Juliana Cunha da Cruz

Itaconic acid production by A*spergillus terreus* using feedstock from the sugarcane industry: experimental investigation and mathematical modelling

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Doctorate thesis presented to the postgraduate program of "Engenharia de Processos Químicos e Bioquímicos" from Escola de Química – Universidade Federal do Rio de Janeiro, as a requirement for obtaining the Doctorate degree in Science (D.SC.).

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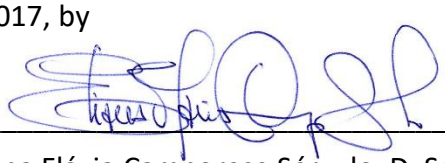

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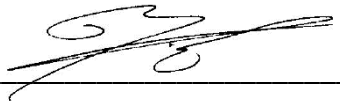

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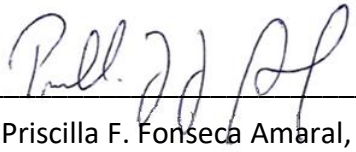

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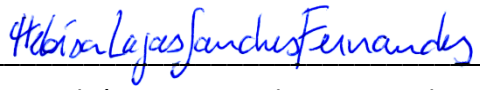

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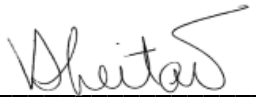

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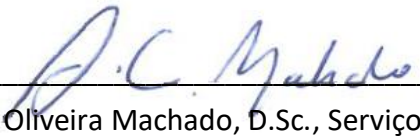

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To the Mother Earth

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“Quem não gosta de fungo bom sujeito não é/ É ruim da cabeça ou tem fungo no pé”
Version of Dorival Caymmi’s song

“It is only through education and increasing the intelligence of the masses that we can have WORLD PEACE”
GMCKS

“Step by step, oh babe!”
Maurice Starr (New Kids on the Block)

RESUMO

CRUZ, Juliana Cunha da. Produção de ácido itacônico por *Aspergillus terreus* usando matéria prima da indústria de cana de açúcar: investigação experimental e modelagem matemática. Orientadores: Eliana Flávia Camporese Sérvulo, Aline Machado de Castro, Philippe Bogaerts. Rio de Janeiro, 2017. Doutorado (Programa de Engenharia de Processos Químicos e Bioquímicos) – Escola de Química, Universidade Federal do Rio de Janeiro, Rio de Janeiro, 2017

O mercado mundial de ácido itacônico (AI) é esperado que exceda 200 milhões de dólares até 2020 devido à crescente demanda por produtos químicos de origem renovável. Esse composto, comercialmente produzido por fermentação, principalmente por *Aspergillus terreus*, pode ser utilizado como superabsorvente biodegradável em fraldas descartáveis, utilizado na indústria de transporte ou agrícola, para dispositivos eletrônicos e muitos outros. Uma possível estratégia para atingir o potencial mercado de AI, que é atualmente de nicho, é diminuir o custo da fermentação. O uso de matérias primas da indústria de cana de açúcar, como o açúcar cristal, ou a sua forma menos refinada, conhecido por "*very high polymerization*" (VHP), poderia ser uma alternativa menos dispendiosa comparado a substratos puros (sacarose e glicose), utilizados para obter altos rendimentos IA (cerca de 0,5 g IA/g de glicose). Além disso, o desenvolvimento de um modelo matemático dinâmico que descreva o processo fermentativo de produção de AI é uma estratégia que proporcionaria grande avanço no bioprocesso, não apenas para a melhor compreensão da produção do ácido, mas também para aplicar o modelo no controle do processo industrial. Este estudo avaliou sete cepas de *A. terreus* para a produção de AI com substratos puros (glicose e sacarose) e selecionou *A. terreus* NRRL 1960 (52 g/L IA; 0,43 g IA/g de glicose; 63% de eficiência) como a cepa mais apropriada para as investigações do processo. A avaliação que comparou a produção de AI em meios com açúcar cristal ou açúcar VHP – 114 g/L – e diferentes fontes de nitrogênio (nitrato de amônio (NH_4NO_3) – 3g/L – ou ureia comercial – 2,3g /L), indicou que a combinação de açúcar cristal e NH_4NO_3 foi a mais satisfatória na produção de AI (41 g AI/L). A combinação de VHP com NH_4NO_3 resultou em menor produção (25 g/L). Entretanto, a inovação inerente ao uso de VHP para produção de AI incitou a utilização dessa combinação para os estudos posteriores. Com o objetivo de verificar a produção em outras concentrações

das fontes de carbono (VHP) e de nitrogênio (NH_4NO_3), as fermentações foram feitas com as seguintes concentrações: 76, 95 e 114 g/L de VHP e 2, 4 e 6 g/L de NH_4NO_3. A aplicação da maior concentração de cada fonte resultou em maiores valores de produção de AI (46 g AI/L e 67% de eficiência). Ademais, o modelo matemático cinético proposto nesse estudo, desenvolvido com o auxílio do software MatLab®(versão R2016b student) a partir dos resultados de diferentes concentrações de VHP e de NH_4NO_3, descreve a produção de AI por três reações que representam o crescimento celular, a produção de AI e o consumo de AI quando o substrato sacarino é esgotado. A identificação dos parâmetros do modelo foi satisfatória (r^2 = 0,93) considerando todas os experimentos de combinações de carbono e nitrogênio. Este estudo apresenta o grande potencial de aplicação de VHP como matéria prima de menor custo que as fontes puras, com alto rendimento e produtividade, assim como estabelece um modelo matemático dinâmico que define a produção de AI por *A. terreus* NRRL 1960.

Palavras-chave: Ácido itacônico; *Aspergillus terreus*; Processos fermentativos; Material de origem renovável; Modelagem matemática dinâmica.

ABSTRACT

CRUZ, Juliana Cunha da. Itaconic acid production by *Aspergillus terreus* using feedstock from the sugarcane industry: experimental investigation and mathematical modelling. Advisors: Eliana Flávia Camporese Sérvulo, Aline Machado de Castro, Philippe Bogaerts. Rio de Janeiro, 2017. Doctorate (Programa de Engenharia de Processos Químicos e Bioquímicos) – Escola de Química, Universidade Federal do Rio de Janeiro, Rio de Janeiro, 2017

The global market of itaconic acid (IA) is expected to exceed $ 200 million by 2020 due to growing demand for chemicals from renewable sources. This compound, commercially produced by fermentation, mainly by *Aspergillus terreus*, can be used as a biodegradable superabsorbent in disposable diapers, used in the transportation or agricultural industry, for electronic devices and many others. One possible strategy to reach the potential IA market, which is currently niche, is to lower fermentation cost. The use of raw materials from the sugarcane industry, such as crystal sugar, or its less refined form, known as "very high polymerization" (VHP), could be a less expensive alternative compared to pure substrates (sucrose and glucose) applied for obtaining high IA yields (about 0.5 g IA/g glucose). In addition, the development of a dynamic mathematical model that describes the fermentative process of IA production is a strategy that would provide a great advance in the bioprocess, not only for the better understanding of the acid production, but also apply it to control the industrial process. This study evaluated seven *A. terreus* strains to produce IA with pure substrates (glucose and sucrose) and selected *A. terreus* NRRL 1960 (52 g/L IA, 0.43 g IA/g glucose, 63% efficiency) as the most appropriate strain for investigations in this study. The analysis that compared the production of IA in media with crystal sugar or VHP sugar – 114 g/L – and different nitrogen sources (ammonium nitrate (NH_4NO_3) – 3 g/L – or commercial urea – 2.3 g/L) indicated that the combination of crystal sugar and NH_4NO_3 was the most satisfactory for IA synthesis by *A. terreus* NRRL 1960 (41 g/L IA). The combination of VHP and NH_4NO_3 resulted in lower production (25 g/L). However, the innovation inherent in the use of VHP for IA production prompted the use of this combination for subsequent studies. The synthesis of IA was also evaluated in different concentrations of carbon (VHP) and nitrogen sources (NH_4NO_3), with the following concentrations: 76, 95 and 114 g/L of VHP, and 2,4 and

6 g/L of NH_4NO_3. The application of the higher concentration of each source resulted in higher AI production values (46 g/L AI and 67% efficiency). In addition, the kinetic mathematical model proposed in this study, developed with the use of the software MatLab® program (version R2016b student) with the results of different concentrations of VHP and NH_4NO_3, describes the production of IA by three reactions that represent cell growth, the production of IA and the consumption of IA when the sugary substrate is exhausted. The identification of the parameters of the model was satisfactory (r^2 = 0.93) considering all the experiments of combinations of carbon and nitrogen. This study presents the great potential of applying VHP as a lower cost raw material than pure sources with high yield and productivity, as well as establishing a dynamic mathematical model of IA production by *A. terreus* NRRL 1960.

Key words: Itaconic acid; *Aspergillus terreus*; Fermentative process; Bio-based material; Mathematical modelling.

LIST OF FIGURES

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LIST OF ABBREVIATION AND ACRONYM

A.	*Aspergillus*
ACO	Aconitase
ARS	Agricultural Research Service
ATCC	American Type Culture Collection
CBS	Convention of Biological Diversity
CA	Citric acid
CAD	*Cis*-aconitate decarboxylase
cadA	*Cis*-aconitate decarboxylase gene
CIS	Commonwealth of Independent States
CTC	Centro de Tecnologia Canavieira
DES	Deep eutectic solvents
DSM	Dutch State Mines
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DWPI	Derwent World Patent Index™
E&A	Eastern and Southern
EU	Europe Union
F	Force
g	Earth's gravitational acceleration
HPLC	High performance liquid chromatography
IA	Itaconic acid
IBGE	Instituto Brasileiro de Geografia e Estatística
IEA	International Energy Agency
LaBiM	Laboratório de Biotecnologia Microbiana
LCD	Liquid-crystal display
m	Mass
MFS	Major facilitator superfamily proteins
MA	Malic acid
MMA	Poly(methyl methacrylate)
Mtt	Mitochondrial tricarboxylate transporter

mtt	Mitochondrial tricarboxylate transporter gene
NRRL	Northern Regional Research Laboratory
P(AM-co-IA)	Poly(acrylamide-co-itaconic acid)
PAI	Poly(acrylonitrile-co-itaconic acid)
PBA	Poly(butyl acrylate)
PDA	Potato dextrose agar
PIA	Poly(acid itaconic)
PSt	Polystyrene
RID	Refractive index detector
SAP	Superabsorbent polymer
SBR	Styrene-butadiene rubber
ASTM	Active standard test method
sp.	Species
SP	São Paulo
TCA	Tricarboxylic acid
TRS	Total reducing sugars
UNICAMP	Universidade Estadual de Campinas
UPR	Unsaturated polyester resin
VHP	Very high polymerization
VVHP	High quality VHP
W&C	West and Central

LIST OF SYMBOLS

A	Number of the components j responsible for activation effect
Ef	Efficiency
F	Fisher information matrix
H	Number of the components j responsible for inhibition effect
I	Itaconic acid concentration
j	Reaction component
J	Cost function
k	Reaction number
K_M	Parameter representing the concentration of component at which the rate is half the maximum
$K_{y\ inhibition\ j}$	Inhibition coefficient
$K_{y\ activation\ j}$	Activation coefficient
M	Number of experiments
n	Number of measurements of each experiment
N	Total number of components of the reaction k
P	Final (or maximum) concentration of product
P_k	Product of the reaction k
P_0	Initial concentration of product
Q_i^{-1}	A positive-definite symmetric weighting matrix
R_k	Reactant of the reaction k
S_0	Initial concentration of substrate
$S_{i\,i}$	Confidence matrix
s	parameter sensitivity
S	Substrate concentration
t	Time instance of each measurement
t_f	Fermentation period
X	Cell concentration
X_0	Initial concentration of cells
$Y_{\frac{I}{X}}$	Itaconic acid/cell yield = pseudo-stoichiometric coefficient

$Y_{P/S}$	Yield of product in relation to substrate
$Y_{X/S}$	Yield of dry cells in relation to substrate
y_i	Reactants (or substrates) of the reaction k
y_j	Component j (reactants or products)
$y_{activation\ j}$	Component y that activates the reaction i
$y_{inhibition\ j}$	Component y that inhibits the reaction i
$y_{j\ measured\ max}$	Maximum concentration of each component for normalization purposes
$\mu_{\max k}$	Maximum specific rate of the reaction k
μ_X	Specific rate for cell growth
μ_k	Specific rate of the reaction k
μ_{max}	Maximum specific rate of cell growth
$\nu_{i,k}$, $\nu_{j,k}$	Respectively the pseudo-stoichiometric coefficients the reactant or the product of the reaction k
σ^2	Standard deviation
φ_k	Rate of the reaction k

SUMARY

LIST OF PUBLICATIONS

Abstract

Cruz JC, Castro AM, Sérvulo EFC. Itaconic acid bioprocess from different carbon and nitrogen sources. Green Chemistry and White Biotechnology International Conferences, Mons - Belgium, 22nd and 23rd May 2017

Oral presentation of full paper

Cruz JC, Farias JP, Castro AM, Sérvulo EFC. Otimização da produção de ácido itacônico por via microbiana. XX Congresso Brasileiro de Engenharia Química, Florianópolis – Brazil, 19 to 22 October 2014

Book Chapter

Cruz JC, Sérvulo EFC, Castro AM (2017) Microbial Production of Itaconic Acid In: Microbial production of food ingredients and additives, Grumezescu A and Holban AL (Ed.) Academic Press, 291-316.

Publication

Cruz JC, Dias DSB, Castro AM, Sérvulo EFC (2017). Itaconic acid production by *Aspergillus terreus* from low-cost carbon and nitrogen sources. International Journal of Engineering and Technical Research (IJETR) 7(9)

Chapter 1

INTRODUCTION

The world demand for eco-friendly products is constantly growing. Many bioprocesses are under development to align the environmental and economic aspects of manufacturing renewable products, which are not all feasible yet (Bailey 2016). The continuing effort and research, associated with government policies that promote sustainable programs, effectively nurture the growth of bio-based products market (Report Linker 2017). Significant research has been done to obtain products that are economically feasible and from renewable sources.

Bio-based organic acids are part of the portfolio of profitable and renewable chemicals. The combination of those two important factors results in an increasing demand of those acids. Moreover, the stringent restrictions imposed by governmental regulators in many countries have been encouraging companies to seek alternative renewable products and biotechnological processes (Report Linker 2017). The challenge is not only to obtain eco-friendly products, but to have equivalency in quality and quantity for competing with the products that are already available on the corresponding market (Bailey 2016).

Itaconic acid (IA) is a bio-based chemical with great potential for the chemical market and attractive end use applications (Weastra 2012). Even though the chemical properties of the organic acid enable a vast possibility of applications, such as for the agricultural and transportation industry, personal care sector, for components of electronic devices, among others, IA is currently a niche market (Transparency Market Research 2015). The expected expansion of IA on the appropriate market depends on the development of the technologies for producing IA and its derivatives. Innovation, price competitiveness and global expansion are key components for any product to achieve success in the renewable market. Improvements in production medium, as well as the application of the most appropriate fermentation conditions for achieving high IA yield, are some of the investigations regarding the develop of technologies for IA production (Krull et al. 2017).

Commercial IA is obtained by microbial fermentation, mainly by *Aspergillus terreus* with glucose (Saha 2017), however, sucrose is also a possible substrate (Lockwood and Moyer 1945). The high sensitivity to impurities, inherent IA production by *A. terreus*, impairs the use of some raw materials without previous pretreatment (Hiller et al. 2014) such as molasse (Nubel et al. 1962) or lignocellulosic material (Sieker et al. 2012; Pedroso et al. 2017). The requirement of a step of purification would increase the end-product cost. Moreover, the use of feedstocks of pure quality, such as glucose or sucrose, increase the end-product cost, especially because that bioprocess demands a high initial concentration of substrate – over 100 g/L for sugars (Karaffa et al. 2015). Lower cost feedstock from sugar carbon source may be applied as an alternative for obtaining high IA yields without the need of previous treatment of the feedstock.

The most exported sugar in the world (Platts 2016), very high polymerization (VHP) sugar is a more processed sugar than the crude one from sugarcane, developed and produced

in Brazil. VHP is used in the food industry to obtain different products, including granulated sugar and others refined sugars (Usina Atena 2017). However, the strong recommendation for reducing sugar consumption by humans (World Health Organization 2015) has decreased the demand of different types of sugars in food industry over the years in some countries such as Norway, Canada, India and Brazil (Reuters 2017). That and other facts have already been a subject to the preposition of the European Union to redirect part of the sugar for bioethanol production and to other fermentations processes (COFALEC 2015).

This study investigated IA production in different conditions with *A. terreus* strains. It was studied the use of two sugary carbon sources (granulated sugar and VHP) and two nitrogen sources (ammonium nitrate and unpureed commercial urea) to select the two sources of carbon and nitrogen. The selected ones were applied in different initial concentrations to evaluate the IA production. Moreover, a dynamic mathematical model was developed and its kinetic parameters were estimated, presenting the reaction scheme that describes the consumption of substrate and ammonium and the production of IA by *A. terreus* in a medium with non-pure sugar source.

Chapter 2

ITACONIC ACID

This chapter introduces IA potential regarding its market and applications. It also presents IA large scale process. The description about IA are, then, addressed to the biochemical aspects, which are important for understanding the bioprocess. The information should be a guide toward a better understanding of the obtained results in further chapters. Finally, a brief analysis of IA scientific publications and patents trends is presented as an estimation of the current state of art of IA. This chapter intend to provide context for justifying this study.

2.1 IA origin and definition

Itaconic acid (IA) may be synthesized either chemically or biochemically. The former has never been produced commercially due to the numerous stages for IA synthesis and the low efficiency of the process, while the latter is obtained by fermentation mainly using filamentous fungi with a significant production yield (Pfeifer et al. 1952; Kuenz et al. 2012). A more detailed description about IA production is presented in later sections.

IA is presented in the form of white crystals and it is chemically defined as an unsaturated dicarboxylic acid with one of the carboxylic groups conjugated to a methyl group (Figure 2.1). Some of IA characteristics are listed on Table 2.1.

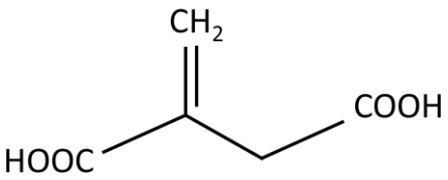

Figure 2.1 Molecular structure of IA.

Table 2.1 Properties of IA (Zhang et al. 2013; Pubchem 2017).

IUPAC name	2-methylidenebutanedioic acid
Synonyms	Itaconic acid; 2-methylenesuccinic acid; methylelesuccinic acid; Propylenedicarboxylic acid; Methylenebutanedioic acid
Abbreviation	IA
EC Number	202-599-6
CAS Number	97-65-4
Molecular formula	$C_5H_6O_4$
Molar mass (g/mol)	130.09874
Melting point	165 – 168 °C
Appearance (Color)	White
Appearance (Form)	Powder or crystals
Density (g/cm³ at 25°C)	1.573
Solubility in water (g/100 mL, 20°C)	8.31
Acidity (pK_a)	pK_{a1} = 3.84 pK_{a2} = 5.55

2.2 A brief history about IA

IA was discovered in 1837, described by Baup as a product obtainable from the pyrolysis of citric acid and it was named citricic acid (Turner and Liebig 1841; Kane et al. 1945; Tate 1967). In 1840, Crassus described it as the product of the third stage of thermal decomposition of citric acid, and proposed the name itaconic acid (Turner and Liebig 1841). At that time, the chemical route was the only one known.

The IA chemical synthesis is as follows:

- Distillation of citric acid;
- Oxidation of isopropene, or from mesityl oxide to citraconic acid and subsequent isomerization;
- Carboxylation of acetylene derivatives, for example, propargyl chloride or butynoates;
- Condensation of succinate or succinic anhydride with formaldehyde to generate citraconic acid with subsequent isomerization.

This sequence of chemical reactions concerning the chemical route, however, is not economically feasible. The requirement for several stages resulted in an unsatisfactory yield and used components that were not readily available (Merger and Liebe 1991).

IA was first polymerized by Swarts as a form of ethyl ester in 1873 (Tate 1967). Dialkali esters polymers were developed with properties close to glass at a process that lasted about three days (Hope 1927). Despite the interesting properties, it was only possible to obtain IA at a small scale due to the already mentioned low efficiency of the production by chemical route.

In 1931, Kinoshita first reported the production of IA by the microbial route. In the mentioned study, a filamentous fungus isolated from salted prune juice was cultivated under surface fermentation conditions in the presence of concentrated solutions of sugars and high concentrations of chlorides, reaching yield of up to 0.24 (g IA/g substrate). Because the microorganism used was an IA producer, the filamentous fungus was named *Aspergillus itaconicus* (Kinoshita 1931). The production condition of that study, however, was never developed commercially (Kane et al. 1945).

Calam et al. (1939) presented that some *A. terreus* strains produced IA in Czapek-Dox medium containing 50 g/L of glucose. That was the first study which demonstrated that *A.*

terreus was able to produce IA (0.12 g IA/g substrate after 25 days). The authors also showed that most strains – 5 out of the 6 strains tested – did not produce this organic acid to the extracellular medium at the conditions used (Calam et al. 1939).

The homopolymerization of IA was described in 1958, which was done with hydrochloric acid and potassium persulfate (Marvel and Shepherd 1959). Because of the interesting properties of IA polymers, further investigation was done to reach higher final concentrations of IA from different strains.

According to Miall (1978), in 1945, Moyer and Coghill evaluated 30 *A. terreus* cultures from the traditional microbial cell bank Northern Regional Research Laboratory – NRRL (currently Agricultural Research Service – ARS), identifying the strain NRRL 265 as the only IA producer. In the same year, Lockwood and Reeves analyzed 308 strains isolated from soil samples and *A. terreus* NRRL 1960 was chosen for pilot scale processes (Willke and Vorlop 2001). Since then, *A. terreus* strain NRRL 1960 has been the most studied among researchers to obtain IA. *A. terreus* NRRL 1960 is stored in different international cell banks with the following codes: ATCC 10020, ATCC 20589, DSM 826, CBS 11646, among others (NRRL 2017).

The production of IA by microbial route was first patented in 1945 (Kane et al. 1945). Pfizer Company accomplished 28% of the theoretical yield for producing IA with sucrose after 14 days of fermentation (Kane et al. 1945). IA was included in the company's product portfolio in 1945 (Okabe et al. 2009).

The use of other bioprocess conditions allowed Lockwood and Moyer (1945) to obtain 30 g of IA from 100 g of glucose (42% of the theoretical yield). In the following years, the strain *Aspergillus terreus* NRRL 1960 was used for a larger scale production in a 20 L bioreactor (Nelson et al. 1952). At that stage, studies were done on a large scale (between 1130 and 2270 L), and in semi-continuous fermentation (Pfeifer et al. 1952). Other microorganisms have been reported as IA producers, such as *Ustilago zeae* (Miall 1978), and by different *Candida* sp. strains (Horitsu et al. 1983).

In 2004, IA was listed as one of the 12 most promising chemicals available from biomass, according to the United States Department of Energy report (Werpy and Petersen 2004). The document selected IA and 11 others from an initial list of more than 300 bio-based building blocks regarding the potential markets of the chemicals and their derivatives, and the technical complexity in producing those chemicals. Since that report, which included succinic,

fumaric and malic acid among other chemicals, IA gained significant interest in the scientific community and it stimulated vast research regarding the improvement in IA production and its applications (Kuenz et al. 2012; Klement and Büchs 2013).

The homopolymerization process of IA on a large scale was a challenge in the early 21th century (Werpy and Petersen 2004). The improvement of the economic feasibility for obtaining IA depended on the development of the polymerization techniques that would decrease production cost to obtain the homopolymer. That barrier was overcame by researchers from the University of New Hampshire, and the technology was licensed to Itaconix®, which proceeded to develop IA products from the poly(itaconic acid) (PIA) (Figure 2.2) (Durant 2011).

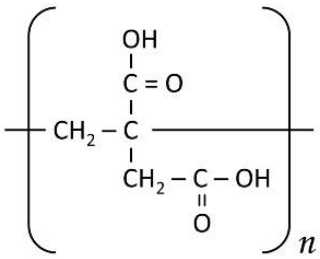

Figure 2.2 Representation of poly(itaconic acid) (PIA).

More recently, IA was identified to be secreted by mammalian immune cells, such as macrophages, responsible for the antimicrobial activity by those cells in situations of inflammatory conditions (Sugimoto et al. 2011). IA was previously detected in the lungs of mice infected with tuberculosis, but it had been assumed that the metabolite was produced by the contaminating bacteria (Shin et al. 2011; Cordes et al. 2015). Michelucci et al. (2013) identified that, in mammalian cells, IA is produced by the immunoresponsive gene 1(*Irg*l), a highly expressed gene by macrophages in inflammation. That IA characteristic was explored by Bajpai et al. (2016) as a component of antimicrobial biofilm with potential application in the biomedical field. A summary of the historical facts is presented on Figure 2.3.

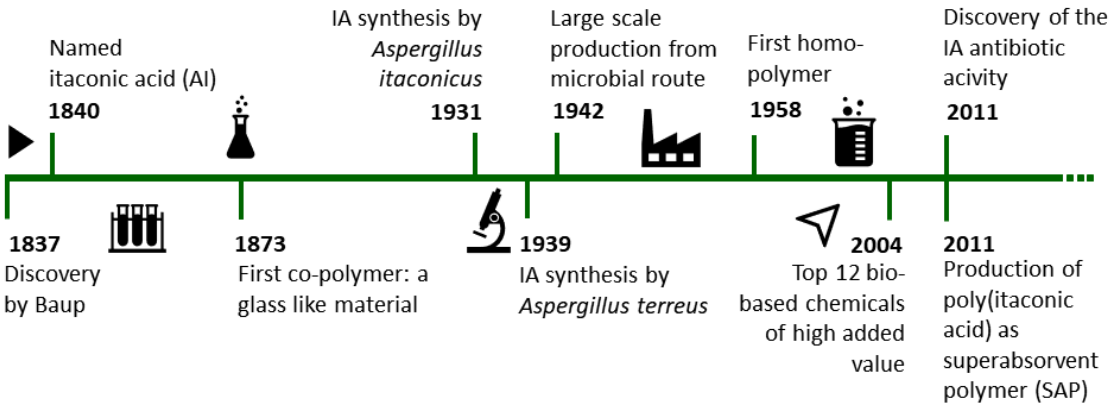

Figure 2.3 Itaconic acid chronological history.

2.3 IA-producer microorganisms

Different microorganisms are able to synthesize IA, but with different production capacities. The main IA producers are from the species *A. terreus*, which are applied on a commercial scale (Saha 2017). The requirement for systems that result in higher IA productivity and higher yields (product/consumed substrate) encourages researchers to investigate further possible IA-producers (Voll et al. 2012). Table 2.2 lists some of the producing microorganisms, as well as the characteristic of the process.

Most of the studies of IA production presented on Table 2.2 were done with filamentous fungi. *A. terreus* is frequently used for scientific research since the beginning of IA studies, and it was obtained high IA yield. However, most studies listed and many others from the literature used glucose as substrate. Despite the high yield and productivity achieved with that carbon source, the use of less expensive material should be reconsidered for producing an end-product more competitive to the bio-based market. Nonetheless, the studies that applied residual feedstocks resulted in lower values of IA production.

It is noticed still from Table 2.2 that the filamentous fungus *A. terreus* produces the highest IA concentrations in glucose medium. However, the technical difficulties regarding the fermentation with filamentous fungus, compared to the processes with bacteria or yeast, encourages the research for different IA producers. The bioprocess with filamentous fungi is usually sensitive to hydro-mechanical stress in submerged fermentation (Voll et al. 2012) and its filamentous growth characteristics can be operationally more complicated than other microorganisms mentioned. Therefore, the use of *Ustilago maydis* may be a promising microorganism for IA synthesis.

Table 2.2 Bioprocesses of IA production with different microorganisms and some of the processes conditions.

Microorganism (reference)	Feedstock	Working volume and fermentation type	IA (g/L)	Yield (IA/substrate)	Productivity (g IA/L/h)
A. terreus NRRL 265 [a] (Lockwood and Moyer 1945)	Sucrose	50 mL, submerged fermentation	48.7	n.d.	0.18
Mutant of *A. terreus* NRRL 265 and 1960 [a] (Nubel et al. 1962)	Beet and sugarcane molasses	2L, submerged fermentation	50	n.d.	-
A. terreus NRRL 1960 [a] (Kautola et al. 1985)	Xylose and glucose	14 L, immobilized cells in submerged fermentation	30	0.54 g/g	0.31
Mutant of *A. terreus* ATCC 10020 [a] (Tsai et al. 2001)	C6 sugars mannose, starch hydrolysate and molasse	45 mL, semi-solid state fermentation	n.d.	0.55 g/g	n.d.
A. terreus TN484-M1[a] (Dwiarti et al. 2007)	Corn sorghum	3L, submerged fermentation	48	0.34 g/g	0.33
A. terreus DSM 23081[a] (Kuenz et al. 2012)	Glucose	100 mL, submerged fermentation	90	n.d.	0.28
A. terreus CECT 20365[a] (Vassilev et al. 2012)	Glucose and glycerol	100 mL, immobilized cells in submerged fermentation	27	0.27g/g	0.22
A. terreus CECT 20365 [a] (Vassilev et al. 2013)	Sugar beet press-mud, olive residues and glycerol	10 g, solid-state fermentation	n.d.	44 g/kg	n.d.
A. terreus NRRL 1960 [a] (Kocabas et al. 2014)	Corn cob, cotton twig and sunflower stalk with glucose	100 mL, simultaneous enzymatic hydrolysis and fermentation	18	n.d.	0.06
A. terreus DSM-23081[a] (Hevekerl et al. 2014a)	Glucose	1 L, fed-batch submerged fermentation	130	0.58 g/g	1.08
A. terreus DSM-23081[a] (Krull et al. 2017)	Glucose	15 L, fed-batch submerged fermentation	150	0.56 g/g	0.62
Mutant of *A. niger* ATCC 1015[a] (Blumhoff et al. 2013)	Glucose	100 mL, submerged fermentation	1.2	n.d.	0.003
Mutant of *Candida* sp. [b] (Tabuchi et al. 1981)	Glucose	25 mL, submerged fermentation	35	n.d.	0.29
Mutant of *E. coli* [d] (Okamoto et al. 2014)	Glucose	1.5 L, submerged fermentation	4.3	0.13 g/g	0.04
Pseudozyma antarctica Y-7808 [c] (Levinson et al. 2006)	Glucose	1L, submerged fermentation	30	0.38 g/g	0.21
Ustilago maydis MB215 [c] (Maassen et al. 2014)	Glucose	2 or 6 L, submerged fermentation	44.5	0.24 g /g	0.31
Ustilago maydis MB215 [c] (Carstensen et al. 2013)	Glucose	2L, submerged fermentation	4	n.d.	n.d.
Ustilago maydis MB215 [c] (Klement et al. 2012)	Glucose	20 mL, submerged fermentation	25	n.d.	n.d.
Mutant of *Yarrowia lipolytica* PO1f [b] (Blazeck et al. 2015)	Glucose	1.5 L, submerged fermentation	1.2	0.058 g/g	0.007

The microorganisms are from different kingdom and types. [a] Filamentous fungus, [b] yeast, [c] basidiomycetes or [d] bacteria.

