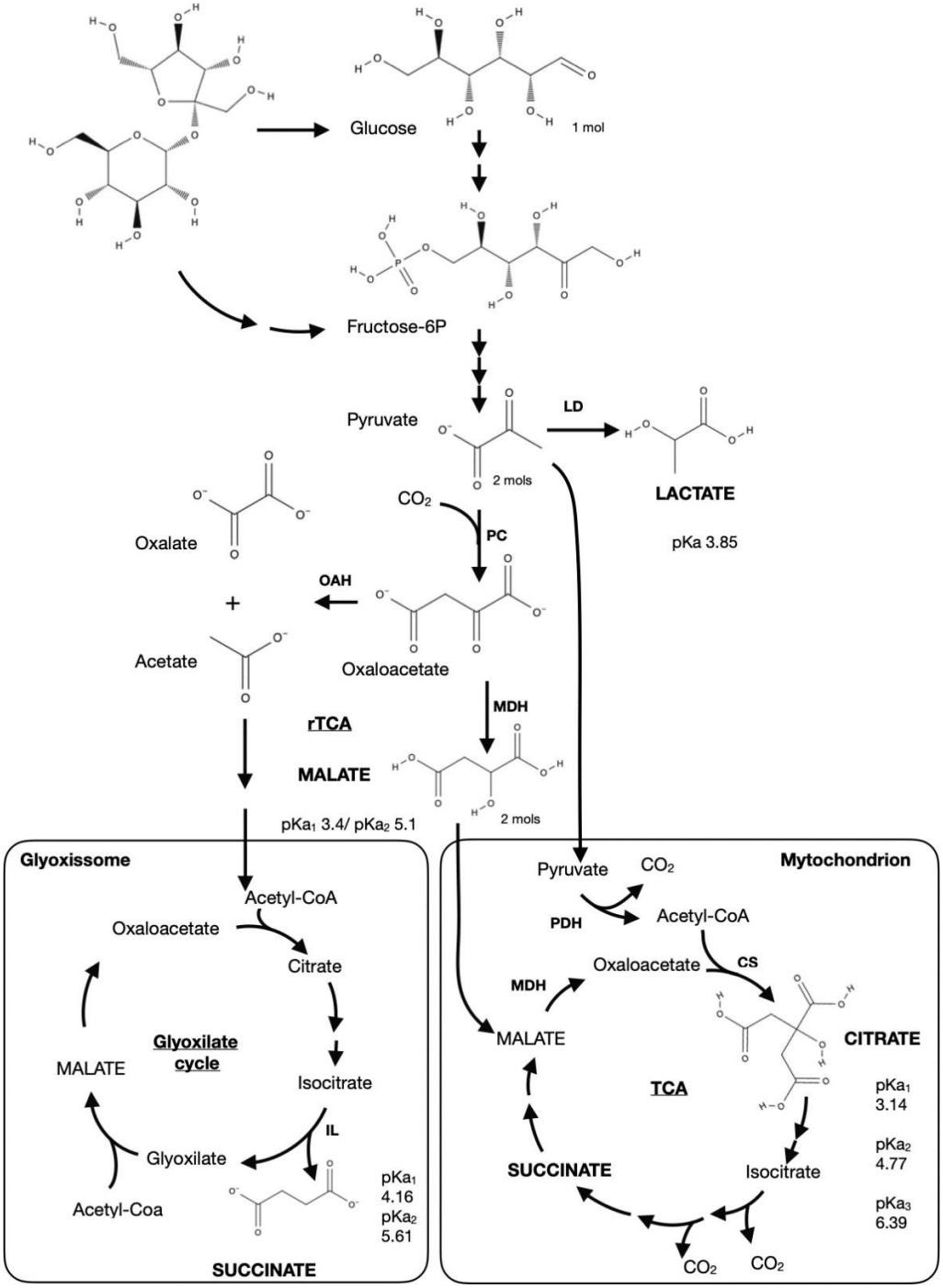
Figure 7: Metabolic pathways for organic acid production



The acids produced by Aspergillus tubingensis AN1257 are written in capital letters, and their structures and pKas are shown. Molecular structures were obtained from Molview (molview.org). Some key enzymes and metabolic pathways for obtention of organic acids are indicated. The metabolic route starts with sucrose being converted to glycolytic substrates. Conversion of 1 mol glucose yields 2 mols malate; 1 mol sucrose yields 4 mols malate. Only the most important metabolic reactions are shown. TCA, tricarboxylic acid cycle; rTCA, reductive TCA cycle; LD, lactate dehydrogenase; PC, pyruvate carboxylase; MDH, malate dehydrogenase; OAH, oxaloacetate acetil-hydrolase; PDH, pyruvate dehydrogenase complex; CS, citrate synthase; IL, isocitrate lyase.