Ustilago maydis is a basidiomycete, which is a non phytopathogenic microorganism when presented as a free-living yeast-like cell, and phytopathogenic as the filamentous form (Levinson et al. 2006; Rafi et al. 2014). Despite the advantages of using basidiomycete, the highest production obtained from that microorganism is about 0.2 g IA/ g glucose (Maassen et al. 2014), which is still much lower than the highest concentrations produced by *A. terreus* (0.48 IA/ g glucose) in batch fermentation in laboratory scale (Kuenz et al. 2012).

Although research tends to focus on *Aspergillus* and *Ustilago* strains, different studies have demonstrated that IA may be produced by other microorganism species. *Helicobasidium mompa* produces IA at low values – 0.25 to 0.5 g/L IA (Araki et al. 1957). *Candida* sp. was genetically modified and it produced about 0.35 g IA/g glucose (Tabuchi et al. 1981). Despite the potential results, to the best of the authors knowledge, no further studies were presented regarding IA production by that *Candida* sp. *Pseudozyma antarctica* was also reported as IA producer, which synthesized 0.1 g IA/g substrate in medium containing either glucose or fructose (Levinson et al. 2006). Genetically modified *E. coli* was also less efficient than *A. terreus*, with final production of about 0.14 g IA/ g glucose by the 4th day of fermentation (Okamoto et al. 2014). Despite the different strategies applied for IA production with other microorganisms, *A. terreus* is still the current, dominant choice for IA production on a commercial scale.

The urge of increasing IA production also drives research on metabolic manipulation of microorganisms (Kuenz et al. 2012). Genetic modification has been done on *Aspergillus niger*, which is expected to produce higher IA concentrations than the bioprocess done with *A. terreus*, since the latter produces over 200 g/L of citric acid (also an organic acid) and has a very similar metabolic system to its parental strain (Blumhoff et al. 2013). The efforts done to induce the capacity of producing IA by *A. niger* has been an important tool for the acknowledge of the IA pathway in *A. terreus* (Steiger et al. 2016).

2.4 Metabolic system of IA production by *Aspergillus terreus*

Different studies regarding the metabolic pathway of IA synthesis have been done mainly with *A. terreus* (Tevz et al. 2010; Huang et al. 2014a; Huang et al. 2014b). Currently, it

is highly accepted that *cis*-aconitate decarboxylase (CAD) is responsible for the final transformation of *cis*-aconitate to itaconate (Hossain et al. 2016; Jiménez-Quero et al. 2016).

In IA synthesis from glucose, the substrate enters the cell and it is degraded mainly via the glycolysis route. Both malate and pyruvate produced in the cytosol enters the mitochondria to the TCA cycle, where *cis*-aconitate is produced. *Cis*-aconitate is transported to the cytosol through a mitochondrial tricarboxylate transporter (Mtt), where CAD synthesizes the itaconate production (Li et al. 2013). Finally, itaconate is externalized through major facilitator superfamily proteins (MFS). The kinetic profile of IA production shows that a slow or null cell growth rate prevails during IA production (Kuenz et al. 2012). This is explained by the deviation of *cis*-aconitate from the mitochondria to the cytosol, i.e., some of the reactions of the TCA cycle are interrupted, thus, cell growth is limited or null. Figure 2.4 illustrates the metabolic pathway from glucose.

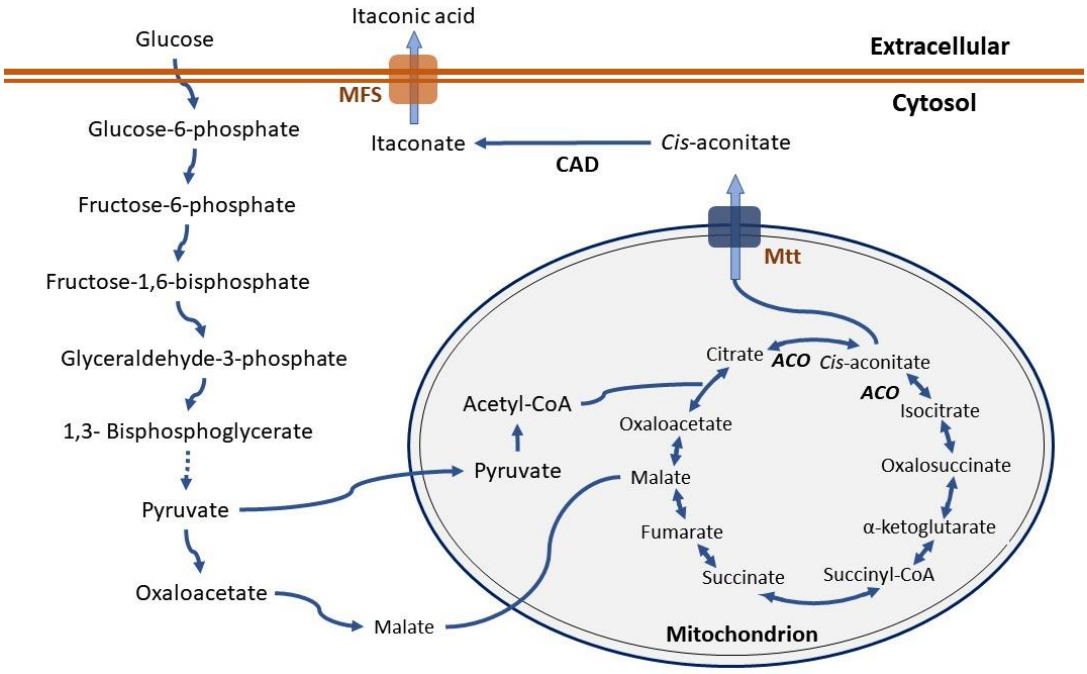

Figure 2.4 Itaconic acid biosynthesis pathway in *Aspergillus terreus*. Mtt: Mitochondrial tricarboxylate transporter; MFS: major facilitator superfamily proteins; CAD: *cis*-aconitate decarboxylase; ACO: aconitase. (Adapted from Klement and Büchs (2013) and Huang et al (2014b)).

Dwiarti et al. (2002) were able to characterize the enzyme CAD for the first time and Kanamasa et al. (2008) identified the gene responsible for its coding. Those findings confirmed the CAD enzyme function in synthesizing IA and identified the region where the gene is located (Lai et al. 2007). (Bentley and Thiessen (1957) identified that the enzyme is inhibited by heavy

metals. Despite these conclusions, up to now, the regulation of IA synthesis is not yet completely known (Klement and Büchs 2013).

A deep understanding of the effect of nutrients on cultivation and production is of paramount importance given that the regulation of CAD is possibly related to the limitation of the essential element other than carbon (Welter 2000). Further knowledge about *A. terreus* metabolic regulation, as well as the effect of medium components and operation conditions, is of great importance for increasing IA production.

2.5 IA: a bio-based chemical with potential market growth and a wide range of applications

The technical barriers of IA synthesis over 10 years ago concerned the formation of other co-products during the fermentation and the need to increase the fermentation's yield and productivity (Werpy and Petersen 2004). Despite the technological advances, there are still limitations regarding the production process, and the decrease of industrial costs are still desirable (Weastra 2012).

The IA market is characterized as a niche among other chemicals mainly due to limited assimilation of IA products in the market and the large availability of substitutes for those. The decrease of production costs would increase IA economic feasibility and expand the commercial interest for IA (Transparency Market Research 2015). Because of its market potential in a world scenario with increasing demand for bio-based chemicals, the IA market is expected to exceed 216 million of dollars by 2020 (Global Industry Analysis 2016).

The advantages of IA products over other chemicals from fossil source include its biodegradability (as homopolymer), non-toxicity and a variety of possible derivatives as polymers. Some IA applications are under development, some of them still need further research and development (R&D) to be economically feasible, while some others are closer to reaching the commercial market. Some of the vast number of end-products available in the literature are presented on Table 2.3.

Currently, the most promising applications of IA are as synthetic latex, methyl methacrylate (MMA), unsaturated polyester resins (UPR) and superabsorbent polymers (SAP) (Global Market Insights 2016), illustrated on Figure 2.5. Synthetic latex represents over 50% of the global market share of IA products, mostly used for polymer stabilization – SBR latex

(styrene-butadiene rubber). As this market niche is already the most demanding for IA, it is expected to have a less prominent growth over the next years (Global Market Insights 2016).

Table 2.3 Characteristics and application of polymers produced with IA.

Polymer	End-product properties	Applications
Poly(itaconic acid) (PIA) (Sadeghi and Hosseinzadeh 2008).	This homopolymer produces superabsorbent polymer (SAP) with hydrophobic properties due to functional crosslinks. SAP can absorb hundreds or thousands of times of its mass, although it is soluble in water.	Disposable diapers and feminine absorbents, retention agents in agriculture, concrete additives, soil correctives, controlled drug release system. It is a potent candidate for replacing SAP from acrylic acid.
Poly(acrylamide-co-itaconic acid) P(AM-co-IA) (Shi et al. 2014)	It forms superabsorbent hydrogel microspheres with high absorption capacity, similar to poly(itaconic acid).	Soil amendments, water shutoff agents, and drug delivery carriers.
Poly(acrylonitrile-co-itaconic acid) (PAI) (Nguyen-Thai and Hong 2014)	(PAI) has high electronic conductivity.	Carbon-based soft electronic devices.
Polystyrene-core(PSt)/ poly(butyl acrylate)(PBA)-co-itaconic acid)-shell and PBA-core/(PSt-co-IA)-shell polymers (Aguiar et al. 1999; Rabelero et al. 2013)	It may be presented as shell-core polymer produced with polystyrene. It changes from elastic to rigid by increasing the amount of IA incorporated into the shell, with properties close to those of a rigid plastic.	Coating, adherent material or high strength plastics.
Poly(itaconic acid–co–bisacrylamide) (Bednarz et al. 2014)	Hydrogels formulated with deep eutectic solvents (DES) composed of high density cross-links with the ability to absorb metal ions.	Absorbents of metal cations (Cu^{2+}, Co^{2+}, Ni^{2+}).

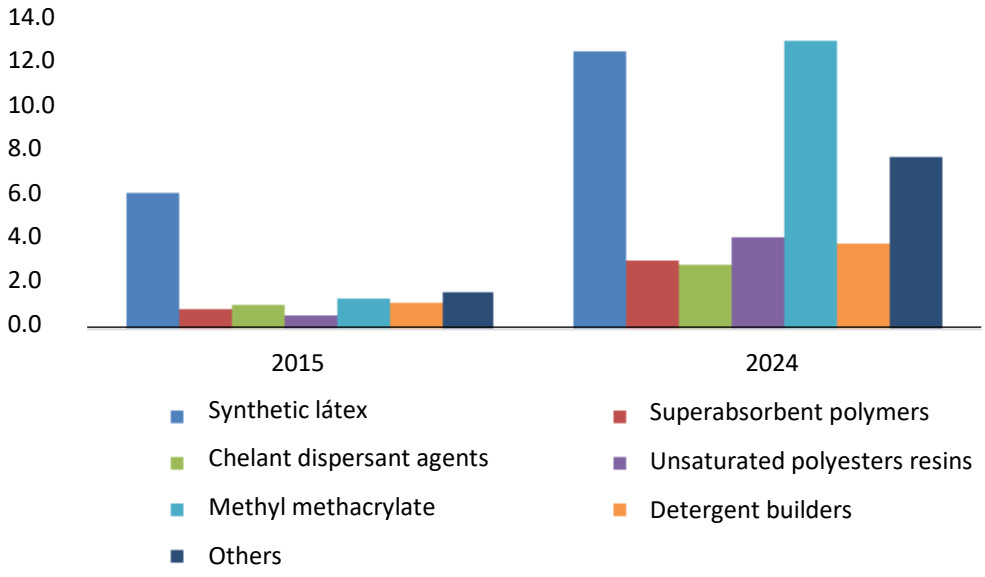

Figure 2.5 USA IA market size in 2015 (real values) and 2024 (projection) of each application. Vertical axis in millions of USD (Global Market Insights 2016) .

The expansion of the MMA market, currently produced from acetone cyanohydrin , is expected to further fuel IA demand. MMA requirement in liquid-crystal display (LCD) screens, smartphones screen and video equipment are some of the mostly likely applications of IA (Global Market Insights 2016).

The use of IA as UPR, which can also be produced with maleic anhydride (Weastra 2012), is directed to marine, construction and transportation industries. Because of its similar structure to maleic anhydride, IA is a potent bio-based chemical for substituting non-renewable chemicals (Global Market Insights 2016).

2.6 IA large scale production

The process of IA production was well described by Okabe et al. (2009). According to the authors, the industrial production of IA is a five-step process. The fermentation step concerns in IA synthesis from microorganism, whose cells are removed at the end of the process by filtration process. The solid-free broth goes to the concentration step where a liquid of over 350 g/L of IA is obtained. The concentrated liquid passes through two series of crystallization processes, at 15°C. The crystals formed are decolorized by treatment with activated charcoal at 80°C. In the case of a large-scale industrial process, the decolorization process can be optimized. The decolorized broth is evaporated and recrystallized before going to the drying and packing steps. If the production requires a high degree of purity, the product goes through a purification process, such as solvent extraction, ion exchange and a new decolorization. Each stage has high efficiency for recovering IA: 95% in the filtration step, 98% of the concentration process and 95% in the crystallization and drying. The total recovery of the process is approximately 80%. The production steps are illustrated on Figure 2.6.

2.7 IA global production

IA was first commercially produced by Pfizer Company, in 1945. Since then, other companies such as Iwata Chemical (started at 1970, in Japan), Rhodia (started at 1995, in France) and Cargill (started at 1996, in the USA) have been great producers (Okabe et al. 2009). The interruption of IA production by Cargill, Pfizer and Rhodia increased the significance of

the other companies worldwide and it resulted that China is currently the country with the largest IA production (El-Imam and Du 2014).

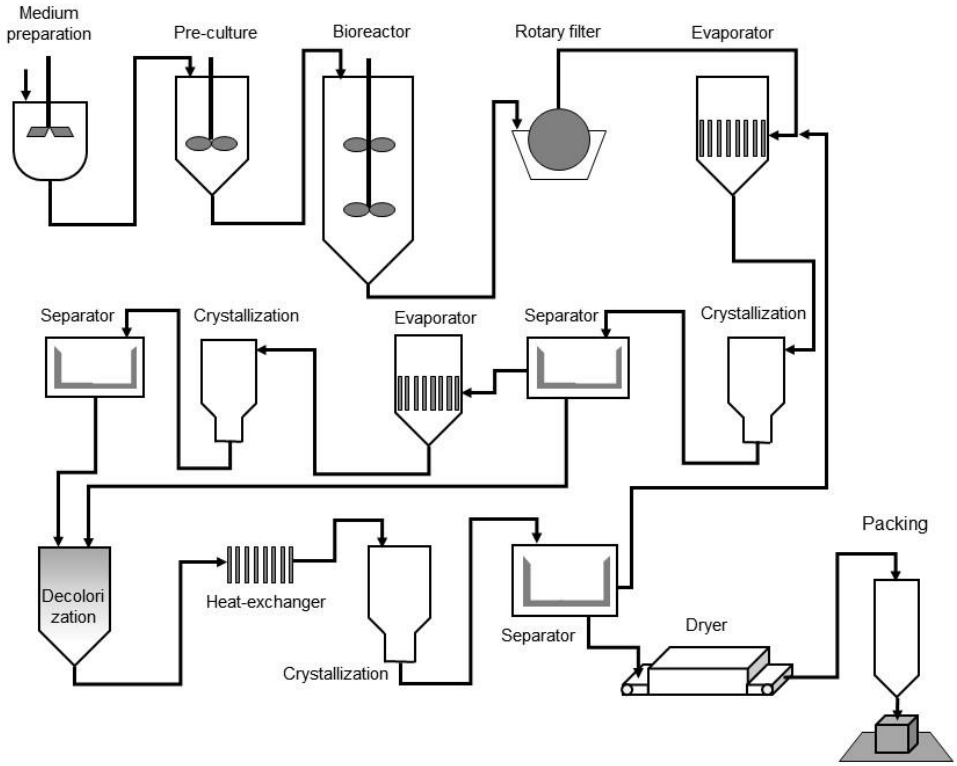

Figure 2.6 Diagram of itaconic acid production by *A. terreus* (adapted from Okabe et al. (2009)).

China has been receiving robust investments from companies and from the Chinese government, including in bioprocesses industries. The increasing research background, human resources and financial support has provided the biotechnology industry growth over recent years in that country (Huang et al. 2010).

Among many Chinese companies that produce IA, the Qingdao Kehai Biochemistry Company is responsible for about 50% of the total capacity of IA production in China, or 18% worldwide, with 10,000 Mt/year (Huang et al. 2010). That company is part of the Qingdao Langyatai Group, and exports IA mainly to North and South America, Western and Eastern Europe (China 2017).

The last reports show that only three countries are currently responsible for the world production: China, India and the USA (Global Industry Analysis 2016). The main current players are Alpha Chemika (India), Chenggdu Jindai Biology Engineering Co. Ltd (China), Jinan Huaming Biochemistry Co. Ltd (China), Qingdao Kehai Biochemistry Co. Ltd (China), Shandong Kaison

Biochemistry Co. Ltd (China), Zhejiang Guoguang Biochemistry Co. Ltd (China) and Itaconix Co. (USA).

In fact, the Asia-Pacific region market should serve as an example for developing the IA market in other countries. The existence of many domestic manufactures among IA players in that region represent the moderately fragmented IA market. The development of technologies and applications addressed to those niche and local markets may be the key strategy to expand IA production in other countries (Global Market Insights 2016).

One example of targeting local opportunities is the possibility of growth in Europe because of product control by the government. Currently, European Union regulations to stop the manufacture of detergents produced from sodium tri(poly)phosphate (STPP) may be substituted with IA derivatives. In Germany, the IA market benefits from environmental government practices and its size reached 2.8 million in 2015. South Africa, Saudi Arabia and the United Arab Emirates may also be targets for IA applications as a result of rising preferences for bio-based products in those countries (Global Market Insights 2016).

2.8 Medium requirement for a high yield IA production

Multiple parameters influence metabolites production, such as medium composition, pH, temperature, the presence or absence of trace elements, and many others (Vrabl et al. 2012). Among them, the carbon source used is very important for an economically feasible process of IA production. The requirement of high initial concentration of sugars to obtain high yields reflect on high cost with feedstock if pure substrates, such as glucose or sucrose, are used.

The knowledge about the sufficient concentration to be used for high IA production without the use of excessive substrate affects the production final cost and depend on the strain. The highest IA yields (> 0.55 g IA /g glucose) are achieved with over 100 g/L of glucose by *A. terreus* NRRL 1960, without significant increase neither decrease in the final yield with substrate up to 200 g/L (Karaffa et al. 2015). Different results were obtained with *A. terreus* NRRL 1963, which presented an inhibition effect with concentrations higher than 160 g/L of glucose (Welter 2000). Kuenz et al. (2012), however, showed that similar concentrations are

obtained by *A. terreus* NRRL 1993, *A. terreus* NRRL 1960, and *A. terreus* DSM 23081 (0.5 g IA / g glucose).

Considering the kinetics properties of CAD, an essential enzyme for IA production, its K_M value for its main substrate, *cis*-aconitic acid, is 2.45 mM at pH 6.2 and 37°C (Dwiarti et al. 2002). The low affinity to its substrate in IA synthesis indicated by the high K_M value demonstrates the need for high substrate concentration for achieving high production yields (Cordes et al. 2015).

Different nitrogen sources, such as yeast extract or corn steep liquor, were used in early studies (Pfeifer et al.1952), but the complexity and varied composition of those reactants are undesirable factors for developing a stable production platform. IA fermentation with urea or ammonium nitrate resulted in low fermentation rates according to Nelson et al. (1952) and Pfeifer et al. (1952). However, ammonium nitrate (NH_4NO_3) has been used as nitrogen source in many other studies with high IA yield (Kautola et al. 1991; Kuenz et al. 2012).

Regarding the nitrogen source concentration, Vassilev et al. (1992) showed that, for immobilized cells, the rate of IA production in absence of nitrogen is higher than with an initial concentration of 4g/L of NH_4NO_3. Those results indicated that the nitrogen consumption is related to cell production rather than IA synthesis (Vassilev et al. 1992). Welter (2000) evaluated the combination of NH_4NO_3 with KH_2PO_4 and it was observed that, for initial 94 g/L of glucose, minimal cell growth and high IA production is obtained with 0.08 g/L KH_2PO_4 and 2 g/L of NH_4NO_3. Kuenz et al. (2012) chose to use 3 g/L NH_4NO_3 rather than 1.5 g/L to avoid insufficient nitrogen source supply, even though both initial concentrations of NH_4NO_3 resulted in similar results.

Other medium components can influence IA production such as Fe, Mn – it should be below 5 µg/L to result in high yields of IA (Karaffa et al. 2015), Mg, Cu, Zn, P, N and carbon source concentration (Miles Laboratories and Elkhart 1963; Kautola et al. 1991; Willke and Vorlop 2001; Li et al. 2012; Karaffa et al. 2015).

2.9 IA production with low cost feedstock

Studies have shown that some residues are suitable as carbon source for IA production, with some examples presented on Table 2.2. The limitations of IA production in some media

are related to *A. terreus* sensitivity to medium impurities, which are not yet well defined (Hiller et al. 2014). However, the literature does not detail which components and at which concentration they impair IA production. Despite that sensitivity, some studies show the capacity of *A. terreus* to produce IA from waste material. The importance in evaluating IA production from residues relies on the possibility of IA production in different countries depending on the abundance of the specific residue. By using low cost feedstock from local source, IA production economic feasibility may promote further application to the market.

Reddy and Singh (2002) showed that 20 and 30 g/L IA was produced, respectively, from market refuse fruits and hydrolyzed corn starch with *A. terreus* mutant. Petruccioli et al. (1999) obtained 18 g IA/L from corn starch feedstock, while (Dwiarti et al. 2007) obtained about 50 g IA/L using hydrolysate sago starch. The use of molasse medium requires a previous treatment for removing the impurities for a high IA yield process (Maassen et al. 2014). The values obtained when residues are used which are still lower than the IA concentrations produced in glucose medium in batch fermentation (about 90 g/L IA (Kuenz et al. 2012)). That reinforces the difficulties in applying a medium with impurities.

Corn cob, a lignocellulosic residue, was used in a two step process: first, xylanase was produced by *A. terreus*, which was further used on the second step of the process concerning the hydrolysis of the lignocellulosic feedstock (with addition of commercial xylanase) for obtaining fermentable sugars for IA production, also by *A. terreus* (about 8 g/L IA) (Kocabas et al. 2014). A different lignocellulosic material, beech wood hydrolysate, was used for IA production and about 13 g/L IA was produced by *A. terreus* in solid-state reactor after the removal of phenolic components with anion and cation exchangers (Sieker et al. 2012).

Sieker et al. (2012) showed that IA production was only achieved when beech wood hydrolysate was detoxified by a mixture of anion and cation exchangers (among other pretreatment analyzed), achieving maximum concentration of almost 4.5 g IA /L for a submerged culture (glucose and xylose from hydrolyzed wood). The treatment used almost completely removed the phenolic compounds and organic acids and decreased the salt ions. Whereas, rice husks hydrolysate pretreated with CaO(s) produced 1.9 g/L IA (Pedroso et al. 2017).

IA production from residues should consider the cost for feedstock treatment to evaluate a real feasibility of the material. A wider knowledge of the potential inhibitors is

important for using less expensive carbon sources with minimal pretreatment (Klement and Büchs 2013).

2.10 *Aspergillus terreus* oxygen strict requirement for IA production

Different studies described the direct relation between aeration and IA production, and the requirement for continuous oxygen supply throughout the bioprocess is of significant importance. Nelson et al. (1952) and Pfeifer et al. (1952) were probably the first to report the need for continuous aeration to reach high IA yields. Nelson et al. (1952) described that a 20 minute interruption in the air flow after 54 hours of fermentation was enough to drastically decrease IA production rates (the values were not detailed). Pfeifer et al. (1952) described that it was only possible to reverse the damage of no IA production (related to 15 to 60 minutes interruptions in air flow) if extra nutrients were added to the medium. Despite the occurrence of further IA production, the final IA concentration was lower compared to the assay which was continuously aerated.

The aeration requirement for maintaining the cell's capacity of producing IA is so important that Larsen and Eimhjellen (1955) conducted the separation of IA-producing *A. terreus* cells – non-proliferating mycelia – from the fermentation broth with constant aeration. The authors described that if the aeration process was not maintained throughout the separation process, the endogenous IA was not expelled to the extracellular medium (acidified tap water).

Gyamerah (1995) showed that *A. terreus* cultivated in glucose medium had different behavior in IA production after 1, 3, 5 or 10 minutes of interruption of oxygen supply after 100 hours of fermentation. The reestablishment of aeration after stopping air supply for 10 minutes resulted in, at most, only 52% of the IA produced on the assay with continuous oxygen supply at the third day. That behavior was similar to the observations by Lin et al. (2004). The shorter interruption periods (3 and 5 minutes) resulted in less severe decrease of IA production (respectively about 77% and 66% less IA compared with the assay without interruption) (Gyamerah 1995). This indicates that the capacity of IA production after the pause in oxygen supply is also related to the duration of the interruption period. The effect of the interruption of aeration in the metabolic pathway of the cell is not yet well understood,

despite the efforts of some authors to describe that phenomenon (Gyamerah 1995; Lin et al. 2004).

2.11 IA production and medium pH

In IA production systems, environments in which the pH is not regulated during the fermentation, the microorganism tends to acidify the medium to a very low pH (<2). IA synthesis is strongly related to the initial pH, as the entire or part of the enzymatic system responsible for IA production may function in an acid environment (Larsen and Eimhjellen 1955). Different metabolites were produced depending on the pH value considered for regulating the entire fermentation process. In pH 2.1, the main products by *A. terreus* was IA, carbonic gas and cells, while the fermentation in pH 6 produced L-malic, succinic, fumaric acids, carbon dioxide and cells (Larsen and Eimhjellen 1955).

Among the existing hypotheses for the transport of organic acids to the extracellular medium by microorganisms, three of them are described below (Vrabl et al. 2012).

- Hypothesis of overflow metabolism – The expulsion of organic acids out of the extracellular medium is considered one of the mechanisms employed by the cell to release energy in a situation in which growth is limited by a non-carbon nutrient and a carbon source is in excess. The hypothesis is subdivided in relation to the location of the bottleneck causing this release, which may be glycolysis, TCA cycle or respiratory chain. In several studies, the phenomenon of overflow metabolism is associated to the increase of glycolytic flow;
- Hypothesis of charge balance - It is considered that when the H^+/substrate transport system is prevented, the transport of the organic acid anions is the main form of compensation of the ion flow for the excretion of H^+ by the enzyme H^+-ATPase. This operation prevents the plasma membrane from being hyperpolarized in a detrimental way to the cell. In an environment where pH is low, most of the excreted protons return to the interior of the cell via the protons of nutrients. In an environment with high pH, especially in cultures with NaOH addition as a control of the excreted protons, the entrance of the proton into the cell is impaired, requiring a new charge flow. The release of organic acids in the medium can balance the proton flow almost stoichiometrically.

- Hypothesis of aggressive acidification - the hypothesis, stablished for *A. niger* strain, describes that the filamentous fungus releases the acid in the extracellular environment, and the acid environment results in a medium with less probability of contamination from other microorganisms. Assuming that the organic acids transported through the membrane are completely protonated (uncharged), these compounds would be the major source of acidification of the medium.

Krull et al. (2017) demonstrated that the need for an acid environment, with fermentation broth pH under 2, is only essential in the beginning of the fermentative process. Their findings with a genetically modified *A. terreus* strain showed that, after the initial drop to 1.6, which is necessary for IA production, the rise and maintenance of the pH at 3-3.4 increases the final IA concentration (around 150 g/L IA). The optimized condition that allowed such concentration involved not only pH adjustment, but also a fed-batch operating system. The final yield of 0.58 g IA/ g glucose is, thus, not higher than other studies without pH regulation.

Chapter 3

SUGAR SOURCE

The choice of carbon source for fermentative processes is important for achieving high product concentration with low expenses with the feedstock. Regarding IA production, the feedstock origin should be a prior concern due to the high initial concentration of substrate required for the processes (over 100 g/L of carbohidrates) and the sensibility to impurities for fermentaions with *A. terreus*. Non sugary sources, such as glycerol, or recalcitrant feedstocks from lignocellulosic materials have been applied as feedstock for IA production with low yield results compared to the the use of carbohydrate source. This study proposes the application of unpure carbon source, such as granulated sugar or very high polymerization (VHP) sugar, composed by almost 100% sucrose. Those feedstocks are low processed source with strong possibility of applying in a biorefinery concept. It chapter presents the definition of VHP and granulated sugar, VHP market, where it is mostly produced and why it is a promissing carbon source for IA production.

3.1 Definition of VHP sugar and granulated sugar

The acronym for very high polymerization, VHP sugar[1], is a non-refined sugar with high concentration of sucrose (99.4% w/w). VHP was developed in 1993 by Brazilian scientists to facilitate the feedstock transportation with minimal previous processing of the sugarcane. The advantages of high sucrose concentration and low presence of contaminants made it very attractive among exporters in the world (Usina Atena 2017). VHP is not fitted for human consumption. In order to be consumable, the feedstock undergoes further processes of removal of other chemical and microbial contaminates at the food factory that purchases VHP.

Once VHP arrives to the destination, it is transformed into other products after refining or other processes of industrialization for human consumption. The VHP is melted and filtered, which results in a viscous sugar syrup. Different processes of washing, centrifugation, filtration and evaporation produces various finished refined sugars (SUCDEN 2017a). The granulated sugar produced undergoes a separation step to differentiate the different sizes of sugar crystals. Each type of sugar is packed for the according specification of industrial customers.

3.2 Production of granulated sugar and VHP

As mentioned previously, granulated sugar is a refined form of the VHP. The production of VHP consists of a few steps. First, sugarcane is harvested and prepared for milling. The cane is grated and cut, and sent to crushing equipment. The residues from sugarcane, bagasse, are usually burned and used as fuel for electricity in the sugarcane plant. The extracted broth is concentrated under vacuo and crystallized. Separation of the solid and the liquid occurs in a high turnover centrifuge. The formed sugar crystals are separated from the first molasses (liquid formed in the first crystallization), which is defined as VHP. The first molasse undergoes a second crystallization process and the result is a molasse (second) with lower concentration of sucrose. The second molasse is crystallized again, producing the third molasse. In some cases, the sugars from the crystallization of the second and third molasses are resolubilized and recrystallized to produce high quality VHP (VVHP).

[1] For simplicity, VHP sugar is called VHP in this study.

Large amounts of sugarcane are processed to obtain VHP sugar. It is estimated that for each hundred tons of processed sugarcane it generates twelve tons of VHP sugar. The VHP produced contains relatively few contaminants, with a brown appearance and it may have ICUMSA[2] between 600 and 1200 – comparatively, while the highly refined sugar is white and its ICUMSA value is 45 (Usina Atena 2017).

The refined sugar is processed at the refinery, and it consists in removing the contaminants of VHP and to improve the product content for the human consumption. It is also done for bleaching and sanitizing the final product (Castro 2013). Figure 3.1 shows the visual appearance of some different commercial sugars, Figure 3.2 presents the process of VHP production, and Figure 3.3 presents the process of a sugar refinery.

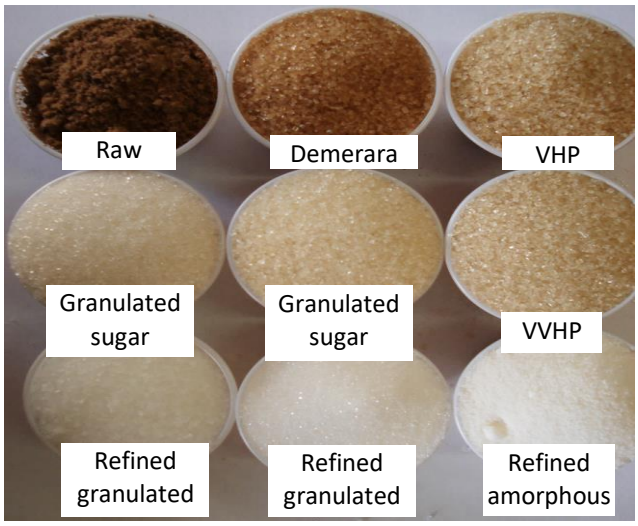

Figure 3.1 Different commercial sugars. Adapted from FineTex (2016).

3.3 Sugar production in Brazil and in the word

Brazil is the largest sugarcane producer and sugar in the world. The harvest of 2016/2017 produced over 650 million tons of sugarcane plant. In that harvest cycle, part of the sugarcane was transformed in 38 million tons of sugar (20% of world production) and 28 million tons was exported (40% of world exports) (Unica 2017a; Unica 2017b).

[2] The ICUMSA classification is an international unit to express the purity of the sugar in solution, which is directly related to the color of the sugar. The lower is the value, the lighter, or the whiter is the sugar. The presence of color is related to the presence of sugarcane solids and particles from sugar processing (Inmetro 2017).

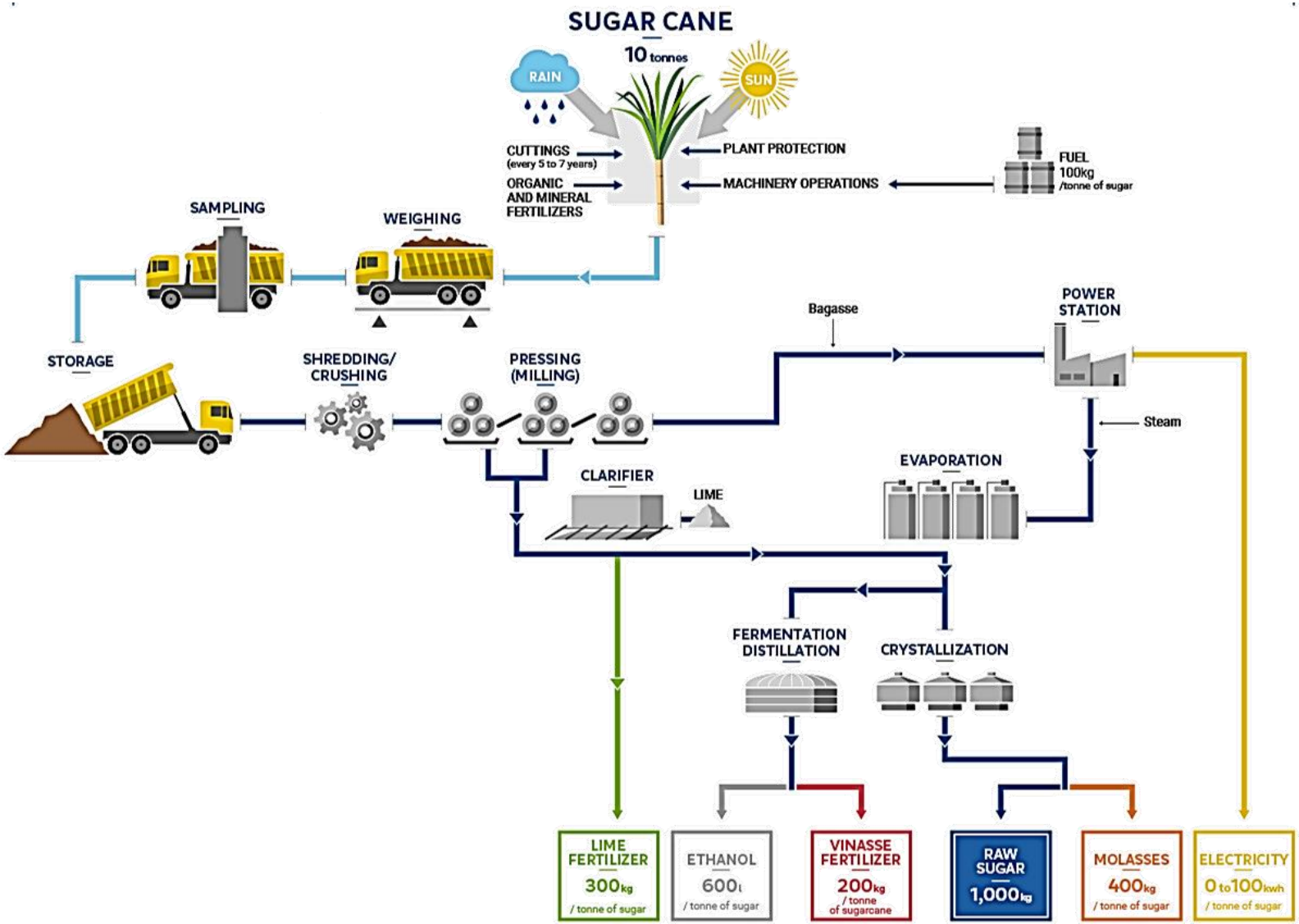

Figure 3.2 Simplified flow diagram of raw sugar, ethanol and electricity production at a sugarcane mill (SUCDEN 2017a).

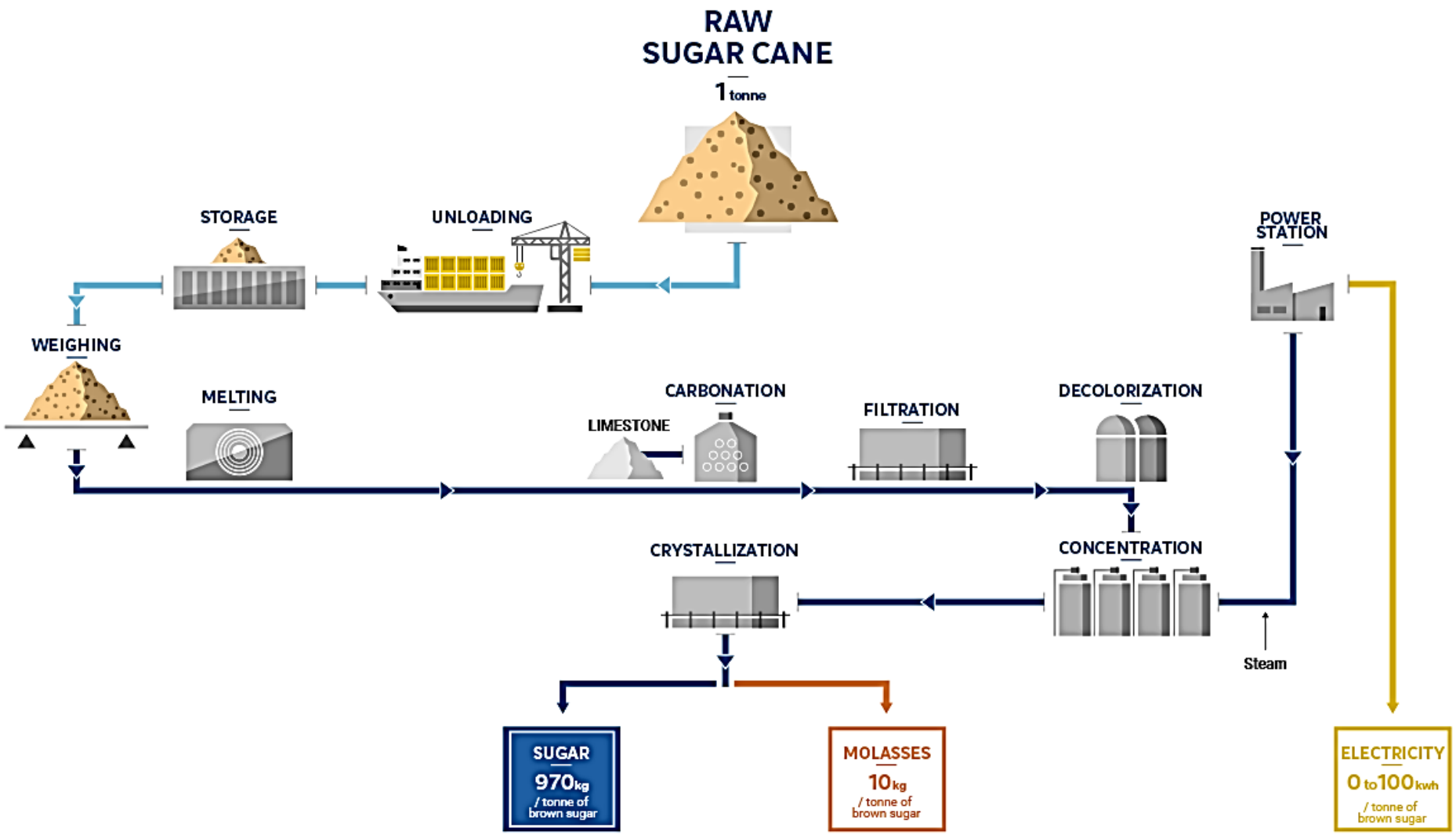

Figure 3.3 Simplified flow diagram of refined sugar, molasse and electricity production at a sugar refinery (SUCDEN 2017a).

São Paulo is the Brazilian State with the largest sugarcane production, with over 60% of the total production. Other areas in Brazil such as Minas Gerais, Paraná and Zona da Mata in Northeast are also great producers (Unica 2017c). Figure 3.4 illustrates the largest Brazilian areas where sugarcane is produced.

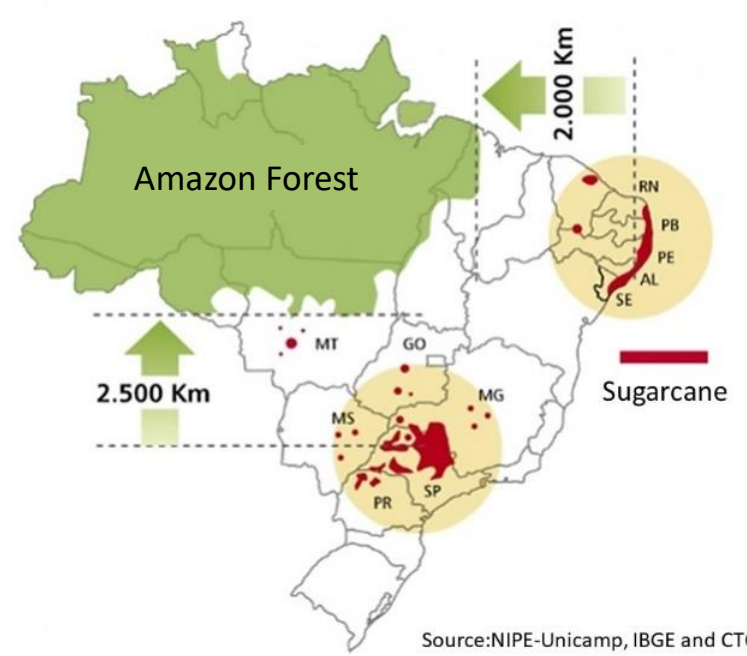

Figure 3.4 The production of sugarcane is concentrated in the Center-South and Northeast regions of Brazil. The map below shows in red the areas where plantations and sugar, ethanol and bioelectricity producing plants are concentrated, according to official data from IBGE, UNICAMP (Universidade Estadual de Campinas – SP) and from CTC (Centro de Tecnologia Canavieira) (Unica 2017d).

The world production of sugar (sugarcane and beet) takes place in countries like India, China, Thailand. Of all sugar produced in the world, approximately 80% is of sugarcane origin and in countries from tropical regions. The remaining 20% is obtained from beets, usually produced in temperate zones in the Northern Hemisphere. Brazilian exports include several countries, such as the Middle East, North Africa, Indonesia and China (Platts 2016).

Figure 3.5 shows the location and quantity of VHP exported and imported in the world in 2016 and Figure 3.6 represents the global flow of white sugar. The most promising locations for implementing IA production by VHP is where there is the highest production. However, the places where smaller productions occur are also potential places to be introduced IA as an end-product to the biorefinery concept. As previously mentioned, the implementation of IA fermentation in local markets and applications may be a strategy to be considered for increasing the size of IA global market.

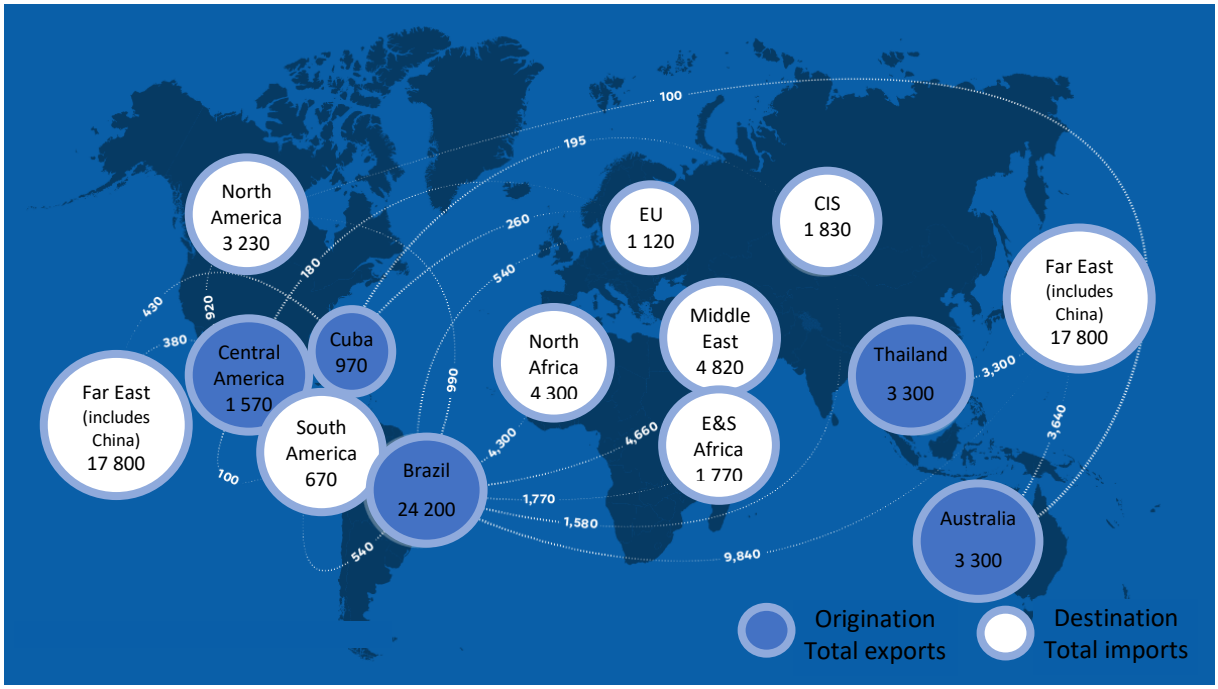

Figure 3.5 Main global trade flows of VHP in the world, 2016. The values are in kilo metric tons (SUCDEN 2017b).

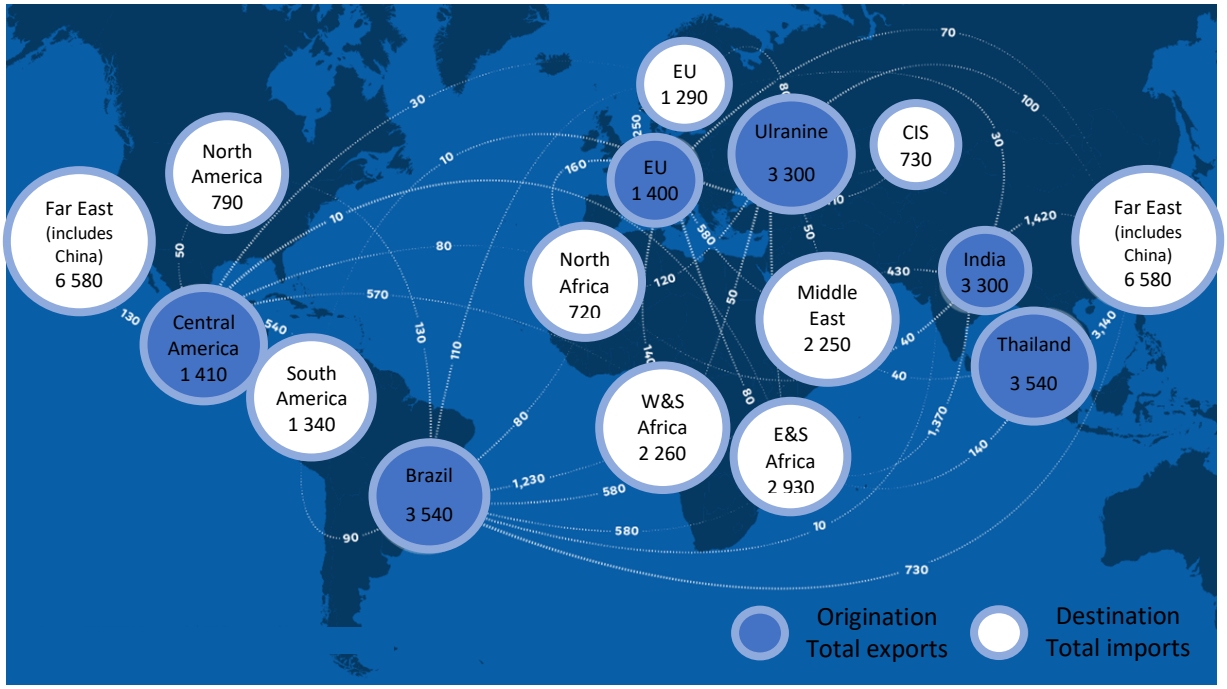

Figure 3.6 Main global trade flows of white sugarin the world, 2016. The values are in kilo metric tons (SUCDEN 2017b).

3.4 The application of sugar in the bioprocess industry

The strong recommendation for reducing sugar consumption by humans (World Health Organization 2015) has decreased the demand of that product in food industry over the years in some countries such as Norway, Canada, India and Brazil (Reuters 2017). 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 (COFALEC 2015). The application of easily assimilated carbon sources, such as those containing sucrose, at a biorefinery concept could be applied to the sugar mill.

One the most complete definition of biorefinery is the one by IEA Bioenergy Task 42 (Cherubini 2010; IEA Bioenergy 2013; Rama Mohan 2016): “Biorefining is the sustainable processing of biomass into a spectrum of marketable biobased products (food/feed ingredients, chemicals, materials) and bioenergy (biofuels, power and/or heat)”. In a biorefinery, there is an integration of conversion processes and equipment to produce biobased products, a concept analogous to petroleum refineries. The possibility of introducing different fermentative processes to the portfolio of a biorefinery would be a strategy to the decrease of sugar demand in the food industry, besides being a way to obtain alternative products from non renewable sources.

Chapter 4

MATHEMATICAL MODELLING AND KINETIC PARAMETERS ESTIMATION IN BIOPROCESS

A model may have different applications depending on the subject area. In biotechnology, the description of a biological phenomenon by a dynamic mathematical model may be used for basic science, but it may also provide information to control operation conditions of an industry process. Moreover, the description of a process may be applied to optimize a bioprocess. In that matter, the development of a dynamic mathematical, which involves theoretical models and empirical results, may be effective for scaling up of a process, despite the time consumption for developing it. The definition of a dynamic model is a wise strategy as an attempt to reduce production costs. The combination of a low-cost feedstock and the mathematical description of the biological system increases the potential of decreasing the end-product cost. This chapter presents the definition of a model and the steps commonly applied to create a dynamic mathematical model in bioprocess.

4.1 Model definition

Models may be found in everyday life, such as at which angle one may turn the car steering wheel to make the car turn right or left, or how strongly one should kick a ball to reach the desired distance. Those systems are not described mathematically, but different experiments, of trial and error, build an empirical model, even in an unconscious way.

A mathematical model may be defined as the representation in mathematical terms of a phenomenon or behavior. The motivation for describing either one may be at least one of the following reasons: to describe the behavior or results observed of a phenomenon; to explain the reason for the observed result of a phenomenon; or to predict the future behaviors of unseen or unmeasured phenomenon (Dym 2004). The complexity of the model depends on the aim of building it, which may be a compromise between a very good description of the system and the limitations of time and work to build and apply the model. In case of a complex system, the set of equations may be a simplified representation of the overall process and its behavior (Richelle 2014).

Models may be defined as theoretical or empirical. Theoretical models cover a wide range of situations and they are able to predict a phenomenon for a wide range of situations, usually at an ideal condition (Koronowicz and Szantyr 2013). For instance, the model of the acceleration of an object dropping at a vacuum environment on the Earth surface may be described as $F = m * \mathrm{g}$ (F: force; m: mass; g: Earth's gravitational acceleration). Empirical models are experimental data based models, obtained by running a set of experiments multiple times and describing the results obtained. For instance, the required time and temperature to bake a specific cake, or, at a solid state treatment station, the quantity of gas and tar produced depending on the components of the waste. The two types of model are not contradictory, as theoretical models are, overall, built from experimental data. One of the differences between both is the range covered by the empirical model and its reproducibility for it to become a well stablished and accepted as a theoretical model.

In bioprocesses, those two types of model may be used to describe a fermentation system. Regarding empirical models, they usually describe a system without a physical interpretation, and are often called black box models. The mathematic equations are, usually, polynomial equations with numerous parameters that may describe the system in the

conditions evaluated. However, they may lead to ineffective extrapolation properties (Can et al. 1998).

Some bioprocess models may combine both empirical and theoretical modelling to describe, for example, a fermentation process. There are theoretical models describing, for example, cell growth or gas transfer to a liquid medium, which may be used to explain phenomena of systems combined with information from experimental data. Those are called hybrid models and they are very useful to be applied in process control or process optimization, as, at the same time they predict the behavior of a system, they may be physically interpreted, in a deeper understanding of the system.

4.2 Example of a simple model identification

A simplified scheme of the model construction procedure is illustrated at Figure 4.1.

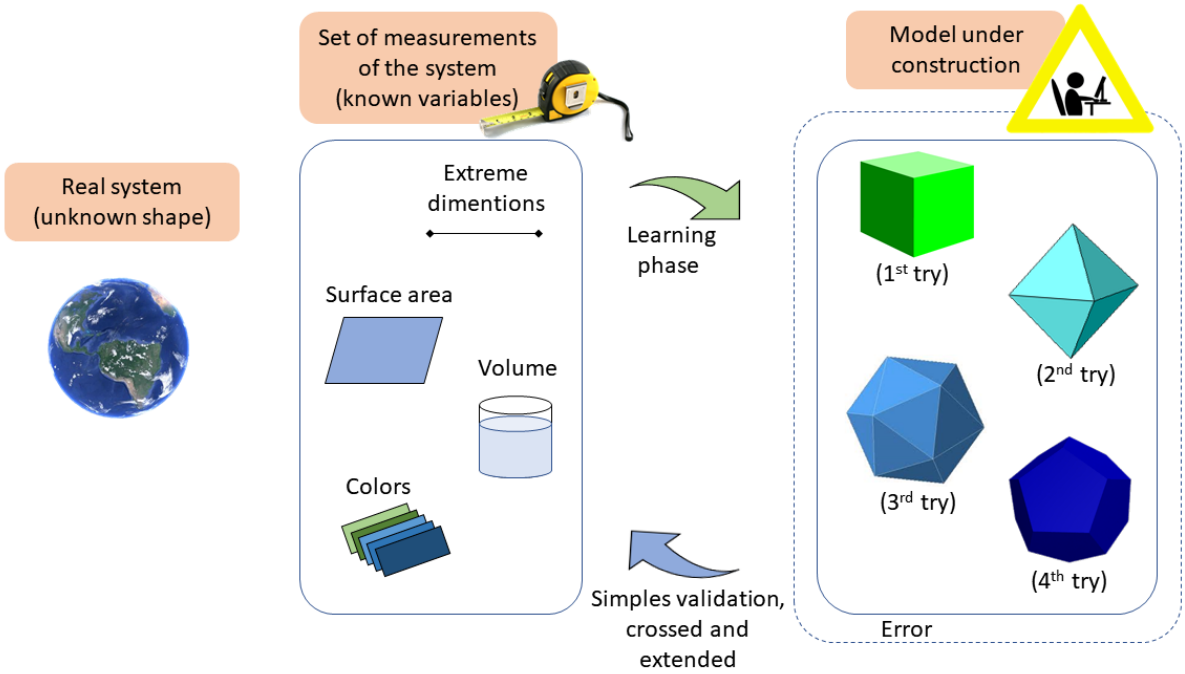

Figure 4.1 Illustration of the several steps related to building a model. In the very simple illustrated case, the real system's shape is unknown, but there is information available about the real system.

In this example, the goal of the model is to identify the shape of the system. Some measurements, however, are available, such as the extreme dimensions, surface area, volume and a range of colors. From previous possible models of physical shapes, it is applied a first

possibility of model, i.e., the cube model. A validation method is applied to verify if the first assumption is consistent with the measurements, which can be if the cube model satisfies simultaneously surface area and volume. If the validation shows unsatisfactory results, a second, third, fourth possible model should be evaluated, with a validation step for each new model proposed. A series of trial and error, from the application of a possible model and its simple validation, increase the knowledge about the system and may indicate a “good” model, that satisfies the aim of building that model.

The prediction of fermentative processes can be very complex regarding those multiple metabolic bioreactions involved in a microorganism cultivation. This is a challenge for successfully monitoring and controlling the product synthesis in large scale productions (Can et al. 1998). A mathematical model has a remarkable advantage to the anticipation of bioprocess responses. The issues regarding time consumption for obtaining offline information of the process, such as metabolites measurements in HPLC and dry mass determination for some microorganisms, or the requirement for a constant sterile environment to obtain those samples may be decreased by using an appropriate mathematical description of the system (Sonnleitner 2013). Moreover, in the case of a sudden malfunction of an equipment responsible for measuring the online information, the existence of a prediction model can allow the prosecution of the process at some extent, or even the model may help in the decision process to apply different fermentation strategies.

There are advantages and disadvantages in applying a dynamic model rather than a static one. Despite the time consumption of defining a dynamic model, it may be more descriptive of the system than a static model, as they allow a wider application of the model. The representation of a phenomenon by differential equations concerning the evolution of a state variable, such as cell growth, substrate and product, enables a more realistic description of a phenomenon than equations describing the process at a single time instance (Can et al. 1998).

Moreover, the construction of a mathematical model for fermentative processes is an important factor to match the increasing demand of sustainable materials. The possibility of better predicting the synthesis of a bio-based product can represent a sharper control and estimation at a market level. In this way, market predictions can have a more accurate basis for estimating production capacities and long-term forecasting.

4.3 Goals for building a model

Two categories may represent a model: model that describes what the system *is*; model that describes what the system *does* (Bogaerts 2016). A model of a spherical Earth, such as mentioned on the section 4.2, defines what is the shape of the planet, while a model of the gravity force on the planet defines the effect of the Earth's gravity force on bodies and objects. Either type may be applied for the simulation of a process, process optimization, process control, software sensor, process design and many others.

A mathematical model should be able to describe a system in a more or less simplified way, depending on the purpose of the model. On the one hand, a model that describes (almost) perfectly a system may contain an excessive information from a variety of mathematic equations. That type of model could represent an overestimation of the phenomena and may require excessive computation process and time consumption. Thus, a "perfect" model can be an unnecessary overestimation of a system. On the other hand, a model with not enough mathematical description of a phenomenon may not be able to sufficiently describe the process and significant information may be lost. The process of building a model should be a compromise between enough information and the cost limitation for building and applying the model. The choice should rely on the aim of the model for acquiring the "good" model (Sonnleitner 2013).

An example of simplicity or complexity of a mathematical description of a phenomenon based on the aim of the model is presented. A simple model for gravity force represents a wide, but limited range of situations, as the model is based on certain assumptions of vacuum environment, for example (which would not affect the gravity force itself, but rather the effect of the object dragged down). A more complete description of the planet's forces acting on an object would be excessive information for a falling apple, but extremely useful for a rocket launch.

4.4 Macroscopic kinetic model for organic acid production by *Aspergillus*

The literature provides few information about macroscopic kinetic model for *Aspergillus* species regarding the production of organic acid. This section presents a brief

description about the dynamic mathematical model available on the literature regarding the production of IA and citric acid (CA) by *A. terreus* and *A. niger* respectively.

To the best of the author's knowledge, there is only one dynamic model describing IA production by microbial route. Riscaldati et al. (2000) applied the model defined by Luedeking and Piret (1959) to describe the kinetics production of IA considering cell concentration:

$$\frac{dI}{dt} = Y_{I/X}\left(\frac{dX}{dt}\right) + \mu_1 X \qquad (4.1)$$

I: itaconic acid concentration

X: cell concentration

$Y_{\frac{I}{X}}$: Itaconic acid/cell yield = pseudo-stoichiometric coefficient

μ_1: specific production rate at zero mycelia growth rate

That model considers two specific production rates for IA production: one occurs during cell growth (first term from the right side of the equation) and the second one is observed when cell is no longer produced (second term from the right side of the equation). In their study, an empirical regression equation was used to describe cell production in relation to limiting reactants (phosphorus and ammoniacal nitrogen).

The equation of Luedeking - Piret was also used to describe CA production, a metabolite produced by *A. niger* commonly compared with IA production by *A. terreus* because of the metabolic similarities between both systems. Papagianni (2004) analyzed some of the deterministic models on the literature that described citric acid production and, according to the author, the profile of citric acid production usually initiated after there was a total consumption of ammonium, and it is observed a lag time for CA production (Papagianni 2004). A similar behavior was observed on this study for some of the assays of IA production.

4.5 Basic principles for building a dynamic macroscopic model of bioprocess

4.5.1 A priori knowledge

When the model goal is clear, *a priori* knowledge of the system should be applied. For instance, considering the application of possible models to describe the trajectory of a falling object on the planet's surface. Depending on the environment where the object is, the model of gravity force may not be appropriate. *A priori* knowledge of the system, i.e., if the studied object is in a liquid, gaseous or vacuum and the knowledge of the assumptions for applying the gravity force model, is required for mathematically describing the phenomenon of a leaf dropping from a tree or a flat rock free falling on a river.

A priori knowledge consist in the combination of information from the literature about similar studies and the applicability of that information on the real studied system. In possession of the system behavior in different conditions – the system data, or measurements – the knowledge from the literature supports initial assumptions, which are verified with the data about the system through methods that validate those assumptions.

In biotechnology, *a priori* knowledge could be the acknowledge of a possible inhibition effect caused by the excess of substrate (Papagianni 2004), or the effect of cell death caused by a strong shear force on mammalian cells (Elias et al. 1995). A basic knowledge of the process, which may be about the process bottleneck that needs deeper understanding, will allow the choice of the experimental variables and the range – or type – of those variables. The choice of variables and the different conditions of the applications of those variables define the experimental field that will provide the experimental data for defining the model.

4.5.2 Defining the experimental field

The different possibilities of experimental field in bioprocesses may contemplate environmental conditions – temperature, agitation, aeration, bioreactor type – or nutritional conditions. The last one may be related to the measurements of reactants – i.e. substrate, nitrogen source, phosphorus source – called inputs, that results in synthesis of products in different concentration – metabolites produced by the microorganism – called outputs (Bogaerts 2016).