After oxidation through glycolysis, 1 mol glucose is converted to 2 mols pyruvate, which are further converted to 1 mol oxaloacetate - by catalysis of the cytosolic pyruvate carboxylase -

and 1 mol of acetil-CoA - by catalysis of the mitochondrial pyruvate dehydrogenase complex. Before condensation of oxaloacetate and acetil-coA in the TCA cycle to form citrate, the precursor oxaloacetate is reduced to malate in the cytosol, and further oxidized again inside mitochondrion (Figure 7). To sustain citric acid accumulation, oxaloacetate acetyl hydrolase (OAH) - which converts oxaloacetate to oxalate, a frequent byproduct of citric acid production - must be inhibited. This can be achieved by decreasing pH and limiting metal ions in media, once OAH is active at pH 5-6 and requires Mn^{2+}, as reviewed by Plassard & Fransson (2009).

Accumulation of succinic acid is less comprehended. However, as it is, like citrate, an intermediary of the TCA cycle, conversion of glucose to pyruvate, and then to oxaloacetate as precursor, may be important (Figure 7). Formation of oxaloacetate occurs in the cytosol by carboxylation of pyruvate and, after reduction to malate, ensures entry in mitochondrion and supply of TCA. As the reactions are reversible, malate could be converted to fumarate, and succinate, or just be converted to citrate to follow the normal flux of the cycle until succinate. Formation in the glyoxylate cycle could also be possible, requiring investigation. Entry of malate into the glyoxyssome with further conversion to citrate, then to succinate, is plausible, once its an alternative pathway for oxidation of acetil-CoA.

Another intermediary in both TCA and glyoxilate cycles, malic acid is instead formed from oxaloacetate in the cytossol, in the reductive TCA cycle (rTCA) as shown in Figure 7. Brown et al. (2013) over expressed the native pyruvate carboxylase and malate dehydrogenase-encoding genes in Aspergillus oryzae, what largely increased malic acid production. This confirms the cytosolic rTCA as the pathway for malate obtention in Aspergillus, whereas investigation in A. tubingensis has not been done yet. For malate accumulation, pH near neutrality is necessary, on the opposite of the acidic condition which allows citric acid production. The question is why pH near 6 or 7 is better, once high activation of OAH could deplete oxaloacetate to form oxalate. Indeed, manipulation of A. niger to contain several copies of an OAH-encoding gene increased oxalic acid production, while deletion of this gene plus insertion of the genes encoding pyruvate carboxylase and malate dehydrogenase resulted in a strain overproducing malic acid (Xu et al., 2019). Malate dehydrogenase also functions in pH near neutrality. Thus, a challenge is to selectively inactivate OAH, not malate dehydrogenase.

The map 00630 of the Kyoto Encyclopedia of Genes and Genomes (KEGG), built on basis of a variety of published work and investigations on biochemistry of several organisms, shows the pathway for tartaric acid production is in glyoxylate and dicarboxylate metabolism (KEGG, https://www.kegg.jp/kegg-bin/show_pathway?map00630). It can be formed from oxaloacetate

originated from pyruvate metabolism, in a reversible reaction catalyzed by tartrate dehydrogenase. It could also be formed from oxaloglycolate, yet by catalysis of a tartrate dehydrogenase, or from meso-tartrate, which are both linked to ascorbate metabolism. The ascorbate route is important for production of tartrate in higher plants, including grapes, where tartaric acid contributes to the acidic component and confers microbiological stability. It is formed in Vitis vinifera from the catabolism of acid ascorbic to L-idonate, which is then converted to D-gluconate, and finally to tartrate (Cholet et al., 2016). Reports of the routes operating in fungi are scarce, thus, we searched fungal genomes for genes coding enzymes for tartaric acid production: tartrate dehydratase - playing a role in the formation from oxaloacetate - and tartrate epimerase - playing a role in the interconversion of the two isomers.

Following the KEGG orthology for the enzyme tartrate dehydratase alpha subunit (Enzyme Commission Number, EC 4.3.1.32, KEGG K03779) we found several entries for bacterial enzymes with the same EC number at the Uniprot page (https://www.uniprot.org/uniprot/A0A087FW24). Searching the protein database of the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) allowed to retrieve 2 fungal proteins, both from Beauveria bassiana. Running BLASTp with the sequence of entry KGQ11123.1, allowed to recover 113 fungal sequences, being 61 from Ascomycota, and 8 from the genus Aspergillus. The 8 sequences from Aspergillus were related to the two component sensing system histidine kinase, but none concerned to tartaric acid synthesis, showing more studies are needed to elucidate this pathway in fungi. A search for tartrate epimerase (EC 5.1.2.5) at the GenBank (NCBI) retrieved 18 proteins, 4 of which were of bacterial tartrate epimerases. BLASTp of the tartrate hydratase from Bradyrhizobium japonicum (PDB 2DW6_D) against the fungal sequences found 189 hits. Among the hundred most significant alignments, none of the proteins retrieved had activity of tartrate epimerase specifically described, whereas most were other enolases, racemases or hydratases, without reaching 30% of identity. All these analyses show that, although AN1257 accumulates tartaric acid in small amounts, the fungal route for its formation requires another study.