If the aim of the model is to describe the different capacities of the fermentative system, or to control the outputs depending on the inputs or the process aspects, the choice of the experimental field should contemplate a sufficient range of possible different fermentation conditions. The consideration of extreme conditions is important to discriminate the limits of the fermentative system.

A realistic response of the phenomenon may be described with a set of data wider than a single experimental condition. An experimental field that considers substrate concentration higher than the usually applied in the fermentative process allows the identification of a possible inhibition effect related to that reagent. Data obtained from the use of reactant concentration at lower values than the usual experimental field may describe at which value the reactant becomes limiting. A good *a priori* knowledge about the system and a well defined goal of the model allow a wise choice of the experimental field.

4.5.3 Defining the structural model

4.5.3.1 Reaction scheme

The experimental data basis is used to define general properties and select a set of structure candidates of the model. For defining a dynamic model of bioprocess, such as the aim of this study, Bastin and Dochain (1990) proposed a system of mass balances which considers the input and output that describes the macroscopic phenomena of that system. The general expression may be represented as (4.2).

$$\sum_{i \in R_k} \left(-\nu_{i,k}\, y_i\right) \overset{\varphi_k}{\rightarrow} \sum_{j \in P_k} \left(\nu_{j,k}\, y_k\right) \tag{4.2}$$

y_i: reactants (or substrates) of the reaction k

y_j: products of the reaction k

$\nu_{i,k}$, $\nu_{j,k}$: respectively the pseudo-stoichiometric coefficients the reactant or the product of the reaction k (negative when related to consumptions-reactant-and positive when related to synthesis-product)

R_k: Reactant of the reaction k

P_k : Product of the reaction k

φ_k: rate of the reaction k

The process of building the structural model evaluates different possibilities of reaction schemes in a trial and error stage. Different reactions may be proposed before there is a satisfactory description of the system. This section presents general structures to describe a dynamic fermentative process.

The reaction rates φ represent the transformation pace of reactants to products. In bioprocesses, there are different theoretical models that define those kinetic functions, which are presented in the next section.

The pseudo-stoichiometric coefficients are called pseudo because they do not represent correlations with respect to the law of conservation of mass of reactants and products, such as the stoichiometric coefficients of chemical reactions. Those values correspond to the quantity of a component in relation to the other at a macroscopic level, not considering other reactants available in the medium also involved in many other bioreactions that are not represented on the global one.

The definition of the dynamic system considers the set of reaction equations that describes the mass balances of each component P_k or R_k, called here y_j, for batch fermentation, where there is no addition or removal of reactant and products during the fermentation, thus, the volume is considered constant (4.3).

$$\frac{d(y_j)}{dt} = \sum_{i \sim j}^{N} \nu_{i\,j}\, \varphi_k\,(y_j, t) \tag{4.3}$$

N: total number of components of the reaction k

t: each time instance

$i \sim j$: The sum is with regards to only the reaction k in which the component j appears

For fed-batch and continuous systems, in which there is addition and/or removal of components during the fermentation, the mass balance considers the influent and effluent

flow rates of liquids and gases. That information may be found in Provost and Bastin (2004) and other sources.

4.5.3.2 Expression of kinetic rate

In chemical processes, there are different mathematical expressions to define the transformation pace of reactants into products. In bioprocesses, due to the cell growth and multiplication, and/or cell death, the quantity of the catalyst changes over time. Cell concentration X is considered in the kinetic model to normalize that effect intrinsic to fermentative processes so the analysis of the behavior of "one cell" can be described. The increasing or decreasing rate φ_k of cell and components in the system (4.4) with respect to the cell concentration (φ_k/X) is called specific rate (μ), and it may be expressed as (4.5).

$$\varphi_k = \mu_k(y_j)\, X \tag{4.4}$$

$$\mu_k(y_{j\,k}) = \frac{\varphi_k}{X} \tag{4.5}$$

φ_k : rate of the reaction k

μ_k: specific rate of the reaction k with respect to the component y of that reaction

y_j: component j (reactants or products)

X : cell concentration

The observation of some patterns related to biological transformation and long years of studies made the kinetics equation defined by Jacques Monod (1949) be widely known as the Monod law. As many theoretical models, Monod law is based on assumptions, which considered only the positive of constant cell growth phases (from the lag phase to the stationary phase) (Monod 1949). The dynamic mathematic expression represented by (4.6) regards cell production, but it may be used for substrate consumption or synthesis of metabolites.

$$\mu_X = \mu_{max} \frac{S(t)}{K_M + S(t)} \quad (4.6)$$

μ_X : specific rate for cell growth

μ_{max} : maximum specific rate of cell growth

S : substrate concentration

K_M : coefficient representing the concentration of component at which the rate is half the maximum

Monod law is one of the most applied description of kinetic rate of microorganisms, but different authors also have built kinetic expressions for different behaviors and different assumptions. Some of the many existing theoretical kinetic models are presented on Table 4.1.

Table 4.1 Some structural models for specific growth rate (Bastin and Dochain 1990).

	Model	Structure
Dependence on the substrate concentration S(t)	Monod (1949)	$\mu(S) = \mu * \frac{S(t)}{K_M + S(t)}$
	Tessier (1942)	$\mu(S) = \mu * (1 - e^{-\frac{S(t)}{K_M}})$
	Haldane (Andrews, 1968)	$\mu(S) = \mu_0 * \frac{S(t)}{K_M + S(t) + \frac{S^2(t)}{K_1}}$
	Ming et al (1988)	$\mu(S) = \mu * \frac{S^2}{K_1 + S^2}$
Dependence on the product concentration P(t)	Aiba et al (1968)	$\mu(P) = \mu * e^{-K_1 P(t)}$
	Jerusaliwski and Engambervediev (1969)	(a) $\mu(P) = \mu * \frac{P(t)}{K_P + P(t)}$ With K_P: saturation constant (b) $\mu(P) = \mu * \frac{K_P}{K_P + P(t)}$
Dependence on the substrate concentration S(t) and the product concentration P(t): μ(S,P)	Ghose and Tyagi (1979)	$\mu(S,P) = \mu_0 * \frac{S(t)}{K_M + S(t) + \frac{S^s(t)}{K_1}} * \left(1 - \frac{P(t)}{P_L}\right)$
	Sevely et al (1981)	$\mu(S,P) = \mu * \frac{S(t)}{K_M + S(t)} * \frac{K_P}{K_P + P(t)} * \left(1 - \frac{P(t)}{P_L}\right)$

Some models relate the cell production rate considering an activation effect of reactant in the product synthesis, represented in Monod law by the term $S(t)/(K_M + S\ (t))$. Analogous approaches may be applied for metabolite production and nutrient consumption, which can be activated by different components in the medium. The opposite may also happen, meaning the possibility of inhibition effect by medium components, which can be reactants or even products. That effect was presented by Jerusaliwski and Engambervediev (1969) in a model with a term of inhibition effect represented by $K_P/(K_P + P\ (t))$ (Table 4.1).

A typical example or activation and inhibition effect in fermentative process is the production of ethanol by the yeast *Saccharomyces cerevisiae*. The TCA cycle pathway is inhibited by high sugar concentration, which activates the pathway of ethanol production. The increase of ethanol concentration at a certain value inhibits cell viability.

The two terms of activation and inhibition are of great value in building kinetic rate expressions and can be applied for describing the specific rate of a reaction scheme with more than one substrate and product, depending on the phenomenon observed within the data of a system. A general application that combines activation and inhibition effects is presented in (4.7).

$$\mu_k = \mu_{\max k} \prod_{j=1}^{A} \frac{y_{\ activation\ j}}{y_{\ activation\ j} + K_{y\ activation\ j}} \prod_{j=1}^{H} \frac{K_{y\ inhibition\ j}}{K_{y\ inhibition\ j} + y_{\ inhibition\ j}} \qquad (4.7)$$

μ_k : specific rate of the reaction k

$\mu_{\max k}$: maximum specific rate of the reaction k

H : number of the components j responsible for activation effect

I : number of the components j responsible for inhibition effect

j : reaction component

$y_{activation\ j}$: component y that activates the reaction i

$K_{y\ activation\ j}$: Activation coefficient

$y_{inhibition\ j}$: component y that inhibits the reaction i

$K_{y\ inhibition\ j}$: Inhibition coefficient

The step of finding the structure of the model usually requires several steps regarding the selection of reaction schemes and the kinetics expression, and verifying its applicability to the obtained experimental data, done with the simple validation step. When a satisfactory correlation between the model and the data is found, further validation steps are required.

Before presenting the validation methods used in this study, a summary of the steps to this point is illustrated on Figure 4.2, with a general example of given inputs $(A(t), B(t))$ and outputs $\big(C(t), D(t)\big)$ resulted from a certain system, that is used to define a model and its parameters ϑ: pseudo-coefficients ν, maximum specific rate of each reaction μ_{max}, activation coefficients k_M and inhibition coefficients $k_{i\,M}$.

4.5.4 Model validation

As any information, a model has intrinsic errors, which may be from different sources. Citing again the model that describes the shape of the globe in section 4.2, the "good" model selected describes a dodecahedron, and not the irregular spherical shape of the Earth. Because the actual shape was unknown, the dodecahedron was a sufficient description of the measurements. The model goal defines the tolerance of errors or uncertainties of the chosen model, but the existence of errors is inevitable.

There are different sources of model uncertainty (Bogaerts 2016):

1. The data available for defining the model. Errors of the method used, human and equipment errors are some factors that result in measurement uncertainties, which can be represented as the standard deviation of the measurement;
2. The choice of model structure. Again, a bad choice or a good choice of model structure depends on the tolerance. A good *a priori* knowledge of the system and the experience accumulated to define the model may decrease the error of that choice;
3. Model identification procedure. Different methods used for minimizing the difference between the proposed model and the data carry different errors. Each identification criterion has certain assumptions, which are also sources of model uncertainties.

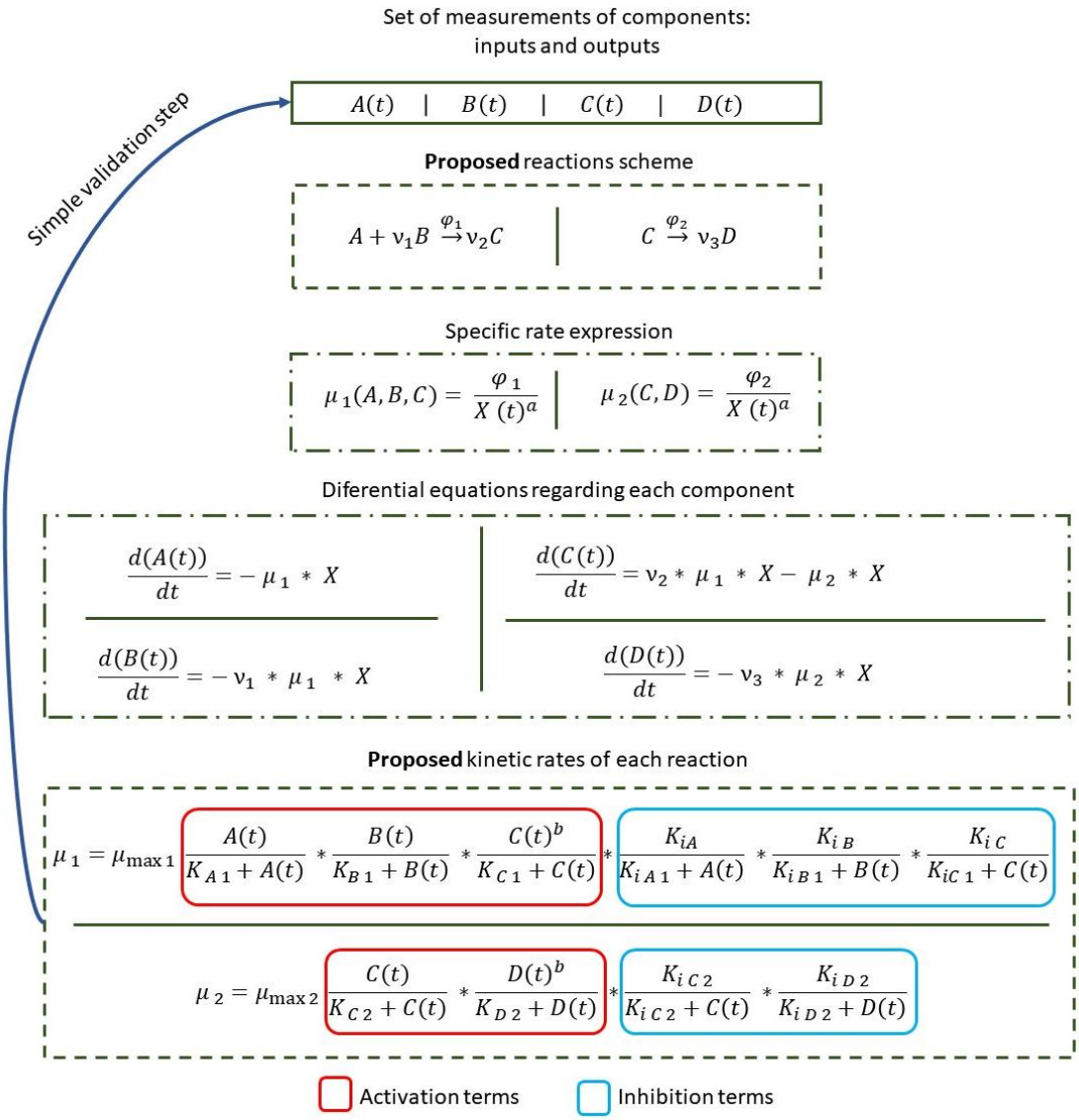

Figure 4.2 General steps of a strategy to find a good model for fermentative processes. The differential equations represent component production with a positive term and component consumption with a negative term. Regarding the proposed kinetic rates, there may be over parametrizations, such as excessive relations of activation and inhibition effects. This example, however, is to illustrate a general definition of model structure considering different possibilities of a system expressed by Monod law and modified Monod law model. |[a] X: cell concentration. Because the catalyst, i.e., cells, changes concentration over time (related to cell growth or cell death), the reaction rate is divided by cell concentration at the time instance. In a real system, X may be included in the reaction scheme as product. | [b] The activation effect of a product, such as C and D, indicates a catalyst phenomenon.

4.5.4.1 Simple validation of the model structure

A model structure that combines empirical results and theoretical concepts provides a physical explanation of the observed behavior at a microscopic or macroscopic level. The design of those types of models requires time and possibly many steps of trial and error before defining a good model, specially concerning those which are nonlinear.

A previous analysis of how well the model proposed corresponds to the real system is often done by superposition of the proposed model curves to the real measurements, such as roughly illustrated on Figure 4.3, in a qualitative analysis. That visual comparison usually also relies on the measurement errors and how the model curve represents the measurements and their error, but a quantitative analysis is also important.

The quantitative response of the model fit to the real data at the stage of trial and error is usually done by an identification criterion. That criterion evaluates the fit of possible model structure to the available experimental data by quantifying how well the model describes the real measured values. In this case, the best model is the one which minimizes the distance between the model and the real system, also called as the cost function J. The most often used identification criterion is the least squared estimation.

Regarding fermentative processes, and some other systems, it should be considered the normalization of the cost function J. That consideration might be necessary because some inputs and outputs may be of different orders of magnitude. That may result in misconception of the minimization of distances between the model and the measured values. The normalization is done by dividing the distance between the measurements and the model by the maximum value of the corresponding input or output, representing, thus, a relative cost function instead of an absolute one (4.8).

$$J = \sum_{t=1}^{M} \sum_{j=1}^{n} \frac{1}{\sigma^2(t)} \frac{(y_{j\,measured}(t) - y_{\,j\,model}(t))^2}{(y_{j\,measured\,max})^2} \qquad (4.8)$$

J: cost function

M: number of experiments

t: time instance of each measurement

n: number of measurements of each experiment

$\sigma^2(t)$: standard deviation

y_j: each component j (reactants or products) measured ($y_{j\,measured}$) or from the model ($y_{j\,model}$)

$y_{j\,measured\,max}$: maximum concentration of each component for normalization purposes

4.5.4.2 Direct validation and cross validation

A perfect validation of a model that describes fermentative process – or other type of system – would be very unlikely as the real system is much more complex, associated with the countless possible unknown parameters within a living system (Can et al. 1998). As stated before, the choice of a good model must be limited to the model goal, and the model validation should be realistic to that goal.

The use of all the available experiments and their values to build a model, concerning the different conditions used for the experimental field, is called direct validation conditions. However, that approach does not validate the model neither its parameters, as the information of all conditions is intrinsic to the model. Direct validation is important at the step of defining the model, but not sufficient to confirm the model structure and parameters.

Cross validation is a more appropriated form of corroborating the model, since only a part of the set of experiments is used to build the model and the parameters. Then, the model obtained is applied to describe the other set of experimental data that was not previously applied. If the model describes well the second set of conditions, it may be considered acceptable to proceed to the parameter uncertainty step (David et al. 2014).

The cross validation should be applied with certain strategy. For example, the set of experiments from extreme conditions may be used to define the candidate model parameters. The set of experiments of intermediate conditions are, then, applied to confirm the validity of the parameters found from the first set of conditions. Other strategies may be applied, and some of them are illustrated on Figure 4.3.

4.5.4.3 Uncertainty analysis of the model parameters

The uncertainties of a model are reflected on the model parameters. Thus, the error propagated though the process of building the model may be identified on the parameters ϑ obtained. The analysis of uncertainty can be done by the knowledge of the sensitivity of a parameter, meaning, how sensible to variation is the identified parameter.

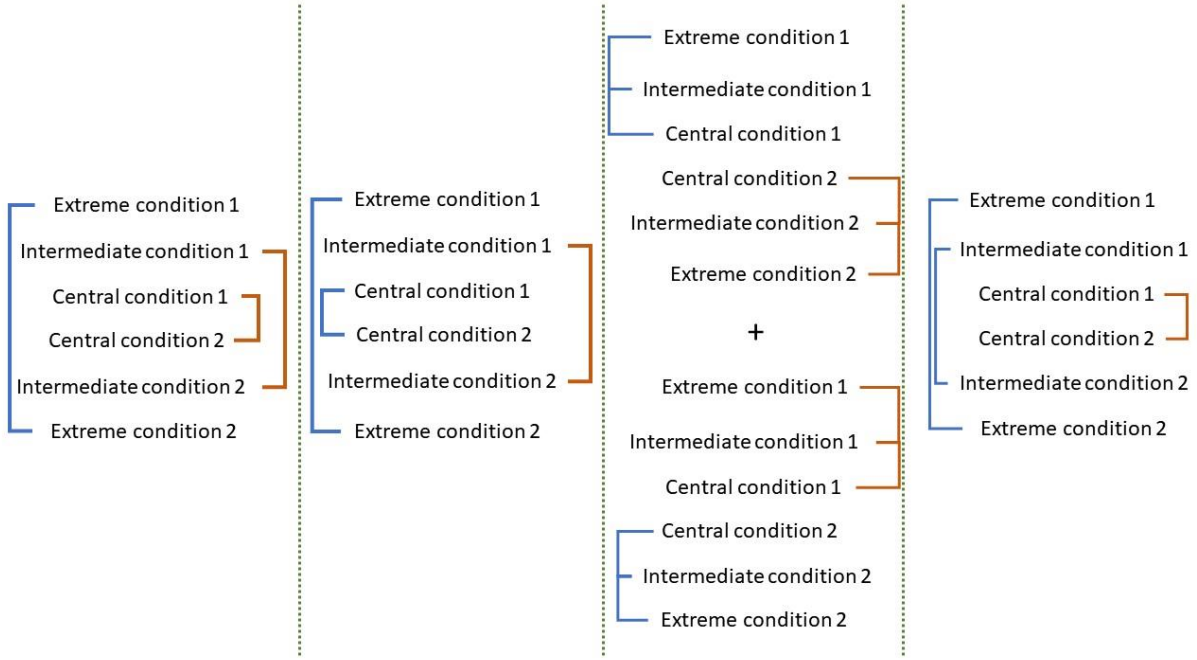

Figure 4.3 Four possible strategies for applying cross validation. Set of experiments used to define the parameters of the model (▬); set of experiments used to validate the model by applying the parameters defined by the first set of experiments (▬).

The Fisher information matrix can provide the local sensitivity analysis, as an information of the model variable (model outputs y_{model}) is influenced by the change in one parameter around its identified value. This local method is applied by exploring small variation of the parameter value, one in relation to another, one by one, without considering the interaction between all the parameters with each other at the same time. Besides, there is the assumption that the system responds linearly to those perturbations. For instance, random parameters ϑ_a, ϑ_b and ϑ_c are evaluated one regarding the other: ϑ_a with respect to ϑ_b and ϑ_c; ϑ_b with respect to ϑ_a and ϑ_c; ϑ_c with respect to ϑ_a and ϑ_b.

The sensitivity function will be used as a mean to compute the covariance matrix of the parameters estimation errors. In addition to that information, the covariance matrix presents the correlation between the parameters. That matrix is useful because redundant information of the identified parameters can be identified. For instance, considering once more the model built to identify Earth's shape, if a sphere was found to be the final model ($V = (4 * \pi * r^3)/3$), the knowledge of the volume and the extreme dimensions, which is twice the radius (r), the existence of the two information would be excessive. In that final model, only one of those parameters is necessary.

The sensitivity functions may identify the overestimation of the model parameters, which less significant parameters may be removed, or their numerical values may be fixed.

For mass balance equations of a bioprocess system, the differential model may be described by (4.9).

$$\frac{d\,y(t)}{dt} = f_y(y_j, \vartheta_{ij}) \tag{4.9}$$

$y \in \mathcal{R}^n$, $\vartheta \in \mathcal{R}^p$

The parameter sensitivity $s(\vartheta_{i\,j})$ is defined as the sensitivity of the state variable y_j to changes $\Delta\vartheta_{i\,j}$ in the parameter $\vartheta_{i\,j}$ (4.11).

$$s(\,y_i\,, \vartheta_j) = \frac{\partial y_i}{\partial \vartheta_j} \tag{4.10}$$

$$s(\vartheta_{i\,j}) = \lim_{\Delta\vartheta_{i\,j}\to 0} \frac{y_i(t,\ \vartheta_{i\,j} + \Delta\vartheta_{i\,j}) - y_i(t,\ \vartheta_{i\,j})}{\Delta\vartheta_{i\,j}} = \frac{\partial y_i(t, \vartheta_{i\,j})}{\partial \vartheta_{i\,j}} \tag{4.11}$$

That parameter sensitivity is presented in absolute values. However, again, the variables of the model y_j may have differences in order of magnitude. This is used to build normalized sensitivity functions, presented in (4.12).

$$S(y_i(t), \vartheta_{i\,j}) = \frac{\partial \ln y_i(t, \vartheta_{i\,j})}{\partial \ln \vartheta_{i\,j}} = \frac{\vartheta_{i\,j}}{y_i}\ s(y_i(t), \vartheta_{i\,j}) \tag{4.12}$$

That expression, however, may cause calculation problems when variables $y_i(t)$ are close to zero, which is, in bioprocess, very usual. Thus, a semi relative parameter sensitivity $\hat{S}(y_i(t), \vartheta_{i\,j})$ is presented (4.13).

$$\hat{S}(y_i(t), \vartheta_{i\,j}) = \frac{\partial y_i(t, \vartheta_{i\,j})}{\partial \ln \vartheta_{i\,j}} = \vartheta_{i\,j}\ s(y_i(t), \vartheta_{i\,j}) \tag{4.13}$$

Therefore, the system presented in (4.9) may be written as the time evolution of the absolute parameter sensitivity as (4.14).

$$\frac{d}{dt}s\left(y_i(t),\vartheta_{i\,j}\right)=\frac{\partial f_i}{\partial\vartheta_{i\,j}}+\sum_{k=1}^{n}\frac{\partial f_i}{\partial\,y_k}s\left(y_k(t),\vartheta_{i\,j}\right) \tag{4.14}$$

The Cramér–Rao bound is an inequality stating that the variance of any unbiased estimator is greater or equal to the inverse of the Fisher information matrix. The set of sensitivity functions (4.15) are applied as a lower bound of that inequality based on the Fisher information matrix (4.16).

$$F=\sum_{t=1}^{N}\left(\frac{\partial\,y_i(t)}{\partial\,\vartheta}\right)Q_i^{-1}(t)\left(\frac{\partial\,y_i(t)}{\partial\,\vartheta}\right)^T \tag{4.15}$$

$$S=\ F^{-1} \tag{4.16}$$

Q_i^{-1} : a positive-definite symmetric weighting matrix defined here as $Q_j=diag\ (\sigma^2(y_{meas,i\,j}))$, where σ^2 are the variances of the corresponding measurement errors. It is assumed that only $y_{meas}(\vartheta)$ involves errors.

The confidence interval of each parameter $\sigma_{\vartheta_i}^2$ obtained from the confidence matrix S (4.17).

$$\sigma_{\vartheta_i}^2=S_{i\,i} \tag{4.17}$$

As stated before, the covariance matrix may be used to identify the linear correlation between two parameters. The matrix S, which is normalized, contains the linear relation between two parameters ϑ_a and ϑ_b. Because they are normalized, that relation ranges from -1 to 1. Values closer to -1 indicates a strong negative correlation. Parameter values closer to 1 represents a strong positive correlation. Parameters that are independent one from the other is 0 in the S matrix.

$$COR\ (\vartheta_a\vartheta_b)=\frac{S_{ab}}{\sqrt{S_{aa}}\sqrt{S_{bb}}} \tag{4.18}$$

Chapter 5

OBJECTIVES

This study's main objective was to obtain high IA yields with substrate from sugarcane industry feedstock by *Aspergillus terreus* and to propose a mathematical model to describe that fermentative process.

The specific objectives were

- The selection of a high yield IA-producer strain of *A. terreus* and estipulate fermentation conditions in agitated flask;
- Investigate the strict requirement of dissolved oxygen for producing high yield of IA;
- Compare IA production in agitated flasks and laboratory scale bioreactor;
- Investigate IA production with different carbon and nitrogen sources;
- Investigate IA production in different initial concentration of carbon and nitrogen sources;
- Propose a mathematical model and the estimation of the kinetic parameter of IA production by *A. terreus* using feedstock from the sugarcane industry.

Chapter 6

MATERIALS AND METHODS

The concentration of medium components and cultivation conditions are one of the bottleneck for high yield IA production. The methods used for selecting the IA producer microorganism, medium components screening for low cost medium feedstock, as well as the details of the fermentation and quantitative analysis of substrates and products are described in this chapter. To simplify the comprehension regarding the fermentation parameters used in each experiment, it is available a detailed list with each experiment condition. The methods used for developing a mathematic model for IA production with VHP is also presented.

6.1 Bibliometrics of scientific articles and patents concerning IA

The compilation of the scientific articles and patent documents published concerning IA, called bibliometrics, were done using two important database respectively: Scopus® (one of the largest abstract and citation database of peer-reviewed literature (Scopus 2017) and Derwent World Patent Index™ (DWPI) (one of the most comprehensive collection of global patent data in English (Clarivate Analytics 2017)). This analysis of the scientific and technological advances regarding IA used mainly the methods presented in Cruz (2012).

The set of articles was selected from the database Scopus ® using the following criteria: scientific articles written in English containing the words “itaconic acid” on the title, published from 1910 (earliest year available on the database) to 2016. The set of patents was selected from the database DWPI with similar method: patent documents containing the words “itaconic acid” on the title published from 1910 (earliest year available on the database) until 2016.

Important information about the articles and the patents such as title, abstract, publication year and priority country of the patents – first country where the invention is filled (OECD 2006) – were used to evaluate the advances of IA technologies. The analysis of the subject area of the articles was done with the information presented on title and abstract of each article to classify them. The following three categories were created to describe the articles main subjects: development or application of IA polymer; development of the fermentative process; research about the metabolic pathway of IA.

6.2 *Aspergillus terreus* strains

The microorganism used for the selection of the strain with the highest capacity of producing IA were from three different cell culture collections: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH Collection – DSMZ collection –, Agriculture Research Service Culture Collection (ARS) – NRRL collection – and Coleção de Culturas de Fungos Filamentosos from Instituto Oswaldo Cruz – IOC collection. The strains of *Aspergillus terreus* and the source from where they were isolated are presented as follows.

- *Aspergillus terreus* DSM 826 – isolated from soil
- *Aspergillus terreus* DSM 5770 – isolated from soil
- *Aspergillus terreus* NRRL 260 – isolated from soil
- *Aspergillus terreus* NRRL 265 – isolated from soil
- *Aspergillus terreus* NRRL 1960 – isolated from soil
- *Aspergillus terreus* IOC 4276 – isolated from dry sand
- *Aspergillus terreus* IOC 4582 – isolated from dry sand

The strains from the DSMZ bank (*Aspergillus terreus* DSM 5770 and 826) were already available at the research laboratory. The others were obtained from the culture collections for the purpose of this study.

6.3 Media composition

6.3.1 Liquid Sabouraud medium

The liquid Sabouraud medium was prepared with (per liter of distilled water): 40 g dextrose, 10 g of peptone, pH 5.6. The medium was sterilized in test tubes at 1.0 kgf/cm^2 for 15 minutes.

6.3.2 Solid media composition

The solid media used in this study were potato dextrose agar (PDA), Sabouraud agar, malt extract and Czapeck Dox agar. The media compositions were as follows (per liter of distilled water):

- PDA medium: 39 g/L PDA medium Difco®;
- Sabouraud agar: 40 g dextrose, 10 g of peptone, 15 g/L of bacteriological agar.
- Malt extract: 48 g/L malt extract medium Sigma®;
- Czapeck Dox Agar: 20 g dextrose, 2 g $NaNO_3$, 0.5 g K_2HPO_4, 0.5 g $MgSO_4$, 0.01 g $FeSO_4$, 20 g bacteriological agar.

The media were sterilized in Erlenmeyer flasks at 1.0 kgf/cm^2 for 15 minutes.

6.3.3 Standard medium composition

The main medium used in this work was described by Kuenz et al. (2012) (Table 6.1). This culture medium was chosen because of its chemically defined composition – medium that did not contain any complex component such as corn steep liquor, present on other studies (Pfeifer et al. 1952; Park et al. 1994). Also, that study described one of the highest IA yields available in the literature in the beginning of this study (about 90 g IA/L and 75 g IA/g glucose with *A. terreus* DSM 23081 in batch fermentation and agitated flasks). The concentration of sucrose was defined considering the mass of carbon in the concentration of glucose used in the reference study (Kuenz et al. 2012) and the correspondent mass of sucrose was calculated.

Table 6.1 Composition of the standard medium used for IA production by *Aspergillus terreus*.

Component	Concentration (g/L)
Glucose or	120
Sucrose	114
KH_2PO_4	0.1
NH_4NO_3	3
$MgSO_4$. 7 H_2O	1
$CaCl_2$. 2 H_2O	5
$FeCl_3$. 6 H_2O	0.00167
$ZnSO_4$. 7H_2O	0.008
$CuSO_4$. 7 H_2O	0.015

The pH was adjusted to 3.1 with 1 M H_2SO_4. The culture medium was autoclaved at 0.5 kgf/cm^2 for 20 min, except for $FeCl_3$ – it reacts with other salts in the culture medium when at high temperatures such as in the autoclave. The $FeCl_3$ solution was be sterilized by membrane filtration of cellulose acetate (pore 0.1 µm).

This standard medium was used for most experiments, except for the experiments of different feedstocks, nitrogen sources, and different initial concentration of carbon and nitrogen sources, described further.

6.3.4 Media with different carbon and nitrogen sources

Some experiments were performed for evaluating IA production from different sources of carbon and nitrogen. The compositions of the media with different carbon and nitrogen sources were the same as the standard medium in concentration of the salts.

The carbon sources were: granulated sugar (purchased from a local supermarket) or VHP (kindly donated by Usina Guarani, Cruz Alta, SP). Both sugary carbon sources were considered to contain 100% of sucrose and the concentration of each carbon source was the same as the concentration used for the assays with sucrose.

The nitrogen sources were ammonium nitrate (NH_4NO_3), and commercial urea (Dimy®, purchased from local supermarket). The commercial urea was composed of 45% of total nitrogen, according to the product description, and it was considered that all nitrogen source was from urea. The compositions of carbon and nitrogen of each medium are presented on Table 6.2.

Table 6.2 Content of carbon and nitrogen sources of the assays using granulated sugar or VHP as carbon sources, and ammonium nitrate (NH_4NO_3) or commercial urea as nitrogen sources. The other medium components concentrations were the same as the standard medium.

Assay	Feedstock	Concentration (g/L)	Nitrogen source	Concentration (g/L)
1	Granulated sugar	114	NH_4NO_3	3
2	Granulated sugar	114	Urea	2.3
3	VHP	114	NH_4NO_3	3
4	VHP	114	Urea	2.3

6.3.5 Media with different concentration of carbon and nitrogen sources

The media compositions of the experiment that evaluated different concentrations of VHP and NH_4NO_3 are described on Table 6.3. Each medium was named according to the content of VHP and NH_4NO_3: low C or N for the lowest concentration applied; high C or N for the highest concentration applied; medium C or N for the mean between the highest and lowest concentrations applied.

Table 6.3 Content of carbon and nitrogen sources of the assays using different concentrations of VHP sugar and NH_4NO_3. The other medium components concentrations were the same as the standard medium and this medium was called modified standard medium.

Assay	VHP (g/L)	NH_4NO_3 (g/L)
Low C, Low N	76	2
High C, Low N	114	2
Medium C, Medium N	95	4
Low C, High N	76	6
High C, High N	114	6

6.4 Manipulation of the microorganisms

6.4.1 *A. terreus* strains activation

The strains from the DSMZ Collection were available in the laboratory, therefore they did not need to be activated from lyophilized. The strains *A. terreus* NRRL 260, 265, 1960, and IOC 4276, 4582 were activated on Sabouraud liquid medium (section 6.3.1). The lyophilized cells were suspended aseptically in 1mL of liquid medium and, after about 1 minute, they were transferred to 5mL of the activation culture medium in a test tube. The tubes were kept in a bacteriological incubator at 30 °C for 24h. The activated cells were transferred to solid Sabouraud medium (section 6.3.2) in test tubes and incubated for 5 days at 30°C, then kept at 4°C.

6.4.2 Cell maintenance and spore growth for inoculum

The cells of *A. terreus* were maintained in Petri dishes and test tubes with PDA medium, and maintained at 4°C. The cells were passed every three months.

The inoculum of each assay was prepared from the Petri dishes by adding about 2-4 mL of sterile saline solution (0.85% w/v NaCl) to a Petri dish with *A. terreus* spores. The spores were detached from the hyphae with a platinum or plastic sterile loop. The concentrated spore suspension formed was uniformly transferred to a sterile Petri dish with PDA medium using a sterile pipette. The Petri dish was placed in a bacterial incubator at 30°C for 5 to 6 days.

6.4.3 Inoculum of spores

The spore suspension used as inoculum was prepared by spreading sterile glass beads (3 mm) and 2-4 mL of saline solution (0.85% w/v NaCl) on the surface of Petri dish filled with the grown *A. terreus* cells to release preferably the spores. To avoid spore lumps, the suspension was transferred to a conical tube containing 1g of glass microbeads (0.05 mm), and vortexed for about 30 seconds. The spore concentration was determined as described further on section 6.6.1. The volume of the inoculum was equivalent to the final concentration of 10^6 spores/mL in the fermentation flask (Erlenmeyer flask or bioreactor) (Kuenz et al. 2012).

6.5 Environmental conditions of the fermentations

6.5.1 Incubator shaker

The experiments were performed in 500 mL Erlenmeyer flasks, with a reaction volume of 100 mL, except for the assay that evaluated IA production with 150 mL compared with 100 mL, described further on section 6.8.3. The flasks were placed in incubator shakers (CIENTEC CT 712 or Tecnal model TE-420). The agitation rate was 150 rpm (60 mm diameter displacement of the shaker), set to 33°C.

6.5.2 Bioreactor

The experiments in bioreactor were done in Multifors 2 with 4 bioreactors on one device, which was kindly provided from *Laboratório de Bioprocessos* (LabBIO) – Coppe – UFRJ. The bioreactor is illustrated on Figure 6.1 and its specifications are presented on Table 6.4.

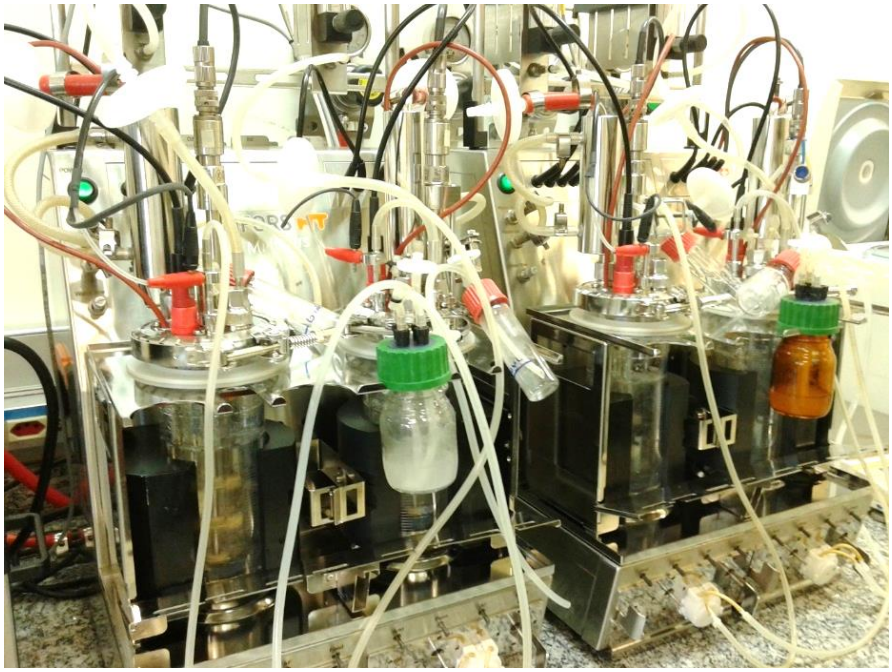

Figure 6.1 Bioreactor Multifors 2 incubated with *A. terreus* NRRL 1960.

Table 6.4 Specifications of the bioreactor Multifors 2, Infors HT®, used for the fermentation with A. terreus for IA production. Source: Infors HT (2017).

Dimensions (WxDxH)	350 x 500 x 850 mm
Total volume per vessel	750 mL
Working volume per vessel	180 – 500 mL
Compact base unit	✓
Fully equipped vessels	✓
Standard parameters	Stirrer speed, temperature, pH, pO_2, antifoam/level, feed, gas mix, gas flow
Vessel type	Flat bottom
Stirrer type	2 Rushton impellers
Sparger type	Ring sparger for microbials
Baffles	Non
Magnetically coupled stirrer system	✓
Stirrer speed	100–1200 rpm
Gas mix	Air/O_2 for pO_2 control Options for other gas mix
Submerse gassing	Gas mix to sparger (approx. 0.02–2 vvm)
Headspace gassing	Optional
Gas flow control	Mass flow control or rotameter
Thermodynamic exit gas cooler	✓
4 High-precision pumps (1 analog + 3 digital)	0.003–3.3 mL/min (standard) 0.001–1.4 mL/min (option) 0.2–17.9 mL/min (option)
Super safe sampler	✓
Touch screen controller with OPC server	✓

Antifoam (204, Sigma) was automatically used when needed. The aeration and agitation rates applied were respectively: 0.5 vvm and 300 rpm; 0.5 vvm and 600 rpm; 1.5 vvm and 300 rpm; 1.5 vvm and 600 rpm. Neither pH nor oxygen were controlled throughout the fermentation.

6.6 Quantitative analyses

6.6.1 Quantification of spores

The spore concentration was determined with mirrored Neubauer chamber and visualized at microscope (400x magnification).

6.6.2 Quantification of dry mass

The cellular quantification for filamentous fungi is done by dry weight, as the formation of mycelium, not single cells, cannot be related to optical density. The determination of cell concentration from dry mass was done during or at the end of the fermentation, depending on the aim of the experiment. For the definition of the mathematical model, presented on section 6.8.13, it was considered a sufficiently small cell mass of 0.01 g/L as initial cell concentration, as the actual initial concentration of cells is experimentally defined by spores/mL, not mass.

The cells were separated from the fermented broth with filtration system operated by vacuum through cellulose acetate membrane filtration of 47 mm diameter and pore 0.45 µm. The cells were washed with distilled water with twice the volume of the filtered cell suspension. The filtered cells on the cellulose acetate membrane were dried at 105°C and weighed until constant mass. The liquid extracted from the filtration before the washing step was maintained at -4°C to further determine the medium components by other methods.

6.6.3 pH measurement

The pH value of the culture media, solutions and fermented medium were determined using a potentiometer (MS Technopon Instrumentação) at room temperature.

6.6.4 Quantification of glucose, sucrose, fructose, total reducing sugars (TRS), IA and other components in HPLC

The analysis of IA, substrates – sucrose, glucose and fructose – and other components (xylitol, ethanol, glycerol, acetic acid, lactic acid, citric acid and succinic acid) was determined by high performance liquid chromatography (HPLC) Agilent Technologies 1260 Infinity with refractive index detector (RID), Aminex HPX-87H column, kindly analyzed by CENPES, at PETROBRAS, or by *Laboratório de Biotecnologia Microbiana* (LaBiM), at Universidade Federal do Rio de Janeiro. The operating conditions of the HPLC are shown on Table 6.5. Figure 6.2 represents a typical chromatogram of IA, sucrose, glucose and fructose, which were the analytes quantified for most of the experiments. The standard reagents for the construction of the standard curves are the pure components.

Table 6.5 Operating conditions of HPLC with refractive index detection (RID) for quantifying itaconic acid, sucrose, glucose and fructose.

Mobile phase	5mM of H_2SO_4
Mobile phase flow	0,7 mL/min
External temperature (oven)	65°C
Internal temperature (detector)	35°C

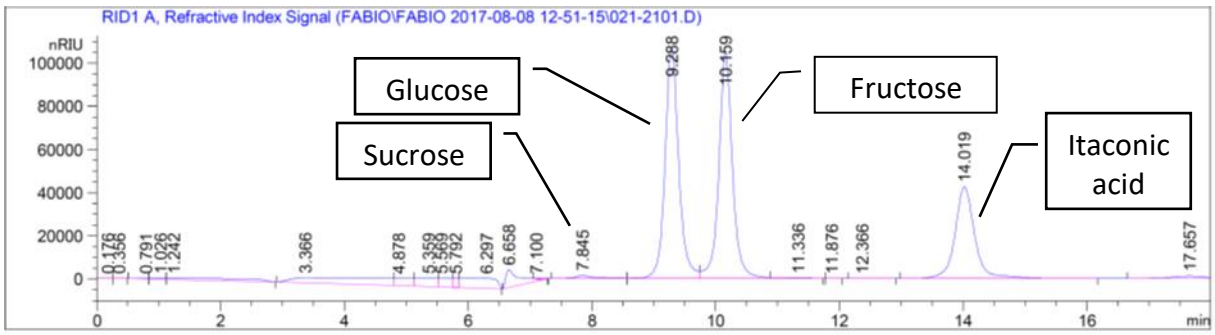

Figure 6.2 Chromatogram of the peaks of IA, sucrose, glucose and fructose with refractive index detection.

The values of total reducing sugars (TRS) were calculated for the assays which sucrose was used as substrate, since sucrose, which is not a reducing sugar. For those assays, the quantified sucrose by HPLC was mathematically transformed in concentration of TRS considering the molecular mass of 1 molecule of glucose and 1 of fructose, both reducing sugars, deduced of 1 molecule of water.

6.6.5 Quantification of ammonium

Ammonium quantification was done from an adapted method of Tabacco et al. (1979). The methodology and the reagents were kindly provided by *Laboratório de Microbiologia Molecular e Proteína*, at Universidade Federal do Rio de Janeiro. Reagent 1 contained (per liter of distilled water): sodium salicylate 9.6057 g, sodium nitroprusside 1.01 g and EDTA 0.50 g, stored in glass amber flask at 4°C; reagent 2 contained (per liter of distilled water): sodium hypochlorite (4-6%) 8.93 g and sodium hydroxide 6.01 g, stored in high-density polyethylene (HDPE) flask at 4°C. Conical centrifuge tubes of 15 mL capacity was used to react 50 μL of the diluted (or not) samples with 2 mL reagent 1 and 2 mL of reagent 2. They were homogenized in vortex, then incubated at 37°C for 5 minutes, with the blank sample done with distilled water. The samples were analyzed in spectrophotometer (DR5000 Spectrophotometer - Hach). For the analytical curve, it was used NH_4SO_4.

6.6.6 Quantification of the components content in VHP sugar

The chemical elements of the VHP sugar were analyzed according to the importance of the component to the fermentation for IA synthesis (Ca, Mg, K, Fe, Cu, Zn, S, P) (Kuenz et al. 2012), or inhibitory for the production when the component is present in high concentration (Mn > 10 μg/L (Karaffa et al. 2015)), and also Co. The method was performed by inductively coupled plasma optical emission spectrometry (ICPOES) with the equipment of Horiba Jobin Yvon, model Ultima 2. Calibration was done with appropriated standard solutions for each element. The procedure was adapted from the norm ASTM-D1976 *Standard Test Method for Elements in Water by Inductively-Coupled Argon Plasma Atomic Emission*

Spectroscopy, usually applied for general aqueous samples. The samples were diluted in water between 10 to 1400 times by weight.

6.7 Yield, productivity and efficiency

Important information of yield, productivity and efficiency are presented in some experiments performed. The definitions are presented as follows.

$$Y_{P/S} = \frac{\Delta P}{\Delta S} = \frac{P - P_0}{-(S - S_0)} \tag{4.19}$$

$$Y_{P/S} := \frac{g\ IA}{g\ substrate} \tag{4.20}$$

$Y_{P/S}$: Yield of product in relation to substrate

P_0: Initial concentration of product

P : Final (or maximum) concentration of product

S_0: Initial concentration of substrate

S : Final (or maximum) concentration of substrate

$$Y_{X/S} = \frac{\Delta X}{\Delta S} = \frac{X - X_0}{-(S - S_0)} \tag{4.21}$$

$$Y_{X/S} := \frac{g\ cells}{g\ substrate} \tag{4.22}$$

$Y_{X/S}$: Yield of dry cells in relation to substrate

X_0 : Initial concentration of cells

X : Final (or maximum) concentration of cells

$$Productivity = \frac{\Delta P}{\Delta t} = \frac{P - P_0}{t_f} \quad (4.23)$$

$$Productivity := \frac{g\ IA}{L\ .\ t} \quad (4.24)$$

t_f: Fermentation period

$$Ef = \frac{Y_{P/S\ real}}{Y_{P/S\ theoretical}} * 100 \quad (4.25)$$

$$Ef := dimensionless \quad (4.26)$$

Ef: Efficiency

6.8 Description of the experiments

In this section, the methods applied for each experiment are presented. The assays for the selection of a strain of *A. terreus* occurred in two steps: the analysis of the IA production by the two strains from the DSMZ cell collection (826 and 5770); the analysis of IA production by the strains from the ARS (260, 265 and 1960) and IOC (4276 and 4582) culture collections. Those experiments are described on sections 6.8.1 to 6.8.7. The selected *A. terreus* was used to define the kinetic profile of IA production in sucrose medium, presented on section 6.8.8. The experiment done in bioreactor is presented on section 6.8.9, being the only one performed in such equipment. Further analysis, presented on sections 6.8.10 to 6.8.12, were conducted in agitated flasks. The section 6.8.13 briefly presents the methods applied for developing the mathematical modelling of this study.

6.8.1 IA production by *A. terreus* DSM 826 and 5770 in glucose medium

The experiment concerning the first step of strain selection used the strains DSM 826 and 5770. The fermentation was conducted in agitated flask with standard medium, with 100 mL of working volume, in a 500 mL Erlenmeyer flask. The inoculation and incubation conditions were such as described respectively on sections 6.4.3 and 6.5.1.

6.8.2 Defining sampling methodology

As mentioned before, IA production by *A. terreus* is highly sensitive to interruption of oxygen supply. An experiment was done to determine if small interruptions of the fermentation for sampling the agitated flasks would decrease the IA production compared to sampling with sacrifice sampling, illustrated on Figure 6.3. The quantification of sacrifice sampling was necessary because the samples from a same agitated flask throughout the fermentation period was of small volumes (2-4 mL), which would not provide trustful mass values for the quantification of dry cells.

The experiment of sacrifice sampling was done with up to 12 flasks with same medium formulation and spore inoculum, and they were placed in the same shaker equipment. For the initial days of fermentation – first to the third day – the dry cell quantification was done by filtering 30 to 50 mL of the fermented broth, with 2 to 3 replicates depending on the volume filtered. From the 4th day, the dry mass was determined by filtration of 10 or 20 mL of the fermented broth.

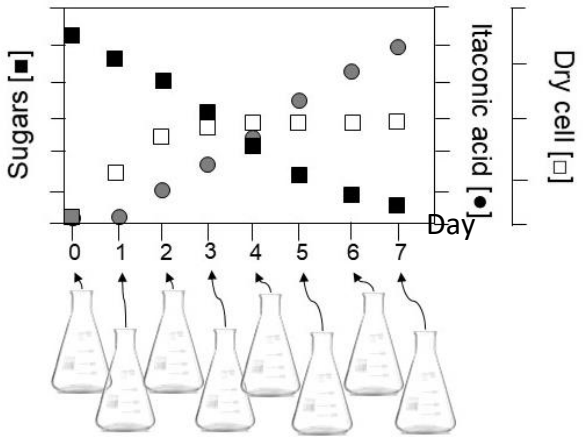

Figure 6.3 Illustration of a kinetic experiment with sacrifice sampling. All fermentation flasks, inoculated with spores from the same batch, undergo the same initial nutritional and environmental conditions and are placed on the incubator shaker at the same time. Each flask is removed from the shaker at a specific day to provide the variables concentrations to the kinetic curves.

The manually agitated sampling was done by removing the Erlenmeyer flask from the incubator shaker with continuous manual agitation, even when the flask was inside the laminar flow cabinet. The fermented broth was sampled (2 to 4 mL) with minimal agitation interruption and the flask was placed back in the incubator shaker. The volume withdrawn was centrifuged in a refrigerated centrifuge (BioVera) at 4 ° C and 2332 g.

6.8.3 IA production and fermentation volume

The analysis of IA production in agitated flasks in different working volumes was done with standard medium with glucose as carbon source. The experiment compared the fermentation results in 500 mL capacity Erlenmeyer flasks containing either 100 or 150 mL of the medium. The inoculation and incubation conditions were such as described respectively on sections 6.4.3 and 6.5.1.

6.8.4 IA production in glucose or sucrose media

The experiment of IA production in agitated flasks in media with glucose or sucrose as carbon source, performed with 100 mL working volume. The inoculation and incubation conditions were such as described respectively on sections 6.4.3 and 6.5.1.

6.8.5 Selection of a new *A. terreus* strain for IA synthesis

The strain selection of the microorganisms acquired lyophilized from the cell culture banks (*A. terreus* NRRL 260, 265, 1960, and IOC 4276, 4582) was initiated with strains activation, described on section 6.4.1. Further, a solid medium was selected to be used for spore formation of each strain. The new selection of the *A. terreus* strain was accomplished by performing the fermentation in media with glucose or sucrose as carbon source, described on section 6.8.7.

6.8.6 Selection of solid medium for spore formation

The sporulation of fungi cells depends on the strain, the solid medium and the incubation condition, which some media may promote a slow or incomplete spore formation. For fermentative processes, those are conditions that could impair fermentation seeding and the time of the bioprocess (Lockwood and Moyer 1945). The literature presents three different solid media used for growing IA-producers *A. terreus* strains: Czapek Dox, PDA and

agar malt extract (Nelson et al. 1952; Elnaghy and Megalla 1975; Gyamerah 1995; Petruccioli et al. 1999; Vassilev et al. 2012). The compositions of each solid media are described on section 6.8.6. One of those media was selected to promote spore production by the 5th day of incubation. The selection of the medium that allowed mostly the formation of spores, rather than hyphae, was done by visualization , as *A. terreus* hyphae are white, cotton-like in color and shape, while the spores have a sandy brown appearance (Calam et al. 1939).

6.8.7 *A. terreus* strain selection: fermentation in glucose and sucrose media

The experiment was performed with standard medium (section 6.3.3), with either glucose or sucrose as substrate, in agitated flask. The inoculation and incubation conditions were such as described respectively on sections 6.4.3 and 6.5.1.

6.8.8 Kinetic profile of IA production in sucrose medium

The experiment was performed with standard media, with either glucose or sucrose substrate in agitated flask. The inoculation and incubation conditions were such as described respectively on sections 6.4.3 and 6.5.1.

6.8.9 IA production in bioreactor

The experiment performed in bioreactor was done to evaluate the effect of different agitation and aeration rates on the production of IA. The description of the bioreactor used in this experiment and the fermentation conditions are described on section 6.5.2.

6.8.10 IA production in different carbon and nitrogen sources in agitated flasks

The experiment for evaluating the fermentation in different sources of carbon and nitrogen was done with granulated sugar or VHP as feedstock, and NH_4NO_3 or commercial urea as nitrogen source, performed in agitated flasks. The media compositions are described

on section 6.3.4. The inoculation and incubation conditions were such as described respectively on sections 6.4.3 and 6.5.1.

6.8.11 Strict oxygen requirement for IA production in VHP medium in agitated flasks

The experiment for confirming the strict oxygen requirement to produce high IA yields was done with VHP and NH_4NO_3, with the concentrations described as the assay number 3 on Table 6.2 (section 5.3). The seeding of all flasks was done with the same inoculum, and the inoculation and incubation conditions were such as described respectively on sections 6.4.3 and 6.5.1. To obtain the dry cell curve, each sample regarding cell concentration corresponds to one Erlenmeyer flask, and the dry mass was obtained with 3 or 4 mass concentration measurements. After 72 hours of fermentation, some of the flasks were removed from the shaker and they were kept static for 30 minutes, while the others were maintained in the moving shaker. After 30 minutes, the flasks were relocated to the shaker and all flasks regarding the two fermentation conditions – continuous (1) and submitted to 30 minutes agitation/aeration interruption (2) – proceeded in the same agitation conditions until the end of the experiment. The quantification of cells throughout the experiment was done with sacrifice sampling.

6.8.12 IA production in media with different concentrations of carbon and nitrogen sources in agitated flasks

The production of IA in media with different concentrations of VHP and NH_4NO_3 was done in agitated flasks. The media composition is presented on section 6.3.4. The inoculation and incubation conditions were such as described respectively on sections 6.4.3 and 6.5.1.

6.8.13 Macroscopicmodeling and parameter estimation of IA production in agitated flask

The steps used in this study to identify the dynamic mathematical model of IA production with different initial conditions of VHP and NH_4NO_3 are presented on Figure 6.4,

and the application of each step is further explained. The mathematical model developed in this study was done at Université Libre de Bruxelles, in Belgium.

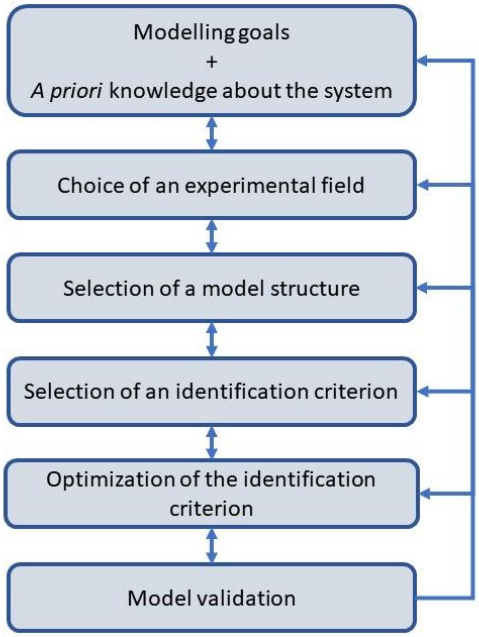

Figure 6.4 General steps for identifying a model (Bogaerts 2016).

The goal of building the macroscopic model was to develop a reaction scheme that defines the production of IA over time, at the expense of substrate and ammonium consumption. Moreover, it was also intended to estimate the values of the kinetic parameters of the reaction scheme proposed in this study. The *a priori* knowledge about the system was acquired during the performance of the previous experiments and during the formulation of the model.

The experimental field was chosen to be the results obtained from the experiment presented on section 6.8.12. The kinetic profiles production and consumption of each assay were used separately (each replicate was used as an assay) as experimental data for the definition of the dynamic macroscopic model.

The steps described as follows were done with the use of the software MatLab® R2016b for student use.

The selection of the model structure was done in a trial and error step, where different reaction scheme was proposed as a description of the macroscopic phenomena observed on the experimental data. This step is specified on section 4.5.3.

The identification criterium, described on section 4.5.4.1, was used to visually identify the fitting to the curves defined by the mathematical expressions proposed during the model formulation and the experimental data.

The model validation was done by cross validation, described on section 4.5.4.2. Further, an analysis of the uncertainty of the parameters defined on the model was performed as described on section 4.5.4.3.

Chapter 7

RESULTS AND DISCUSSION

High IA yields have been obtained in pure sources medium, such as glucose, by different *A. terreus* strains (Kuenz et al. 2012; Krull et al. 2017). The production of about 70% of the maximum theoretical yield (0.72 g IA / g glucose theoretical yield), either in batch or fed-batch fermentations, indicates that further improvement of the fermentation conditions should be directed to the use of lower cost inputs, since the cell requirement of growth and maintenance would hardly allow greater yields than those already obtained in medium of pure carbon sources. In fact, the application of pure glucose as a feedstock for filamentous fungi would probably not be a realistic scenario to expand the IA market, especially considering that IA still has numerous substitutes in the chemical industry. This study evaluated IA production regarding different aspects of cultivation conditions of *A. terreus* strains to obtain a good production of IA. The investigations initiated by selecting a proper *A. terreus* strain that produced IA in high concentrations, to be further applied as the bioagent in fermentations with non purified feedstocks, such as granulated sugar and VHP. Further investigation was also done to define a mathematical model that describes IA production in feedstock from the sugarcane industry.

7.1 Monitoring of IA development by bibliometrics of articles and patent documents

The analysis of the number of scientific research and patents may provide an indication of the trends of a technology or a certain subject of study. This approach, which may be applied as a quantitative analysis of the state of the art, may be also presented as part of the acknowledgement of the scientific importance of the study regarding IA and the current tendencies.

The databases provided 640 articles and 1033 patent documents. The number of scientific articles and patents increased significantly during the period analyzed (Figure 7.1), which confirms the high interest in developing technologies regarding IA production and its derivatives. The frequent world crises that result in drastic fluctuations of oil prices highly motivated the development of alternative technologies to partially substitute products from nonrenewable sources (Macrotrends 2017), including the development of IA technologies. The steep slope observed for the profile of number of patents, which initiated in 2007, corresponds mostly to the numerous Chinese patents about IA published on that period – 76% of the 509 patents published from 2007 to 2016. In that country, the investments from government and companies increased the country's participation in the advances of the world's research (Huang et al. 2010), which is reflected in the high number of patents most recently published (Figure 7.1).

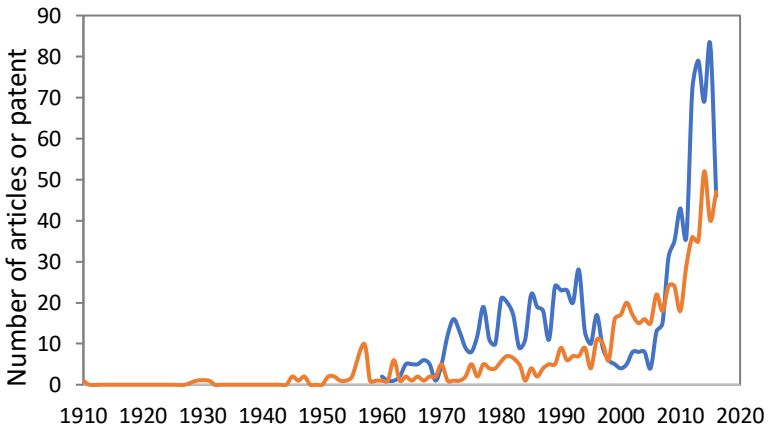

Figure 7.1 Number of scientific articles and patent documents selected from the databases Scopus® and Derwent World Patent Index™ respectively. The documents selection was done considering articles and patents which contained the words "itaconic acid" on the title, published from the initial years available on the databases until 2016. For the articles selection, the documents were restricted to the English language. Scientific articles (—) and patent documents (—).

Figure 7.2 represents a classification of the most recent articles, published from 2012 to 2016 (210 articles), which were separated by the main subject addressed by each document according to the following categories: development or application of IA polymer; development of the fermentative process; research about the metabolic pathway of IA. Those categories were created according to the subject areas of the articles presented on each publication.

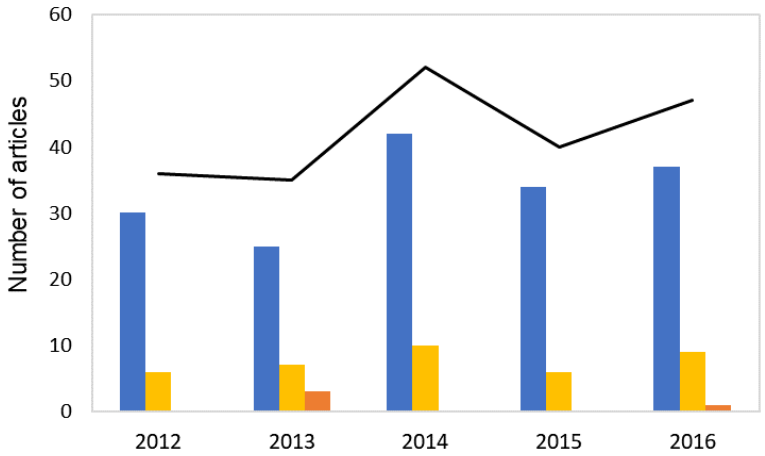

Figure 7.2 Number of scientific articles from the database Scopus containing on the title the words "itaconic acid", published from 2012 to 2016. Each of the 210 articles where characterized by the main subject related to itaconic acid (IA) synthesis or application: development or application of IA polymer (■), development of the fermentative process (■), research about the metabolic pathway of IA (■) and the total number of articles in each year (—).

The studies related to the development of IA derivatives represent 80% of the total articles in that period, ranging from 25 to 40 articles published in each year. The second most frequent subject was the development of IA fermentative processes, with 18% of the studies analyzed. It was not observed a significant fluctuation of the number of articles that concerns those two classifications throughout the period analyzed. The scientific articles about the metabolic pathway of IA production, including the improvement of different strains for higher yield production, were less frequent, with only 2% of the articles from the analyzed period.