4 CONCLUSION

As organic acids are produced by microbial strains, the challenge is to develop a bioprocess by studying physiology and biochemistry. In a trial for organic acid producers, *A. tubingensis* AN1257 showed to have potential for acid secretion either when cultivated at pH 4 or 6, when compared to a standard acid producing strain of *A. niger*. Analysis of *A. tubingensis* AN1257 cultures showed its potential to convert sucrose to malic acid. Even without any molecular

modification, this strain accumulates acid malic when cultivated in high sucrose concentration (12%) and at pH 7, in very simple media, requiring only phosphate and inorganic nitrogen sources in moderate amounts to produce 13.6 . L^{-1}. These results show this species is promising as a producer of malic acid, what paves the way for further improvements. Besides, as a gama of organic acids were also produced, results suggest A. tubingensis as a potential model to investigate organic acid production, especially those whose mechanism of accumulation still requires further investigation.

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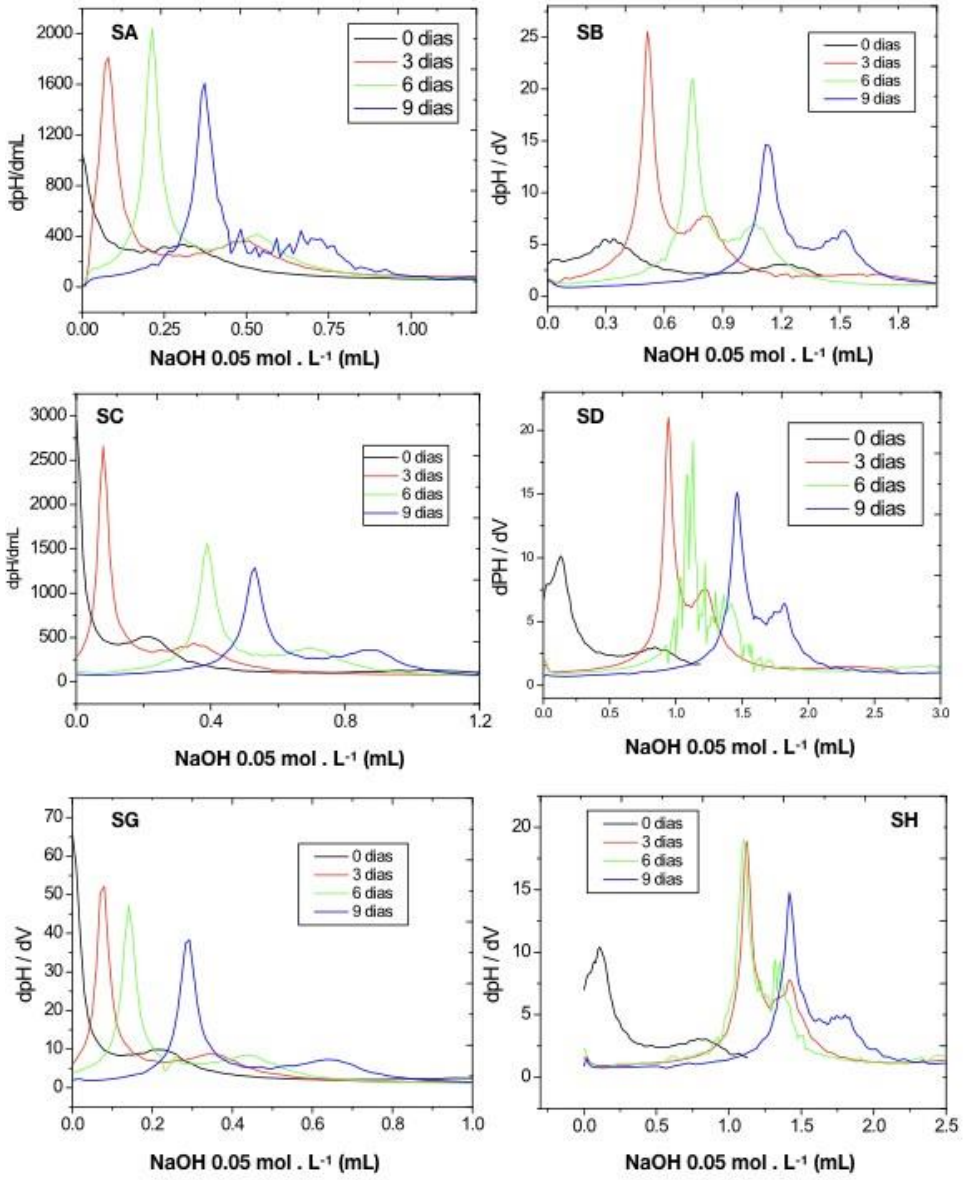
Supplementary material:



Potentiometric titration and derivate of organic acid production in submerged processes. The derivate method was applied after potentiometric titration to estimate the total acid concentration produced by Aspergillus tubingensis AN1257 in submerged processes supplemented with sucrose at 60 (SA, SB), 90 (SC, SD), or 120 g . L^{-1} (SG, SH) and at pH 4.0 (SA, SC), 7.0 (SB, SD), 3.37 (SG) or 7.62 (SH).