The most recent research is substantially more directed for developing IA end-products (80% during the analyzed period, as previously mentioned) rather than for the advances in fermentative processes and microorganism modification for improving the final IA yield. The larger number of articles about the development of the products suggests that the IA studies are in an advanced stage of the overall technology. It is possible that the development of the fermentative process of IA may have reached a stage when further it is no longer required a

significant innovation about the process. However, there are still research concerning that stage of IA production, which indicates that there may still have improvements on this area. Techniques that surpass the usual strategies to improve the fermentation process of IA are required to effectively represent an innovation of the technology.

The most recent patents analyzed, published from 2012 to 2016 (348 patent documents), were separated by the priority country or its region (Figure 7.3). The priority country of a patent is frequently where the requesting institution is located. The analysis indicated that most of the inventions were first patented in China, which is a strong indicator of that country's high interest in developing IA products. The second region where IA patents were mostly deposited is Asia, which Japan is the priority country with more than 60% of those patents. The USA and Europe occupies the third and fourth position respectively. South America was the priority country of only 1% of the patents from the analyzed period. This analysis reflects the robust Chinese investment in IA technologies, and the Chinese position on the current global IA market, since most companies that currently produces IA are located in China (section 2.7).

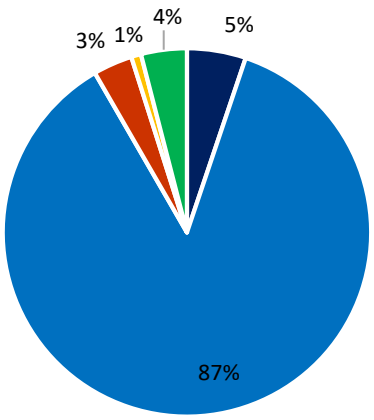

Figure 7.3 Classification of the priority countries or region of the 348 patent documents related to IA, selected from 2012 to 2016 on the database DWPI. Asia ( ), China ( ), Europe ( ), South America ( ), USA ( ).

The analysis of the advances in IA innovations, whether by the published articles or patents, indicate that the IA technology development is currently more directed to the improvement of IA products and their applications. It is important to note that the high number of patent documents evidences the significant interest of the organic acid application, as many patents are deposited by companies or institutions with the intention to apply the

inventions on the market. This is a high indication of the expansion of IA on the available market for renewable sources. Moreover, it shows that improvements for IA processes have been done, and that the interest in IA products concern different counties, but mainly China, which is also the current greater IA world producer (Global Industry Analysis 2016).

It has been previously mentioned that the expansion of IA market requires further efforts for decreasing the end-product cost. The use of abundant carbon sources from residual material or not, which vary according to the country, could be an interesting solution for decreasing the final costs.

7.2 First strain selection and definition of fermentation conditions

In this section, the results of the first selection of the *Aspergillus terreus* strain to be used in this study are presented, which were 2 strains. Moreover, the definition of some conditions of the fermentation in agitated flasks are also presented in this section.

7.2.1 IA production by *Aspergillus terreus* DSM 826 and 5770 in glucose medium

The strains *A. terreus* DSM 826 and 5770 were cultivated in glucose medium in agitated flask and the production behavior of each strain is presented respectively on Figure 7.4a and b. As mentioned previously, the inoculum was done with spores (10^6 spores/mL), and the spores mass, which was not measured, is very low in comparison to the concentration of mycelia formed along the fermentation. Therefore, in this study, the initial values of cell in each assay were represented as 0.001 g/L dry cell.

The highest IA production by *A. terreus* DSM 826 was far below than that obtained by *A. terreus* DSM 5770. In, fact, it may be considered that the fermentation with the former strain resulted in negligible production (1.1 g/L IA) compared with the highest value produced with the latter (31.5 g/L IA), after a 10 day fermentation. Moreover, acknowledging that maximum theoretical IA/glucose yield by *A. terreus* (0.72 g IA/g glucose), the fermentation with *A. terreus* DSM 826 resulted in less than 2% of the theoretical yield by the end of the fermentation, while *A. terreus* DSM 5770 resulted in 42% of the same yield.

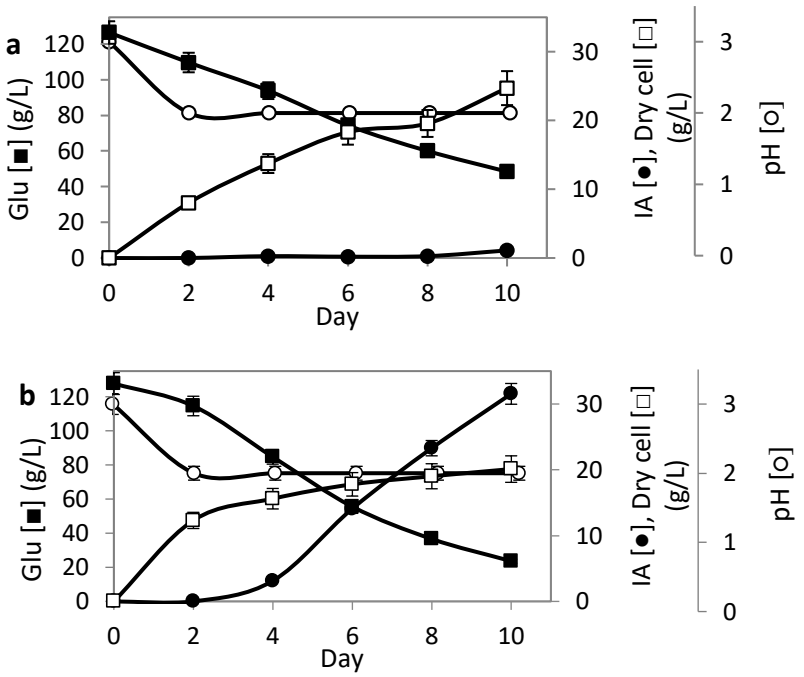

Figure 7.4 IA production by *A. terreus* DSM 826 (a) and 5770 (b) in agitated flasks with standard glucose medium. Both media was initially adjusted to pH 3.1 and not controlled throughout the process. The fermentation occurred at 33°C, with 150 rpm agitation. The seeding of each agitated flask was done with the same inoculum of spores. The dry mass of the inoculum was considered as 0.001 g/L. The sampling was done by sacrifice flasks.

The differences between both fermentations were also observed for the cell growth and remaining substrate profiles. *A. terreus* DSM 826 presented a continuous growth throughout the assay, while *A. terreus* DSM 5770 strain reached a slow cell grow rate, almost stationary, on the second day of fermentation until the 10th day. The glucose uptake by *A. terreus* DMS 826 (Figure 7.4a) was preferably directed to cell production rather than IA synthesis, with a final yield of 0.32 g cell/g glucose. In contrast, for the *A. terreus* DSM 5770 fermentation, it was obtained 0.19 g cell/g glucose, and two distinct cell growth rates were observed (Figure 7.4b): the first one was from the first to the second day of fermentation, reaching 12 g/L cells. From that point forward, cell concentration gradually reached 20 g/L dry cell on the 10th day. It may be noted that the production of IA by *A. terreus* DMS 5770 occurred during the phase of slower cell growth rate.

For both *A. terreus* strains, an initial drop from 3 to 2 in the pH of media was observed after 2 days of the inoculation, and it remained constant until the end of fermentation. Considering the strain that produced IA at higher concentration (Figure 7.4b), the pH and cell growth profiles corroborates previous studies, which have demonstrated that the period of

stationary or slow cell growth phases are coincident with the pH drop to 2 for assays without pH adjustment. Those studies also observed that pH is maintained at 1.5-2 that values throughout the fermentation (Park et al. 1993; Petruccioli et al. 1999; Riscaldati et al. 2000; Kuenz et al. 2012; Krull et al. 2017).

As previously introduced in the section 2.11, the literature reports that an acid environment is required for the synthesis of the IA production essential enzyme system (Larsen and Eimhjellen 1955; Hevekerl et al. 2014b; Krull et al. 2017). However, IA was only produced significantly by DSM 5770, even though the fermentations of both strains reached pH 2 during most the fermentation period. That suggests that the low pH may be important for IA production, but a low pH value does not necessarily result in IA production. Further discussion about the relation of a low pH environment and IA production is done along this study.

Tables 7.1 and 7.2 present the concentrations of some by-products detected in the fermented broth for each strain. After 10 days, the succinic acid content in fermentation broth was 6 times higher than IA for *A. terreus* DSM 826 (Table 7.1). Glycerol was the second highest metabolite among those organic acids and alcohols quantified in medium, and the other metabolites were in less concentration than IA. It is possible that glucose consumption was mostly directed to cell production, as mentioned previously (25 g/L cell after 10 days).

Succinic acid and glycerol were also produced by *A. terreus* DSM 5770, however, at lower levels than those detected by *A. terreus* DSM 826 fermentation (respectively 88% and 92% less production). The other metabolites analyzed were not detected in the fermented medium. Because IA production was much more relevant by *A. terreus* DSM 5770, further investigations were performed using that strain.

Table 7.1 Production of IA and some by-products synthesized by *A. terreus* DSM 826 in glucose standard medium cultivated in shaker. The assay is the same presented on the previous Figure for this strain. All the concentrations are presented in g/L.

Product (g/L) / Day	Itaconic acid	Xylitol	Ethanol	Glycerol	Acetic acid	Latic acid	Citric acid	Succinic acid
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.39	0.00	0.00	0.00	0.44
4	0.27	0.00	0.91	1.00	0.00	0.00	0.00	1.66
6	0.19	0.34	0.71	1.68	0.00	0.00	0.00	3.48
8	0.27	0.24	0.67	2.27	0.00	0.00	0.00	4.31
10	1.10	0.37	0.11	3.68	0.00	0.00	0.00	6.31

Table 7.2 Production of IA and some by-products synthesized by *A. terreus* DSM 5770 in glucose standard medium cultivated in shaker. The assay is the same presented on the previous Figure for this strain.

Product (g/L) / Day	Itaconic acid	Xylitol	Ethanol	Glycerol	Acetic acid	Latic acid	Citric acid	Succinic acid
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.64	0.00	0.00	0.00	0.78
4	3.15	0.00	0.00	1.19	0.00	0.00	0.00	2.42
6	14.10	0.00	0.24	1.42	0.00	0.00	0.00	3.20
8	23.26	0.00	0.00	2.06	0.00	0.00	0.00	3.58
10	31.53	0.00	0.00	2.40	0.00	0.00	0.00	3.58

Even after several repetitions of the same assay, it was not possible to obtain higher concentrations of IA using *A. terreus* DSM 826. Those results, however, were contradictory to the literature, since that strain was previously reported to produce higher concentrations of IA in an optimized medium with glucose as substrate: 33 g/L IA and a yield of 0.18 g IA/g (Jiménez-Quero et al. 2016). Moreover, *A. terreus* DSM 826 was originally from the ARS Culture Collection (Northern Research Regional), deposited as *A. terreus* NRRL 1960, known for its capacity to produce high concentrations of IA (Table 2.2). Kuenz et al. (2012) obtained 88 g/L IA and 0.49 g IA/g glucose for *A. terreus* NRRL 1960 at same fermentation conditions.

A possible explanation for the extremely low IA titer obtained in this stage is the loss of genetic capacity of *A. terreus* DSM 826 to produce IA. Another study has demonstrated the loss of capacity of a IA-producer strain to synthesize the acid. As mentioned before (section 2.2), *A. itaconicus* was the first strain to be identified as an IA-producer. However, according to Miall (1978), Moyer and Coghill (1945) reported that after 14 years of storage of the strain, *A. itaconicus* was able to produce only traces of IA. A similar mutation effect may have occurred to the *A. terreus* DSM 826 strain used in this study. The possible mutation could have led to the loss of the fungus capacity to stimulate the genes *mtt* and *cadA*, which are part of the metabolic pathway to the overproduction of IA. The less production of the MTT mitochondria transporter and CAD (*cis*-aconitase decarboxylase) could have induced cell growth rather than IA production.

7.2.2 Defining sampling methodology with *Aspergillus terreus* DSM 5770

As previously mentioned (section 2.10), due to the strict requirement of dissolved oxygen in the medium throughout the fermentation by *A. terreus* for obtaining a high IA production, even sampling procedures of short duration (about 5 minutes) may result in insufficient supply of oxygen and impair IA production. This behavior was also confirmed during the execution of this work (data not shown). A method of continuous manual agitation during the sampling procedure was tested (described in section 6.8.2), and it was compared with sacrifice sampling to verify the applicability of a continuous manual sampling (Figure 7.5). The dry cell concentration of the manual sampling assay was not presented because the volume of the samples withdrawn from the agitated flask (2mL) did not provide sufficient mass for a reliable quantification of the dry cell mass. It should be mentioned that the initial medium volume was 100 mL, thus, the removal of greater volume from the flasks would compromise the final results.

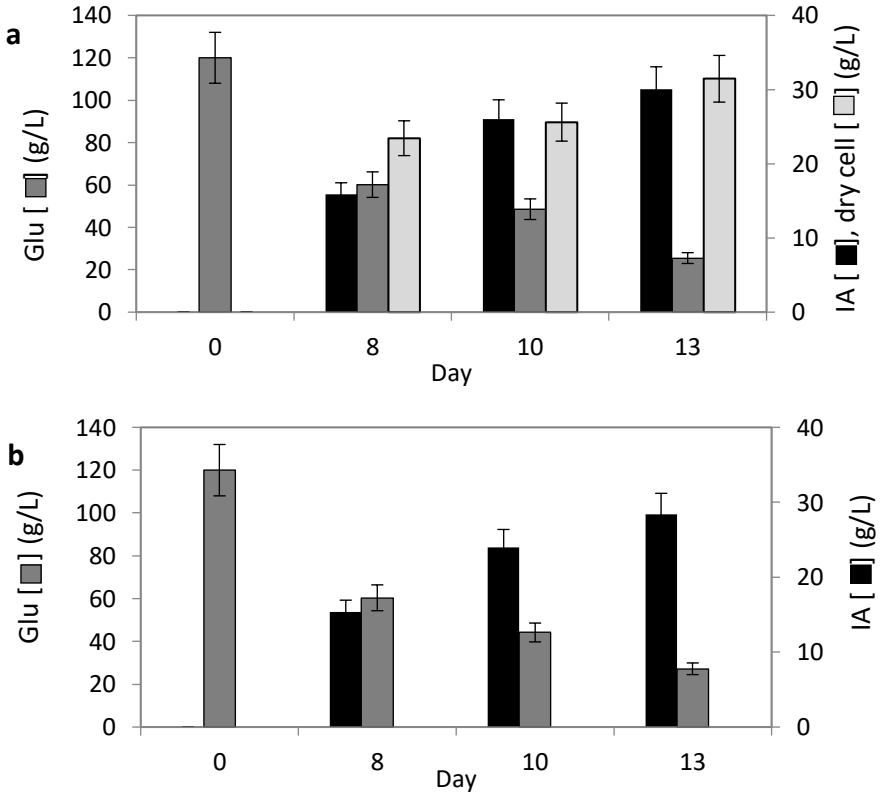

Figure 7.5 Comparison of different sampling methods, performed with the strain *A. terreus* DSM 5770 cultivated in glucose standard medium. Both assays occurred in the same shaker, at 33°C, with 150 rpm agitation. The seeding of each agitated flask was done with the same inoculum of spores. The dry mass of the inoculum was considered as 0.001 g/L. The sacrifice sampling assay (a) presents the cell concentrations, while the manually agitated sampling assay (b) does not.

Figure 7.5a and b shows that the profiles of IA production were equivalent regardless the sampling method, with all mean values within the range of standard deviations. The results indicate that the manual agitation during the sampling procedure was sufficient for maintaining the oxygen supply during the brief moment of sampling. Therefore, it was ascertained that the manual agitation sampling method could be applied for the quantification of medium components during the fermentation.

One advantage of the manually agitated sampling is that each condition of an assay can be done with one agitated flask, added the assay's replicas. On the other hand, that sampling method does not allow the quantification of dry cell concentration. Therefore, if it is required to determine the biomass concentration of an assay, it is used as many agitated flasks as there are the number of points on kinetic profile (section 6.8.2). The choice of one of the sampling methods for the further assays depended on the aim of the experiment: the sacrifice sampling method was used for the assays which required the knowledge of cell concentration; the manually agitated sampling method was used for the assays which was prior to knowledge the concentration of IA, substrate and pH.

7.2.3 IA production and fermentation volume with *Aspergillus terreus* DSM 5770

The previous section mentions the importance of providing sufficient dissolved oxygen during every moment of the fermentation, including during sampling. Considering the fermentation performed in agitated flasks, the aeration occurs by the diffusion of oxygen from the head space of the flask to the medium surface, and the medium bulk. Hence, the fermentations with low working volumes provides a greater air transfer area and air volume inside the Erlenmeyer than high working volumes due to the conical shape of the agitated flasks. Nevertheless, the use of a low working volume leads to a higher evaporation rate, especially considering the long period of fermentation of this study (10 – 14 days fermentation). That could be an issue because the decrease of fermentation medium volume throughout the fermentation by evaporation may change the osmolarity and cell density especially at the last days of the fermentation.

The aim of this assay was, thus, to compare IA production using 100 mL or 150 mL in 500 mL capacity Erlenmeyer flask. The IA production in 100 mL (Figure 7.6a) was almost 60%

higher than that by the fungal cultivation in 150 mL medium (Figure 7.6b). Moreover, the fermentation with greater surface area (100 mL) produced preferably IA than cells, whereas the assay with 150 mL was the opposite, with 35% more cell production than with 100 mL. The final IA yields of the two assays also justifies the employment of 100 mL for further experiments (0.27 and 0.17 g IA/g glucose for the use of respectively 100 mL and 150 mL).

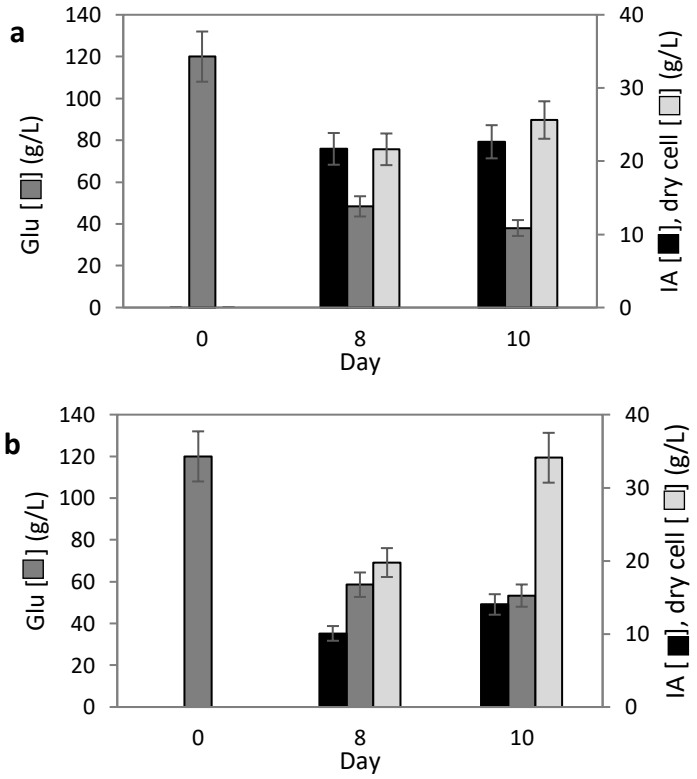

Figure 7.6 Comparison of different working volumes of the fermentation in shaker with *A. terreus* DSM 5770 in standard glucose medium. Reaction volumes: 100 mL (a) and 150 mL (b). The seeding of each assay was done with the same inoculum. The dry mass of the inoculum was considered as 0.001 g/L. The fermentation of the agitated flasks was performed at 33°C with 150 rpm agitation.

It is possible to observe that the use of 100 mL as reaction volume in agitated flask provided more dissolved oxygen than the 150 mL for the same environmental conditions of temperature and agitation rate. This experiment showed that, for the fermentation with *A. terreus* DSM 5770, the use of 150 mL was not an environment as did not provide sufficient dissolved oxygen to produce the same IA concentration as obtained on the first assay (Figure 7.5). Therefore, the assays performed in agitated flasks were performed in 100 mL working volume.

7.2.4 *Aspergillus terreus* DSM 5770 IA production in glucose or sucrose media

Fungi are expected to easily assimilate sugars such as glucose, sucrose or fructose (Papagianni 2004), although the metabolic response to the different saccharides may vary (Saha et al. 2017). According to Mattey (1992), in general, the sugars that are quickly assimilated by the microorganism allow high final yield of organic acids. This experiment evaluated the response of *A. terreus* DSM 5770 to the cultivation in glucose compared to sucrose, since the latter is the substrate of the feedstocks proposed to be used further in this study.

The profiles of IA production by *A. terreus* DSM 5770 fermentation in the different media shows that both IA production and cell growth were similar regardeless the carbon source provided (Figure 7.7). Thus, IA productivities on both substrates were equally 0.09 g/L/h. The pH profiles were also not distint from one carbon source to the other.

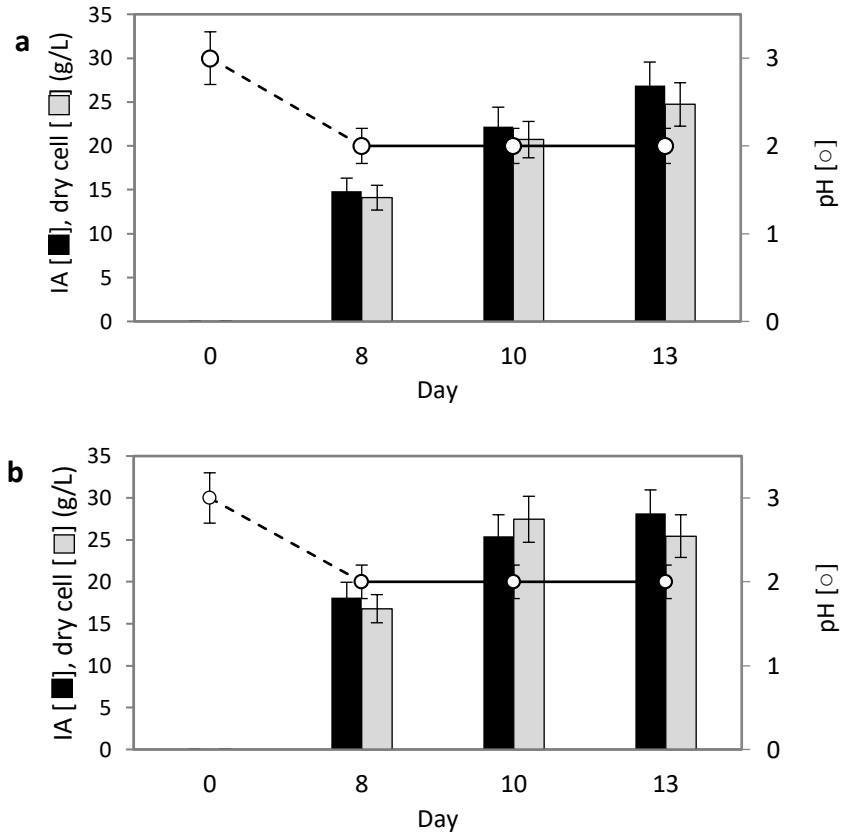

Figure 7.7 Comparison of the cultivation of *A. terreus* DSM 5770 in standard glucose medium (a) or in standard sucrose medium (b) in agitated flasks. The sampling was done by sacrifice flasks. Both media was initially adjusted to pH 3.1 and not controlled throughout the process. The dry mass of the inoculum was considered as 0.001 g/L. The fermentation was performed at 33°C with 150 rpm agitation.

The equivalent IA concentrations obtained with the fermentation by *A. terreus* DSM 57750 in glucose or sucrose medium indicated that further investigations could be considered with feedstocks containing sucrose as substrate. Despite of that, the final concentrations of IA obtained in both media were lower than those produced by other strains, according to other studies (Table 2.2). That finding resulted in the decision to change the strain, thus, a new selection of IA-producer *A. terreus* strain was performed, presented above.

7.3 Selection of new *A. terreus* strain for IA synthesis

Even though *A. terreus* DSM 5770 produced much greater IA concentration than *A. terreus* DSM 826 in glucose medium, the highest value of IA production produced by the former (around 30 g/L) was very low compared with the concentration of up to 70 g/L of IA by other strains in agitated flasks (Kuenz et al. 2012). Considering that further experiments would be performed with carbon and nitrogen sources that are not from pure origin, i.e., with higher impurity content than pure glucose or pure sucrose, those nutrient sources could be an additional impair for achieving high IA yield (section 2.9). It was, thus, decided to search for a different strain that could be more promising for IA production in less pure feedstocks.

The new strain selection was performed with the strains *A. terreus* NRRL 265 and 1960, which have already been demonstrated to be high yields IA-producers (Nubel et al. 1962; Klement and Büchs 2013). The strain *A. terreus* NRRL 260, from the same microorganism collection than the first two, was also tested. Two strains from the national culture collection, the Instituto Oswaldo Cruz, were evaluated for the first time as possible IA producers, to the best of the author's knowledge (IOC 4276 and 4582).

7.3.1 Selection of solid medium for spore formation

The aim of this assay was to select a solid medium that stimulates the formation of spores for the inoculum of the new strains: *A. terreus* NRRL 260, 265, 1960 and IOC 4582 and 4276, which had been provided lyophilized. As previously described (section 6.8.6), three different solid media were applied to analyze the most propitious spore formation: Czapek

Dox, PDA or agar malt extract. The evaluation of the spore production was done by macroscopic visualization of the shape and color of the microorganisms on each solid medium.

Figure 7.8a is an illustration representing the cell growth in solid media that stimulated mostly the formation of mycelia. That image represents the resulting cell growth for all strains cultivated in either Czapek Dox or agar malt extract medium. In contrast, the typical velvety, cinnamon-coloured morphology of *A. terreus* spores (Calam et al. 1939) represented on Figure 7.8b (macroscopically) and 7.8c (50x magnification) was observed in PDA medium for all the strains by the end of the incubation time. PDA medium was, thus, selected for producing the spores that were used for the inoculum of the microorganisms' fermentation.

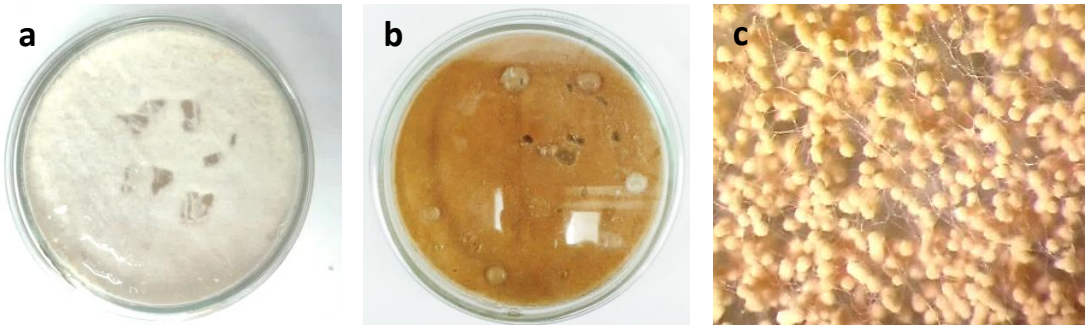

Figure 7.8 Macroscopic morphology of *A. terreus* strains with majority formation of hyphae (a) or spores (b and c). The macroscopic image presented in (a) represents *A. terreus* NRRL 260, 265, 1960 and IOC 4582 and 4276 cultivations in Czapek Dox and agar malt extract solid media, while (b) represents a macroscopic image of solid cultivation of the same strains in PDA medium and (c) represents the spores formed in PDA medium for all the strains (50x magnification).

7.3.2 *Aspergillus terreus* strain selection: fermentation in glucose and sucrose media

The five strains of *A. terreus* were cultivated in standard glucose and sucrose medium for defining each microorganism's capacity to synthesize IA. The comparison between the results defined the new strain to be used in this study.

The analysis of fermentation results reveals a different IA production and cell growth depending on the strain and the substrate used after a 10 day fermentation. Figure 7.9 presents the results of glucose standard medium and Figure 7.10 presents the fermentation results in sucrose standard medium. The IA production by *A. terreus* NRRL 1960 was remarkably higher in both carbohydrates media compared to the other strains. That strain synthesized over 50 g/L IA in both substrate media, with a yield of 0.45 g IA/g glucose and 63% efficiency for glucose. It may be noted that substrate was almost completely consumed by the

end of the assay and cell production was not higher than 6 g/L dry cell. Compared to the previous assays with *A. terreus* DSM 5770 (Figure 7.4b, 7.5, 7.6 and 7.7), this assay demonstrated that *A. terreus* NRRL 1960 was a superior IA producer.

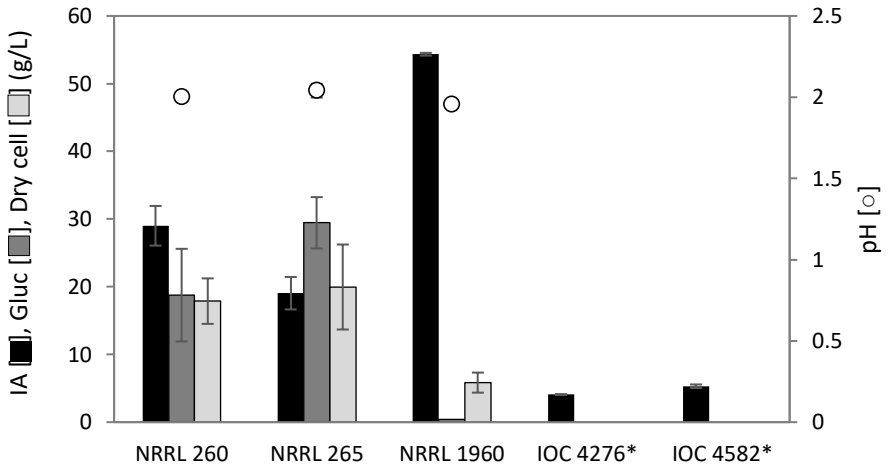

Figure 7.9 Results of fermentation by different *A. terreus* strains in glucose standard medium cultivated in agitated flasks after 10 days. The media initial concentration of glucose was 120 g/L, and the pH was adjusted to 3.1 and not controlled throughout the process. The dry mass of the inoculum was considered as 0.001 g/L. The fermentation was performed at 33°C with 150 rpm agitation. The assays with the IOC strains (*) lasted 7 days and their values of pH and dry cell could not be determined for technical reasons.

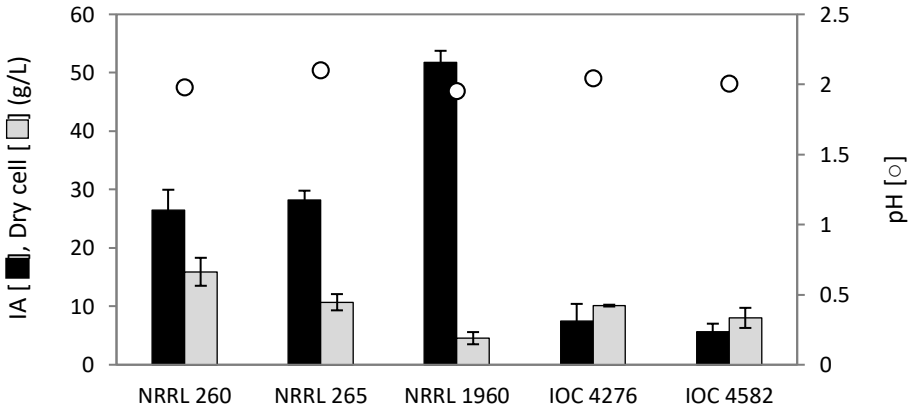

Figure 7.10 Results of fermentation by different A. terreus strains in sucrose standard medium cultivated in agitated flasks after 10 days. The media initial concentration of sucrose was 114 g/L, and the pH was adjusted to 3.1 and not controlled throughout the process. The dry mass of the inoculum was considered as 0.001 g/L. The fermentation was performed at 33°C with 150 rpm agitation. The values of sucrose concentration could not be determined for technical reasons.

A. terreus NRRL 260 produced equivalent IA and cell in both media, either with glucose or sucrose as substrate. On the other hand, *A. terreus* NRRL 265 synthesized higher

concentration of IA in sucrose than in glucose medium, which was the opposite of the cell growth.

The values for glucose and sucrose media for IA production by the *A. terreus* strains from the IOC collection showed that the latter was more favorable to IA production. However, both strains produced very low IA concentrations, even though both produced higher IA concentration than the previously tested strain *A. terreus* DSM 826 (Figure 7.4a). The conditions of the fermentation with sucrose did not promote more than 10 g/L dry mass. To the best of the author's knowledge, this is the first time that *A. tererus* IOC 4276 and 4582 were reported as IA producers, despite the low production values.

The differences in final concentrations of IA produced by each strain corroborates that not all strains are good IA producers. Lockwood and Reeves reported in 1945 that an analysis of over 300 *A. terreus* strains demonstrated that only 11 were able to produce more than 45% of the theoretical yield $Y_{P/S}$ (Miall 1978). More recently, Saha et al. (2017) screened 20 *A. terreus* strains for IA production with pentose sugars and only 6 of them were identified as high yield producers.

The pH values were very similar for all strains (pH 2) regardless the substrate applied or the final concentration of IA in the fermented medium. Once again (Figure 7.4a), the pH values lower than 2 was not an indicative of high IA production.

Table 7.3 presents the yields in relation to the substrate consumed, the productivities and the efficiencies calculated for all strains and carbon sources tested in this study. This comparative analysis supports the choice of the strain NRRL 1960 as the *A. terreus* strain used in further evaluations.

The comparison with all the strains evaluated in this study shows that A. terreus NRRL 1960 is the most promising. The values obtained with *A. terreus* NRRL 1960 in this study are comparable with the highest IA production reported on the literature. Recent examples of fermentations in agitated flasks with glucose medium were reported to reach 0.62 g IA/g glucose with *A. terreus* NRRL 1963 (Welter 2000) and 0.51 g IA/g glucose with *A. terreus* NRRL 1960 and DSM 23081 (Kuenz et al. 2012), which may be higher than the value obtained in this study because of differences in nutritional and environmental conditions.

Table 7.3 Yields, productivity and efficiency of the fermentations of each *A. terreus* strain analyzed in this study. The calculus was done considering the assays cultivated in 100 mL reaction volume, at the 10th day, except for the productivity of the IOC strains, which were calculated for the 7th day of fermentation. The efficiency is the yield obtained in each assay divided by the theoretical yield of *A. terreus* fermentation in glucose medium (0.72 g IA/g glucose), presented in percentage values.

A. terreus strain	$Y_{P/S}$ (g IA /g glucose)	$Y_{X/S}$ (g cell/g glucose)	Productivity (g IA/L/h)		Efficiency (%)
	Glucose medium	Glucose medium	Glucose medium	Sucrose medium	Glucose medium
DSM 826	0.015	0.34	0.005	-	2.1
DSM 5770	0.36	0.35	0.08	0.09	50.0
NRRL 260	0.28	0.17	0.12	0.11	38.9
NRRL 265	0.21	0.22	0.08	0.12	29.1
NRRL 1960	0.45	0.05	0.23	0.22	62.5
IOC 4276	-	-	0.02[a]	0.04	-
IOC 4582	-	-	0.03[a]	0.03	-

The values of IA yield and cell yield, both in relation to the consumption of substrate, show that the high production of cells did not favor the production of IA. Also, the productivity of *A. terreus* NRRL 1960 was at least double the productivity of the other strains, including the strains first analyzed in this study (*A. terreus* DSM 826 and 5770). Regarding the efficiency, certainly that strain is the best among the others to produce IA.

The literature has shown that *A. terreus* also synthesizes other metabolites such as malic acid (Krull et al. 2017), succinic acid (Dowdells et al. 2010), fumaric acid (Jiménez-Quero et al. 2016), lovastatin (Jia et al. 2009; Boruta and Bizukojc 2017), α- ketoglutaric acid (Krull et al. 2017) and gluconic acid (Dowdells et al. 2010). The different responses of IA yields and productivity may be related to the overproduction of one or more of those metabolites mentioned rather than IA synthesis, especially by the lower IA producer strains (*A. terreus* NRRL 260, and IOC 4276 and 4582). The superior performance of *A. terreus* NRRL 1960 in both media determined the use of that strain to the further experiments in this study.

7.4 Kinetic profile of IA production by *A. terreus* NRRL 1960 in sucrose medium

This assay consisted in IA synthesis by *A. terreus* NRRL 1960 with sucrose medium performed in two different incubator shakers. The aim of the assay was to obtain the kinetic profile for IA produced in sucrose medium and to evaluate the reproducibility of the same

experiment in two different shaker equipment. That analysis verified if the shakers were equivalent regulated in the eventuality of the need to use either equipment throughout this study.

The substrate profile is represented by total reducing sugars (TRS), since the substrate – sucrose – was quantified regarding sucrose, glucose and fructose (section 6.6.4). The carbohydrates glucose and fructose were not added to the fermentation medium, but rather they were produced by the hydrolysis of sucrose during the sterilization procedure, which applies high temperature. The heat and the acid environment (pH 3) promoted the hydrolysis of sucrose in D-glucose and D-fructose (Hirschmüller 1953).

Figure 7.11 shows that the same concentration of IA obtained previously in sucrose medium (Figure 7.10) was produced on the 10th day of fermentation, but a slower IA production rate initiated by the 9th day. Also, the IA profile indicates that IA has a lag phase of production, since IA is quantified from the second day of fermentation. As expected, TRS consumption initiated on the first day, as well as cell growth, which had a slower cell growth rate from the 4th day, in comparison to that same curve describing the previous days of the assay.

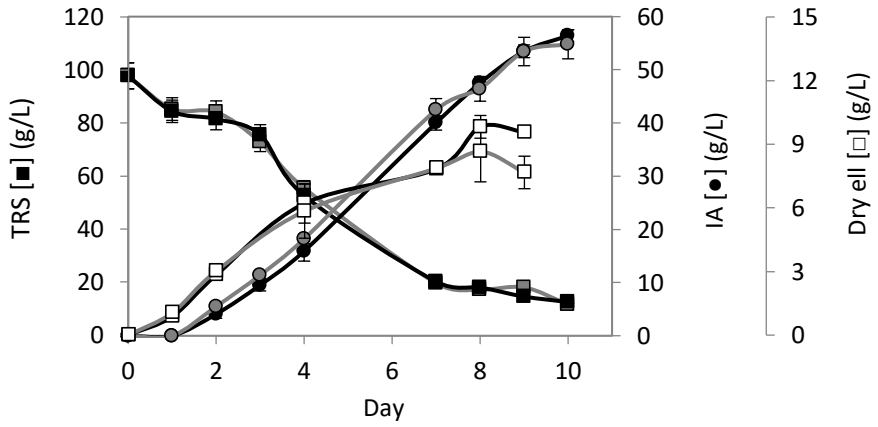

Figure 7.11 *A. terreus* NRRL 1960 cultivation in sucrose medium in incubator shaker 1 (*black line*) and incubator shaker 2 (*gray line*). TRS: total reducing sugars.

The literature states that 1 mol of glucose produces 1 mol of IA (Eimhjellen and Larsen 1955; Klement and Büchs 2013). Considering that 1 mol of sucrose (glucose–fructose) results in 2 mols of IA, the maximum theoretical yield is 0.76 g IA/g sucrose. Therefore, the IA concentration obtained by *A. terreus* NRRL 1960 is equivalent to 75% efficiency for sucrose as substrate. That value is similar to those obtained by the fermentation with three different *A.*

terreus strains in 180 g/L initial glucose medium in 1.5 L stirred tank reactor: DSM 23081 (70% efficiency), NRRL 1960 (72% efficiency), *A. terreus* NRRL 1963 (68% efficiency) (Kuenz et al. 2012).

Cell production rate was slower from the 4th day of fermentation, with a yield $Y_{P/S}$ of 6.13 g IA/g dry cell. It was observed an excess of 12% of sucrose at the 10th day of fermentation, which indicated that the assay could have been done for further days. On the other hand, it was observer that IA production rate was already deaccelerated, and possibly longer periods of fermentation would demonstrate a stagnation in IA production.

Moreover, both profiles concerning the fermentation in different incubators demonstrated that the assays were equivalent regardless the equipment applied. That means that the possible differences in performance of one shaker compared to the other, i.e., temperature and agitation regulation, did not affect the final IA concentrations. The validation of the equivalency between the two incubator shakers allowed the use of either one for the assays depending on the requirement.

7.5 IA production by *A. terrreus* NRRL 1960 in bioreactor

The previous assay showed that the two incubator shakers resulted in equal production of IA. In this assay, the aim was to perform the fermentation with *A. terreus* NRRL 1960 in sucrose medium at a laboratory-scale bioreactor. The differences between the two systems, such as aeration and agitation conditions, temperature regulation, online analysis of the fermentation parameters, usually provide a more satisfactory production results with the bioreactors then with agitated flask. On the other hand, the modification of the fermentative conditions from agitated flasks to bioreactor requires a good definition of the controlling systems and the regulation of the parameters, such as agitation and aeration rates, in order to obtain high yields of the bioprocess product. For bioprocesses with filamentous fungus fermentation, the agitation system could change the shape of the mycelia and metabolites production due to the shear force of the bioreactor (Okabe et al. 1993). A proper determination of the operation conditions is crucial for a satisfactory production.

This study evaluated IA production in a laboratory scale bioreactor of 500 mL capacity and operated with 400 mL working volume. The conditions used was aeration at 0.5 vvm and

agitation of 300 rpm (Figure 7.12). The system used did not enable a reliable sampling of cells due to mycelia aggregation in the bioreactor blades and on some parts of the bioreactor, which impaired a homogeneous sampling of the biomass.

Moreover, the operational conditions of the bioreactor resulted in a decrease of volume throughout the fermentation due to water loss by evaporation, despite the presence of a condenser, which resulted in the reduction of about 50% of the initial value, i.e., almost 200 mL. The values presented on Figure 7.9 represents each concentration of IA and substrate considering the relation between the initial and the actual volume at each moment of the sampling. Further comments regarding that issue is presented later in this section.

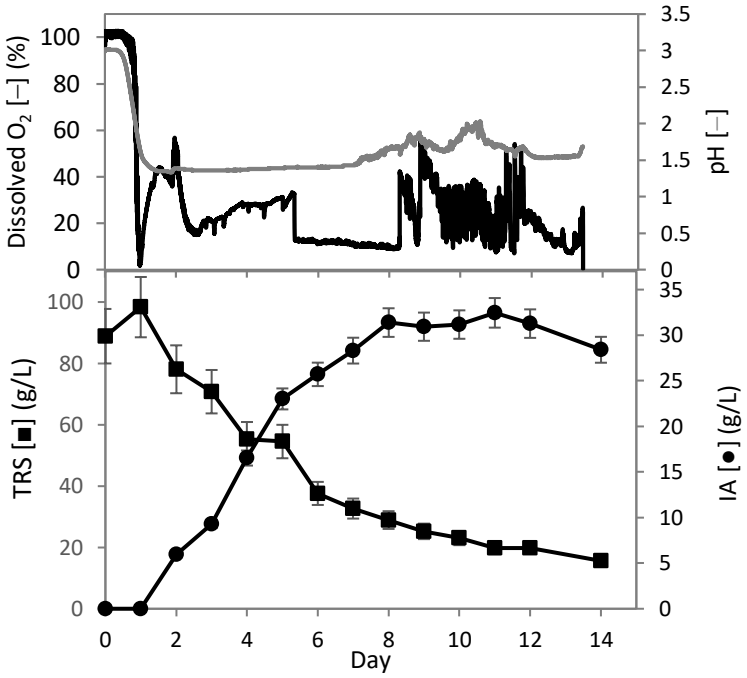

Figure 7.12 Relative values (considering the volume loss due to evaporation) of substrate and IA at fermentation by *A. terreus* NRRL 1960 in laboratory scale bioreactor with sucrose medium at constant agitation of 300 rpm, aeration of 0.5 vvm and temperature (33° C). The pH, aeration and agitation rates were not adjusted throughout the assay.

The observed profile of IA production in laboratory-scale bioreactor is similar to that obtained in agitated flasks (Figure 7.11). Both presented a lag phase of IA production, and IA production was first detected at the second day of fermentation. The TRS profile showed to decrease at different rates, and it was not completely consumed by the end of the assay. Because the assay in bioreactor was conducted for a longer period than the assay with

agitated flasks, it was possible to observe a consumption of IA from the former, from the 11th to the 14th day, despite the presence of carbohydrates in the medium.

An importance difference between the different assays was that the highest IA concentrations were achieved with the agitated flasks (55 g/L IA, Figure 7.11) against a maximum of 32.5 g/L in bioreactor (Figure 7.12). That difference could have been related to the choice of agitation and aeration rates applied in this condition. More probably, the issue regarding the decrease of the working volume along the assay may have increased the viscosity and the osmolarity of the medium. The saturation of medium components and metabolites produced could induce an inhibition effect on the cells, and the result could have been the low production of IA.

The pH, which was not controlled throughout the fermentation, dropped sharply from 3 to 1.5 from the first day of fermentation, even if no IA was produced. From the 7th to the last day of fermentation, it was observed an oscillation of the pH measurements. That fluctuation may be related to the decrease of medium volume related to evaporation, which resulted in an oscillation of the liquid medium level at the height of the pH electrode, thus, impairing the representation of the pH at that period.

The dissolved oxygen values decreased drastically in the beginning of the fermentation, which corresponded to the period of a sharp drop of pH as well. Then, the dissolved oxygen (DO), which was not controlled, increased from the first to the second day, and it decreased again by the end of the fermentation, but at higher levels than the initial drop on the first day. The period when the dissolved oxygen was at 10% (from the 6th to the 8th day), IA production initiated a slower production rate, despite the presence of almost 50% of the substrate. That result of IA production and substrate concentration did not corroborate the values observed for IA production in agitated flasks (Figure 7.11). The decrease of dissolved oxygen at that period is probably due to the increased viscosity and osmolarity of the medium, as mentioned previously, and the probable increase of cell concentration, which would result in a fast uptake of oxygen. The oscillation observed for the DO values from the 8th day probably concerned the presence of mycelia at the end of the sensor, which was not possible to be avoided.

The TRS concentrations detected on the last days of fermentation (16 g/L TRS) were intrigant, since the results showed that IA was consumed rather than the remaining substrate

from the 11th to the 14th day of the assay. To better understand that effect, Figure 7.13 presents the fermentation responses at their actual concentration value during the fermentation, without the correction of the volume lost by evaporation. Therefore, it is presented the concentration of each carbohydrate of the fermentation – sucrose, glucose and fructose – quantified separately. The glucose and fructose detected in the medium at the beginning of the fermentation concerns the partial hydrolysis of the sucrose in the medium due to the sterilization method applied, as mentioned in the previous section. The concentration of the remaining fructose presented on Figure 7.13 by a line with the triangle marker was calculated by subtraction of the fructose concentration from the glucose concentrations, as one molecule of sucrose consist in one molecule of glucose and one molecule of fructose.

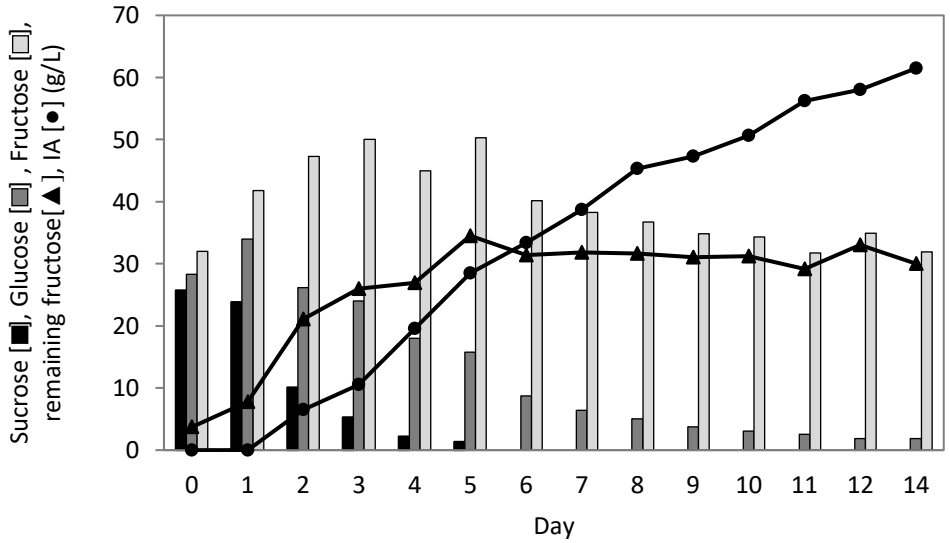

Figure 7.13 Absolute concentrations of the substrates and IA in the fermentative process by *A. terreus* NRRL 1960 in sucrose medium performed in 500 mL capacity bioreactor with 400 mL initial working volume. The bars represent the carbohydrates quantified in the medium at each sample (sucrose, glucose and fructose).

The time zero presented on the Figure 7.13 indicates the concentrations of carbohydrate before inoculation regarding the thermal hydrolysis of sucrose during the medium sterilization, as mentioned previously. The concentrations of the two monomers of sucrose in the beginning of the assay was not equivalent. The detection of almost 4 g/L of fructose more than glucose could indicate that part of the sucrose purchased contained also fructose or it may indicate that part of the glucose was converted to another component.

The sterilization method for this assay required a higher pressure, thus, also higher temperature than the other assays presented (sterilization at 0.5 kgf/cm^2 manometric

pressure for agitated flasks and 1 kgf/cm^2 for the bioreactor) due to the recommendations from the equipment's manual. The use of 121°C instead of 111°C at an acid environment (pH 3) may have transformed the produced glucose in hydroxymethilfurfural (Wierckx et al. 2011; Gomes et al. 2015). That component is known to be toxic to microorganisms at different concentration and depending on the microorganism (Wierckx et al. 2011), and it could have impaired *A. terreus* IA production at some level.

The presence of glucose and fructose by the first day of fermentation may have promoted different consumption rate of each carbohydrate. The curve of remaining fructose shows an increasing concentration of that carbohydrate until the 5th day, throughout the assay and an accumulation of 35-40 g/L from the 6th to the 14th day. The decrease of glucose continues until the end of the assay. From the 11th to the 14th day, most of the carbohydrate available in the medium was fructose, which is when tit was verified a consumption of IA (Figure 7.9).

Besides hydrolysis of the sucrose to fructose and glucose in the beginning of the fermentation, the differences of each carbohydrate concentration throughout the assay may be related to the biochemical reactions for sucrose consumption. The filamentous fungus enzymatically hydrolyzes sucrose to glucose and fructose by the secretion of invertase in the medium (Giraldo et al. 2014), and each carbohydrate may be consumed at different rates by their respective cell membrane transporters (Sloothaak et al. 2015). In this study it was observed (Figure 7.13) that glucose was preferably consumed by the fungus, and fructose accumulated in the medium, with a concentration that varied from 40 g/L at the 7th day to 32 g/L at the 14th day. That accumulation of fructose, added to the change of the medium rheological aspects and ions concentrations could have produced a saturation effect on the cells hexokinase enzymes, causing a preferable consumption of IA rather than the remaining fructose.

Eimhjellen and Larsen (1955) showed that the yield of IA production is much lower in medium with pure fructose compared with sucrose or glucose at initial concentration of 50 g/L of each carbohydrate separately (respectively 0.26, 0.57 and 0.52 g IA/g substrate). To the best of the author's knowledge, no study has been done regarding the fungus highest preference for IA rather than fructose in medium containing both carbon sources at a high concentration of the former.

It is important to note that the increase in viscosity and the osmolarity of the fermentation probably led to a lower oxygen availability. The consumption of substrates when IA was not produced could have been directed to cell maintenance in that environment.

The operating conditions and other factors, such as medium composition, affects the viscosity of the fermentation by filamentous fungi, and those conditions are usually considered to determine biomass concentration and the morphology of mycelia (Papagianni 2004). Regarding filamentous fungi submerged fermentation, the cells morphology may be presented as pellets, as freely dispersed mycelia, as distinct pellets of aggregated biomass or both. Each characteristic has their advantages and disadvantages (Krull et al. 2010).

The microscopic morphology of IA-producing *A. terreus* NRRL 1960 are presented on Figure 7.14 for the 1st and 9th day of fermentation. The submerged fermentation resulted in the formation of both free mycelia and pellets. The former may be observed on Figure 7.14a, b and d, while an example of pellet is presented on Figure 7.14c, which shows the edge of a pellet.

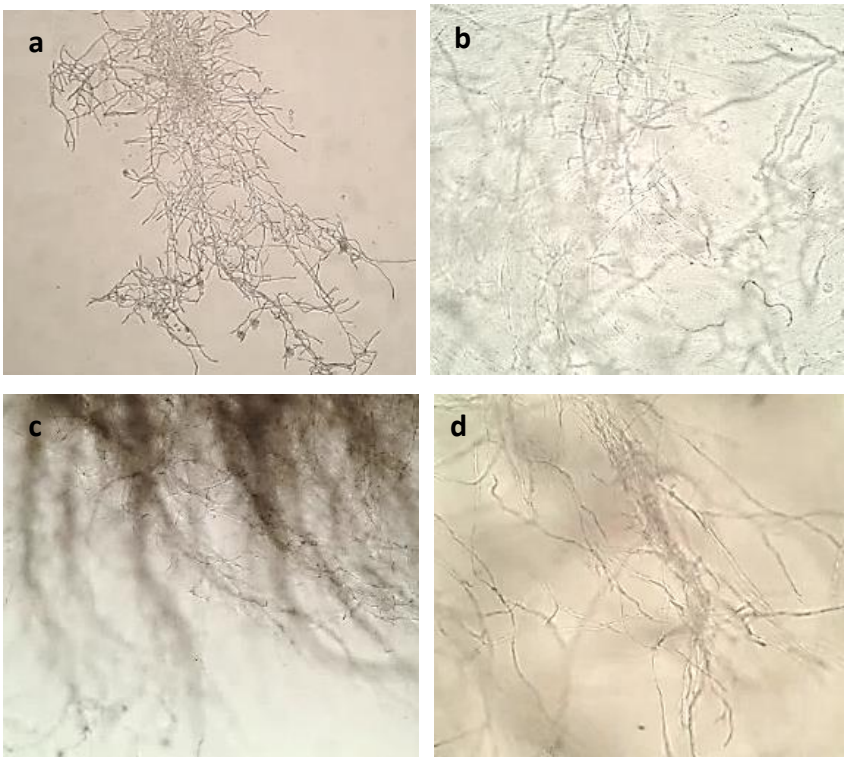

Figure 7.14 Microscopic morphology of *A. terreus* NRRL 1960 cultivated in laboratory scale bioreactor in sucrose medium. Day 1 of assay, 100 x magnification (a); day 1 of assay, 400 x magnification (b); day 9 of assay, 100 x magnification (c); day 1 of assay, 400 x magnification (d).

The formation of pellets or free mycelia is the result of many different factors of the fermentation. The type and the concentration of the inoculum, medium composition, agitation, temperature and pH are some of those factors. However, the bioprocesses which produces pellets encounters the issue of reproducibility of pellets formation, at uniform size and density, is often a bottleneck of productivity in industrial processes (Krull et al. 2010).

It has been suggested that low inoculum concentration, low temperature and nitrogen-limited medium promotes the formation of pellets (Žnidaršič and Pavko 2001). Due to the numerous operational conditions which affects pellet or mycelia formation, it is not possible to speculate which of those conditions could have induced the morphology observed in this study.

The change of the cultivation equipment for the fermentation usually does not promote similar results as the conditions of aeration, agitation, geometry and many other characteristics are discrepant from agitate flasks to bioreactors. Despite many repetitions, the issues concerning the fermentation in the laboratory-scale bioreactor could not be reduced for technical difficulties. Nevertheless, the production of IA in bioreactor should be more promising if the fermentation conditions could have been better controlled, which was not possible in this study.

This study also performed the fermentation at different agitation and aeration conditions: 0.5 vvm and 600 rpm, 1.5 vvm and 300 rpm, and 0.5 vvm and 600 rpm. Nevertheless, despite numerous repetitions considering different strategies to overcome the issues, it was not possible to obtain reliable results which could represent the fermentation for those conditions.

One of the issues observed when 1.5 vvm and/or 600 rpm were applied was that the microorganism grew on the surface of the medium. The fast velocity combined with either aeration flow brought most of the spores to the surface in the beginning of the fermentation and the filamentous cells grew on the walls of the bioreactor, over the liquid phase. Because most of the cells were not on the liquid bulk, the fermentation was interrupted. The strategy to overcome that issue was to start the agitation after one hour after incubation and use antifoam to decrease the formation of bubbles that carry the spores to the surface of the fermentation. However, a better strategy should be considered, such as the initialization of the fermentation at a lower aeration and/or speed rate of the impeller. Another possibility

could be the inoculation of non-proliferating mycelia, to decrease the issues regarding the growth of spores.

The most severe issue was the liquid loss by evaporation. That effect, which also occurred on the mildest condition of aeration and agitation (0.5 vvm and 300 rpm), was even more accentuated for the conditions of 1.5 vvm and 600 rpm. An alternative was to feed the system with sterile water to decrease the problems of evaporation during the fermentation. However, that approach was not enough because of the long period between the feedings (that could not be changed because of technical matters). Therefore, there was an accumulation of cells in some parts of the bioreactor, which prevented the viability of the fermentation at those conditions. The decrease of volume during the fermentation caused by evaporation can also increase the broth viscosity, especially considering the high initial concentration of substrate (114 g/L). Therefore, it is recommended to perform the fermentations for IA production by *A. terreus* at a mild condition of agitation and aeration, with a good control of the evaporation loss. In this study, further experiments were done in agitated flasks for a matter of technical reasons.

7.6 IA production in different carbon and nitrogen sources in agitated flask

The aim of this experiment was to evaluate the combination of two carbon and two nitrogen sources to produce IA with low cost feedstocks as an alternative to the pure sources. The previous carbon sources, which were either glucose of sucrose, were replaced by granulated sugar or VHP, and the use of the nitrogen source used in all previous experiments, NH_4NO_3, was compared with urea from unpurified origin. The four combinations of different carbon and nitrogen sources are presented on Figure 7.15.

The quantification of substrate in the assays with commercial urea used as nitrogen source showed that the values of fructose concentration increased from the 3th day of fermentation, then decreased from the 8th to the 10th day of fermentation (data not shown). Because that is a highly improbable result, it was further investigated it other metabolite could have possibly been produced and interfered with the quantification of fructose. The investigation showed that malic acid (MA) had the same retention time as fructose for the conditions applied for the quantification method, and they could not be identified separately.

To preserve the reliability of the data, it was chosen to present only IA concentration and pH values.

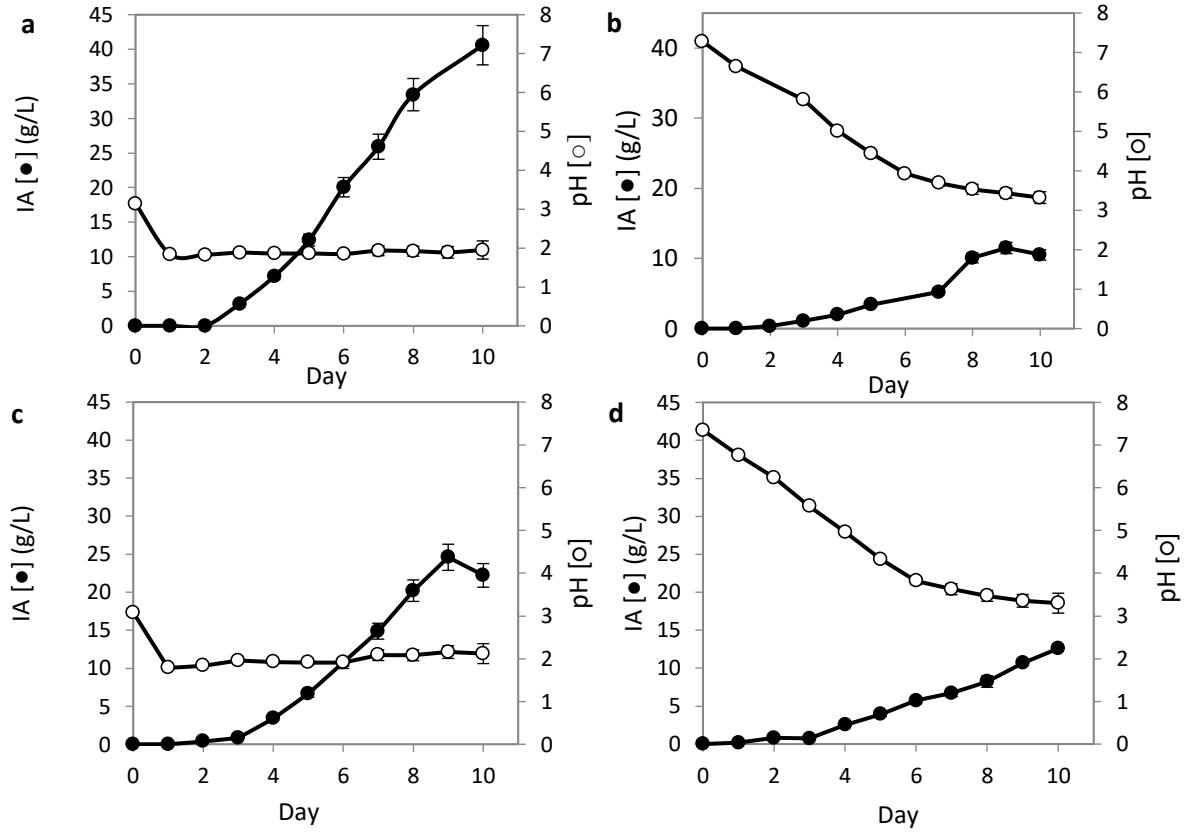

Figure 7.15 Comparison of A. terreus NRRL 1960 fermentation in different carbon and nitrogen sources performed in agitated flask: granulated sugar and NH4NO3 (a), granulated sugar and commercial urea (b), VHP and NH4NO3 (c) and VHP and commercial urea (d). The seeding of each assay was done with the same inoculum. The fermentation was performed at 33°C with 150 rpm agitation.

The combination of granulated sugar and NH_4NO_3 resulted in the highest IA production of 41 g/L IA (Figure 7.15a) among all the combinations tested. That value is almost the same concentrations obtained with pure sucrose in agitated flasks (Figure 7.11). The fermentation with granulated sugar and commercial urea (Figure 7.15b) produced 70% less IA compared to the assay with the same carbon source and NH_4NO_3. That strongly suggest that the nitrogen source is not appropriate for IA production. The medium with the combinations of VHP and NH_4NO_3 (Figure 7.15c) resulted in the second highest IA concentration among the four conditions evaluated, with 25 g/L IA produced by the 9th day. The assay with VHP and urea medium resulted in equivalent IA concentration to that obtained with the same nitrogen source, i.e., 12 g/L (Figure 7.15d).

The possible inconvenience in using commercial feedstocks without previous treatment is the components that may limit IA production (Hiller et al. 2014). Granulated sugar and VHP differs in impurities content, as the first is a refined form of the second. Considering the results with NH_4NO_3, the difference in IA production for the two carbon sources are consistent, since the assay with VHP (Figure 7.15c) produced almost 40% less IA than the assay with granulated sugar (Figure 7.15a).

Regarding the pH profiles, Figure 7.15a and c presented a drop from 3 to 2 at the first day of fermentation, which agrees with the typical pH profile for IA production (section 2.11). It was used the same nitrogen source for both conditions. On the other hand, it was observed a very different profile when commercial urea was used. Figure 7.15b and d indicate that the medium with urea presented a neutral pH begore the inoculation, and they both gradually decreased from 4 to almost 3 from the 6^{th} to the last day of the experiment.

For this study, the result of low IA concentration in urea media could be related to the initial pH. Regardless of the adjustment of the medium to pH 3 before sterilization, the acid environment and the heat during autoclaving resulted in the dissociation of urea in ammonium and carbon dioxide (Brooks et al. 2007). That justifies the detection of high initial pH in the medium after autoclaving.

As mentioned previously, the assays with urea possibly produced malic acid, which was probably due to the pH close to neutral in the beginning of the fermentation. The study of Larsen and Eimhjellen (1955) corroborates that observation, as the authors showed that, in fermentation medium buffered with CaO_3 at pH 6.0, IA was not produced, whereas malic acid (5.2 g/L), succinic acid (2.4 g/L) and fumaric acid (0.4 g/L) were quantified in the medium after 6 days. Although, the highest conversion of the substrate's carbon was to produce dry cells and CO_2 (respectively 40 and 35%). In this study, the medium was not buffered, thus, the pH dropped throughout the fermentation period and the synthesis of IA initiated when the medium pH was between 5 and 6 (Figure 7.15b and d).

It is possible that the use of commercial urea in bioreactor with online adjustment of the pH could enable the use of that carbon source for IA production, which is lower cost alternative than NH_4NO_3.

The pH profile observed in this study for IA production (Figure 7.15b and d) contradicts the hypothesis presented by Larsen and Eimhjellen (1955) and frequently mentioned in

scientific papers (Hevekerl et al. 2014b; Krull et al. 2017). Larsen and Eimhjellen (1955) had concluded from their result that the synthesis of essential enzymes for IA production only occur in an acid environment, which contradicted the study by Lockwood and Reeves, in 1945 (Larsen and Eimhjellen 1955), who described that IA was produced over a wide pH range. Hevekerl et al. (2014) also showed that IA production occurs at 6 different initial pH ranging from 1.9 to 4.9 without adjustment during the cultivation, and that IA production was not different at pH equal or higher than 3.1.

Nelson et al. (1952) and Pfeifer et al. (1952) also reported a low IA yield in fermentation containing urea as nitrogen source, although the authors did not present those results neither present the pH of the fermentation though out the bioprocess. To the best of the author's knowledge, those were the only studies that presented IA production in medium with urea.

Although IA production in granulated sugar was more efficient than in VHP medium, it was chosen to apply the latter to the further studies of IA production for the novelty of the feedstock. Moreover, VHP is produced at sugarcane mills, while granulated sugar is a refined form of VHP, produced by the food industry, as mentioned in section 3.2). The use of the former would be more appropriate for the application of a biorefinery concept than the latter, as the use of VHP as feedstock would represent less production costs.

The selection of VHP as feedstock also considered the analysis of the chemical elements of VHP (Table 7.4). The results of VHP content shows that the elements which compose the fermentation medium of this study (Ca, Mg, K, Fe, Zn, Cu, S, P) are not presented in excess. Moreover, it was not possible to detect Mn within the lowest concentration limit of the method used for quantifying the elements. Karaffa et al. (2015) that the presence of higher than 0.5 µg/L of Mn decreases the IA yield. Concerning nitrogen source, NH_4NO_3 was selected because IA production was more efficient with that nitrogen source.

Table 7.4 Content of some elements present in VHP sugar. The analysis was done for the elements which are also present on the standard medium of IA production in this study.

Element in solid VHP	Concentration (mg/Kg of VHP)
Ca	34
Co	< 0.5[a]
Cu	< 0.5 [a]
Fe	0.83
K	52
Mg	14
Mn	< 0.5[a]
P	2.0
S	27
Zn	0.23

[a] The values were under the detection limit of the method

7.7 Strict oxygen requirement for IA production in VHP medium by *A. terreus* NRRL 1960 in agitated flask

The aim of the experiment was to verify the effect of IA production in VHP medium with interrupted aeration after several days, followed by the interruption of agitation, thus, aeration, in agitated flasks. Figure 7.16 presents fermentation in VHP medium with a brief interruption of oxygen supply for 30 minutes after 3 days of fermentation compared with the profile of the fermentative process performed with continuous agitation.

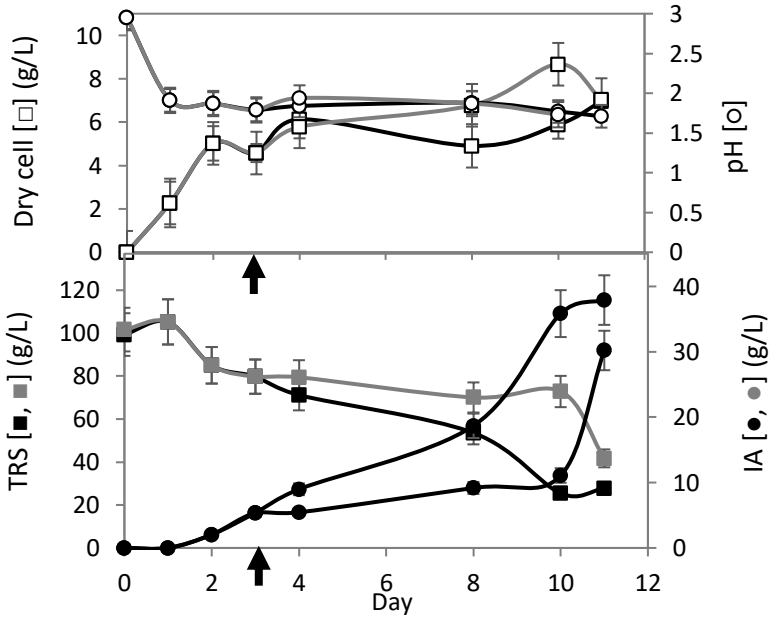

Figure 7.16 *A. terreus* NRRL 1960 fermentation with VHP as feedstock with agitated flask. Fermentation without agitation interruption (1) (*black line*) and 30 minutes agitation/aeration interruption after 72 hours of fermentation (*gray line*). The black arrow indicates the interruption instance of agitation.

The drastic decrease of IA production when agitation/aeration is interrupted compared with the continuous fermentation assay corroborates the effect presented by other studies (section 2.10). IA production was reestablished at very slow rate by the 10th day of aeration interruption fermentation with a IA production peak on the 11th day, although at a concentration slightly lower than the one observed with the continuous fermentation.

It is also observed that the interruption of oxygen supply not only decreased the rate of IA production, but also decreased significantly the consumption of substrate, since sugar consumption was at slower rate compared with the continuous fermentation. Substrate was consumed at a slow rate, thus, it is possible to suggest that the lack of oxygen did not promote the synthesis of other metabolites. This indicates that an inhibition effect occurs inside the cell, that can be reversed after continuous aeration, as observed by the drastic increase of IA production observed from the 10th to the 11th day of fermentation.

Different studies described the direct relation between aeration and IA production, and the requirement for dissolved oxygen at all moments. Pfeifer et al. (1952) and Nelson et al. (1952) were the first to report the strict requirement of continuous aeration for *A. terreus* to reach high IA yields. Nelson et al. (1952) described that a 20 minute interruption in the air flow after 54 hours of fermentation was enough to drastically decrease IA production rates (the values were not detailed). Pfeifer (1952) described that it was only possible to reverse the damage of no IA production (related to 15 to 60 minutes interruptions in air flow) if extra nutrients were added to the medium. The authors reported that that, despite the occurrence of further IA production, the final IA concentration was lower compared to the assay which was continuously aerated (Pfeifer et al. 1952; Nelson et al. 1952).

The aeration requirement for maintaining the cell's capacity of producing IA is so important that Larsen and Eimhjellen (1955) conducted the separation of IA-producing *A. terreus* cells (non-proliferating mycelia) from the fermentation broth with constant aeration. The authors described that if the aeration was not maintained throughout the separation step, the endogenous IA was not expelled to the extracellular medium (acidified tap water).

Riscaldati et al. (2000) showed that during the cell growth phase there is a higher demand for oxygen, as well as for the sources of phosphorous and nitrogen. When cell concentration reached a stationary, the dissolved oxygen (DO) slowly increased from under 20% DO to almost 40 or 80% DO, depending on the assay's initial condition (initial pH or

aeration rate). Kuenz et al. (2012) also described the occurrence of a drastic decrease of dissolved oxygen to 20% DO in the beginning of the fermentation (the first day of a 10 days' fermentation). The continuous need for oxygen supply even when cell growth is at low rates indicates that oxygen requirement is higher for IA production than cell maintenance. Moreover, it has been mentioned (section 2.10) that capacity of IA production by *A. terreus* after the pause in oxygen supply is also related to the duration of the interruption period.

The reason for the significantly lower IA production when oxygen supply is completely interrupted has not yet been clarified. Based on the evaluation of different works, this study states the following hypothesis: the enzymatic system that enables the synthesis of IA is affected by the lack of oxygen, which may be related to an inhibitory effect of the transformation of cis-aconitate to trans-aconitate inside the mitochondria or the cytosol.

A requirement of a readily transportation system of *cis*-aconitate from the mitochondria to the cytosol has been evidenced (Huang et al. 2014). At sufficient oxygen concentration, *A. terreus* promptly transports *cis*-aconitate by Mtt transporter, from the mitochondria to the cytosol, where is further converted to itaconate by CAD enzyme (Huang et al. 2014). In the occasion of lack of dissolved oxygen, the energy applied for transporting H^+ and IA could be impaired. The Mtt transporter would have a lower activity in the absence of oxygen, and *cis*-aconitate would accumulate inside the mitochondria.

In the occasional malfunctioning of H^+ transportation, the pH inside the cell could decrease and promote the formation of *cis*-aconitic to *trans*-aconitate, since the former is unstable in environments with a pH under 7 (Ambler and Roberts 1948) and it is spontaneously converted to the latter, the thermodynamically more stable form of the substance (Steiger et al. 2016). Because t*rans*-aconitate has been described as an inhibitor of at least two enzymes form the TCA cycle enzymes – aconitase (Laube et al. 1994) and fumarase (Rebholz and Northrop 1994), the process could prevent IA production. The longer the period of the absence of oxygen, the greater would be the conversion of *cis* to *trans*-aconitate, thus, the longer would be the time required to reestablish the capacity of producing IA by *A. terreus*.

The hypothesis of inhibition effect inside the mitochondria by the lack of oxygen may be supported by the observations of Gyamerah (1995) studies with *A. terreus*. The author showed that the inhibition of mitochondria membrane transporters, without interrupting oxygen supply, results in more than 90% less IA than the standard strain, while the inhibition

of cell membrane transporters causes less than 9% inhibition effect of IA production. The inhibition of mitochondrial membrane, which also transport H^+, could have caused the effect mentioned previously, and impaired IA production. That assay intended to simulate the effect of the interruption of aeration for *A. terreus* fermentation.

The hypothesis of inhibition effect proposed in this study could also explain the same issue of strict oxygen requirement observed for citric acid production by *A. niger*. Kubicek et al. (1980) described that stopping aeration for up to 20 minutes resulted in complete loss of ability to produce CA, although the *A. niger* viability was not reduced. When the fermentation was reestablished, growth rate increased, and they concluded that aeration must be in excess. The more recent literature cites the study of Kubicek et al. (1980) as the only presenting a detailed information about strict oxygen requirement of CA production by *A. niger* (Papagianni 2007; Max et al. 2010), but, to the best of the author's knowledge, no further discussion about that effect has been presented.

Recently, Steiger et al. (2016) showed that when genes for CAD and MttA expression are introduced in *A. niger*, itaconic and aconitic acid (*cis* and *trans*) are observed in the medium, which does not occur in wild strains (Li et al. 2012; Steiger et al. 2016). That indicates that *A. niger* produces itaconic acid and the lack of those genes in wild strains of *A. niger* prevents the natural transportation of IA to the extracellular environment. Therefore, in the absence of oxygen, the production of CA by *A. niger* could be impaired by the conversion of cis-aconitate to trans-aconitate, and the TCA cycle enzymes could be inhibited.

7.8 IA production by *A. terreus* NRRL 1960 in media with different VHP and NH_4NO_3 concentration in agitated flask

The acknowledge of the IA production by *A. terreus* NRRL 1960 in VHP medium in different concentrations of the carbon and nitrogen sources is important to stablish proper nutritional conditions for high IA yield at low cost. The results of IA production in media with different initial concentrations of VHP and NH_4NO_3 in agitated flasks are presented on Figures 7.17 to 7.21. For simplification, the conditions of high, medium and low concentrations of VHP, and high, medium and low concentrations of NH_4NO_3 are referred as, respectively high C, medium C and low C, and high N, medium N and low N.

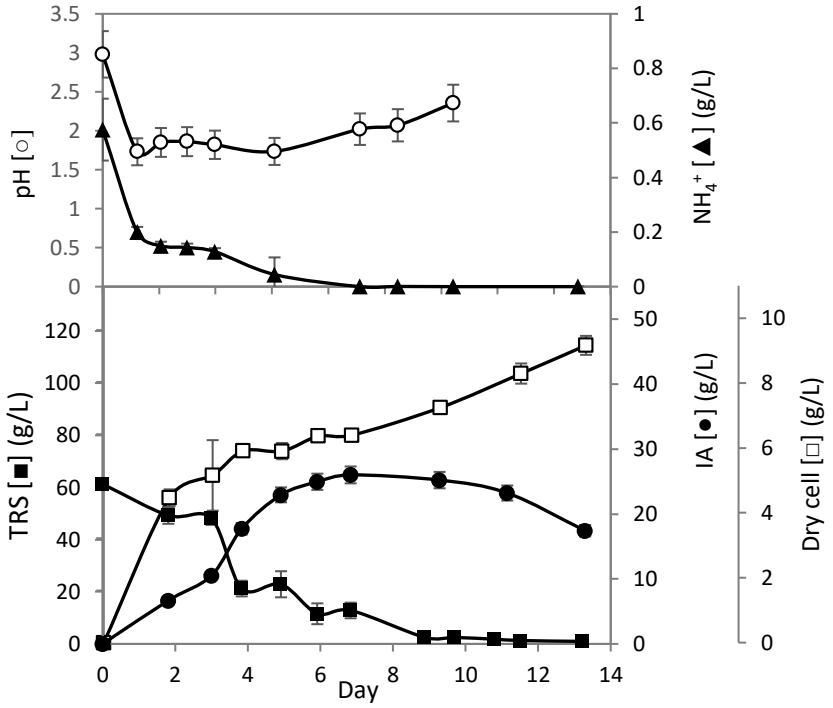

Figure 7.17 Experiment regarding the evaluation of IA production in agitated flask in media with different initial concentration of VHP and NH_4NO_3: 76 g/L of VHP and 2 g/L NH_4NO_3 (low C and low N). The medium pH was adjusted to 3.1 and it was not controlled throughout the process. The dry mass of the inoculum of spores (10^6 spore/mL) was considered as 0.001 g/L. The fermentation of the agitated flasks was performed at 33°C with 150 rpm agitation.

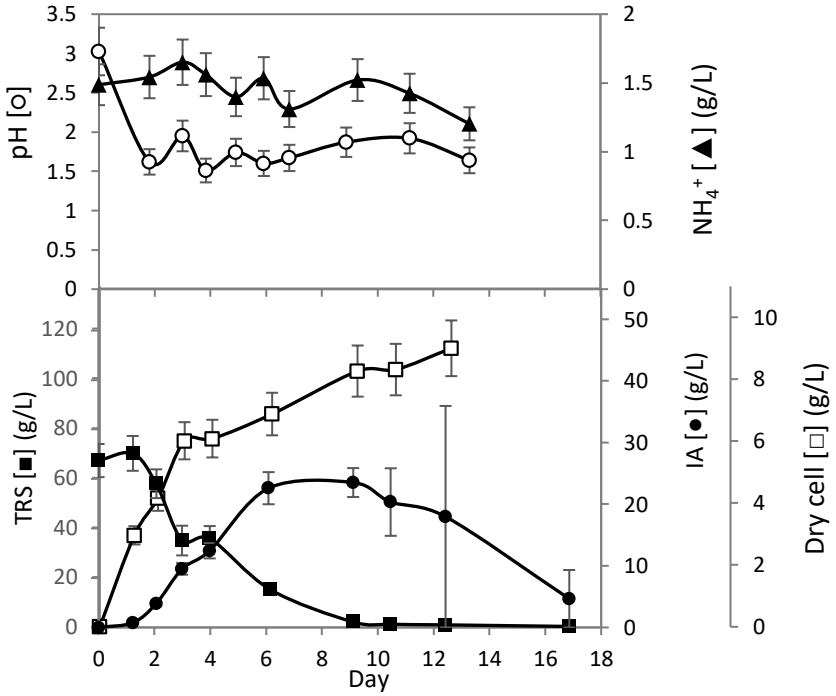

Figure 7.18 Experiment regarding the evaluation of IA production in agitated flask in media with different initial concentration of VHP and NH_4NO_3: 76 g/L of VHP and 6 g/L NH_4NO_3 (low C and high N). The medium pH was adjusted to 3.1 and it was not controlled throughout the process. The dry mass of the inoculum of spores (10^6 spore/mL) was considered as 0.001 g/L. The fermentation of the agitated flasks was performed at 33°C with 150 rpm agitation.

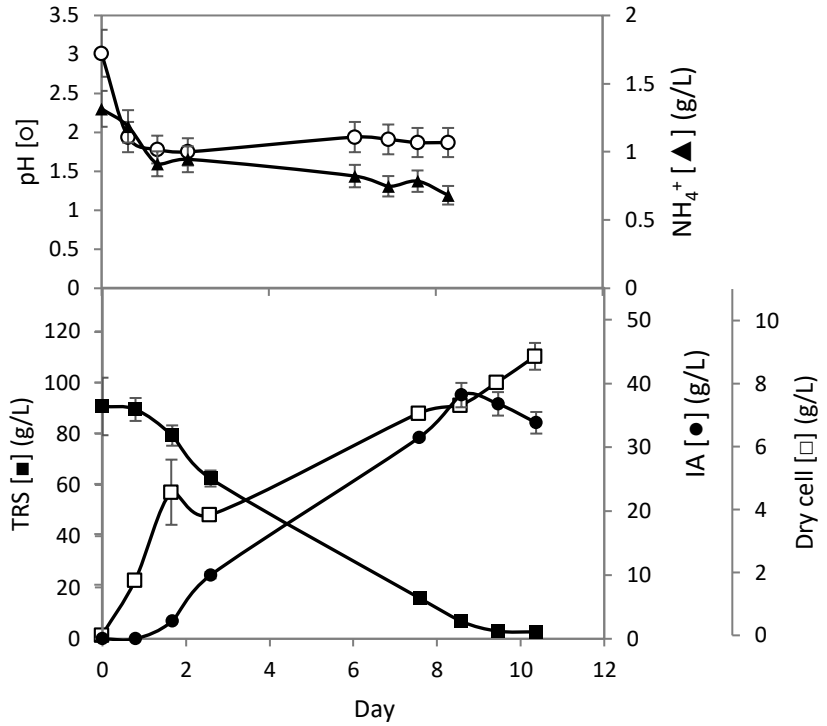

Figure 7.19 Experiment regarding the evaluation of IA production in agitated flask in media with different initial concentration of VHP and NH_4NO_3: 95 g/L of VHP and 4 g/L NH_4NO_3 (medium C and medium N). The medium pH was adjusted to 3.1 and it was not controlled throughout the process. The dry mass of the inoculum of spores (10^6 spore/mL) was considered as 0.001 g/L. The fermentation of the agitated flasks was performed at 33°C with 150 rpm agitation.

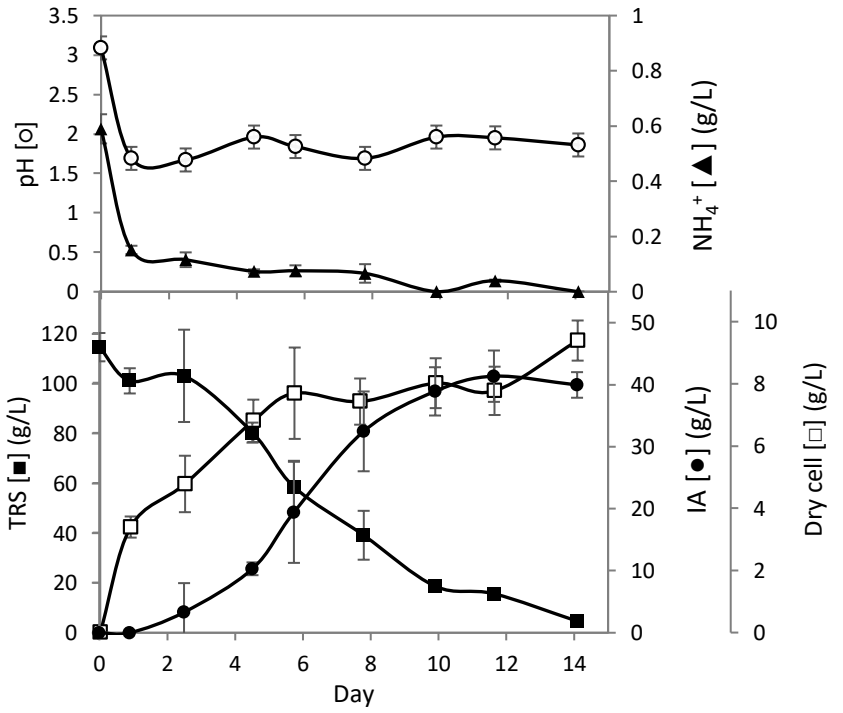

Figure 7.20 Experiment regarding the evaluation of IA production in agitated flask in media with different initial concentration of VHP and NH_4NO_3: 114 g/L of VHP and 2 g/L NH_4NO_3 (high C and low N). The medium pH was adjusted to 3.1 and it was not controlled throughout the process. The dry mass of the inoculum of spores (10^6 spore/mL) was considered as 0.001 g/L. The fermentation of the agitated flasks was performed at 33°C with 150 rpm agitation.

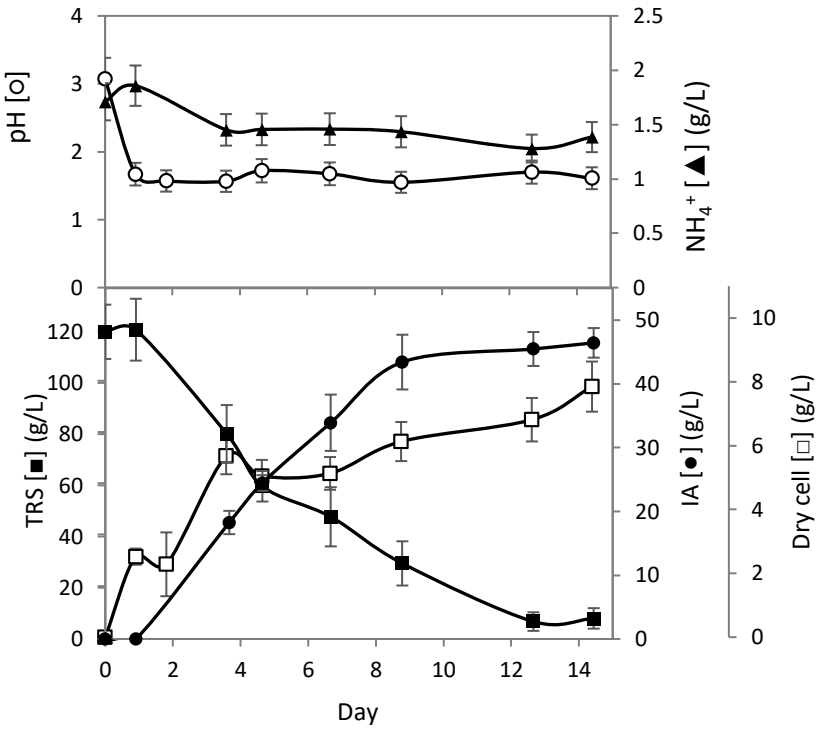

Figure 7.21 Experiment regarding the evaluation of IA production in agitated flask in media with different initial concentration of VHP and NH_4NO_3: 114 g/L of VHP and 6 g/L NH_4NO_3 (high C and high N). The medium pH was adjusted to 3.1 and it was not controlled throughout the process. The dry mass of the inoculum of spores (10^6 spore/mL) was considered as 0.001 g/L. The fermentation of the agitated flasks was performed at 33°C with 150 rpm agitation.

The highest IA concentrations were achieved in medium with high C (Figure 7.20 and 7.21), despite the different initial concentrations of N. The difference between the assays with high C is that high N resulted in a stationary IA production phase at the 9th day (Figure 7.21), while the assay with low N had highest IA concentration at the end of the assay (12th day). This indicates that high N and C induced a faster formation of IA and less cell growth compared with the low N and high C condition (respectively Figures 7.21 and 7.20). Those two effects are probably related, and it may indicate that cell growth was inhibited by the formation of IA in the medium.

The low initial concentration of VHP resulted in the lowest concentrations of IA, and the carbohydrates available were almost completely consumed before the end of the fermentation (Figures 7.17 to 7.19). All the conditions presented that ammonium was consumed at a fast rate in the beginning of the fermentation, when it is also observed a high rate of cell production. This sharp change in nitrogen concentration was not observed for high and medium N assays (Figures 7.18, 7.19 and 7.21).

Regarding the cell growth profiles, it was identified at least two distinct growth rates for all the assays. The fast growth rate for the assays were observed the at the first 2-4 days. The less steep cell growth rate observed in all the assays forms an almost stationary growth phase at about 6-8 g/L dry cell for the periods for the periods when IA consumption was not observed. For the assays which IA was consumed when substrate was close to zero (Figures 7.17 to 7.19), it is observed that the production of cells was accelerated but at a slower rate than the first phase observed.

The pH profile is very similar for all assays, which corroborate the previously data obtained in this study. As observed in most of the experiments in this study, pH rapidly decreases in the beginning of the fermentation, and, as no pH adjustment is implemented, pH is naturally kept maintained at 2 (about 1.8 for most quantifications), except for the assay with low C and N (Figure 7.17), which had a pH increase from 1.7 to 2.4 from the 6^{th} to the 12^{th} day of fermentation. A pH rise was also observed when IA was consumed in absence of glucose, reaching up to 5 with xylose as substrate (Kocabas et al. 2014).

The slower cell growth rate for the assays with high N contradicts the hypothesis of Vassilev et al. (1992). The authors presented results with immobilized cells of *A. terreus* TKK 200-5-1 in medium with different glucose and ammonium concentration and reported that the production of the acid was favored when ammonium nitrate was not added to the production medium. Similar effect was demonstrated by Kuenz et al. (2012) in free-cell submerged fermentation. It has also been suggested that IA high concentration production is related to phosphorus limitation, which is another effect that has been shown to be inaccurate (Hevekerl et al. 2014a; Krull et al. 2017).

There is also the possibility that nitrate ions were also consumed as nitrogen source in this study. The review of Tudzynski (2014) reports that, for filamentous fungi, NH_4^+ is a preferable nitrogen source than nitrate, which is only consumed in the absence of the first. This was not observed with *A. terreus* fermentation by Welter (2000) neither by Kuenz et al. (2012), which showed that nitrate uptake occurs since the beginning of the fermentation and that its consumption is similar to the ammonium uptake profile. In both examples, IA was only produced when nitrogen consumption was no longer detected, even without nitrogen limitation. Tudzynski (2014) also listed different effects of nitrogen regulation regarding

secondary metabolites production, which may be associated with nitrogen repression or induction.

The IA production by *A. terreus* related to a slow cell growth rate have been previously mistaken to be related to nutritional exhaustion due to low initial concentration of ammonium or other nitrogen source (Miall 1978). However, Vassilev et al. (1992) studied IA production with immobilized *A. terreus* and reported that the production of IA was favored when ammonium nitrate was not added to the production medium. Similar effect was demonstrated by Kuenz et al. (2012) in free-cell submerged fermentation. Also, this study showed that high IA production was observed when high concentration of ammonium is still in the medium, which corroborates the results by Hevekerl et al. (2014a) and Krull et al. (2017). It has also been suggested that IA high concentration production is related to phosphorus limitation (Miall 1978), which is another effect that has been shown to be inaccurate (Hevekerl et al. 2014a; Krull et al. 2017). What triggers the overproduction of IA by *A. terreus* is still unknown (Krull et al. 2017).

The kinetic profiles presented in this section and the section regarding the fermentation in bioreactor (Figure 7.9) showed IA consumption, which occurred when substrate was no longer present in the medium for the present assay. Very few works have discussed the consumption of IA (Arpai 1958; Jakubowska and Metodiewa 1974; Chen et al. 2016), however, the comprehension of this pathway may be helpful to improve IA synthesis.

Part of the catabolic pathway of IA was described by Chen et al. (2016) to open the possibilities of understanding IA regulation mechanisms, even though the aim of its degradation by the fungus is still unclear. According to the authors (Chen et al. 2016), *A. terreus* LYT10 was not able to grow in IA medium as the only carbon source (from 5-40g/L). However, the same work showed that when the strain was kept for 20 hours in glucose medium before being transferred to the same medium (155 g/L glucose) with additional 40 g/L of IA, the strain consumed IA for the next 36h. It suggests that the microorganism requires an initial production of enzymes before IA can be used as a carbon source. The authors presented three enzymes responsible for IA degradation, but they do not exclude the possibility of other enzymes to be responsible for IA intake (Chen et al. 2016).

The yields, productivity and efficiency of each assay at the day of the highest value of IA are presented on Table 7.5. The yield values for some of the conditions indicate that VHP

with NH_4NO_3 are a combination that enables high concentration of IA, which are comparable with the yields obtained with glucose and pure sucrose in agitated flask (Figure 7.6 and 7.8). Regarding the values of efficiency, the condition of low C and high N presented the highest value. On the other hand, considering the productivity, it may be noticed that the condition with high C and N could be more feasible for considering the application at an industrial process. The proper choice of the condition, i.e., either low C with high N or high C with high N, should consider the prices of IA and the time-cost of the process.

Table 7.5 Yield, productivity and efficiency of each assay of low C, low N; high C, low N; medium C, medium N; low C, high N; high C, high N. High, medium and low values of N: 6, 4 and 2 g/L of NH_4NO_3. The yields and productivities were calculated considering the highest IA concentrations obtained in each assay, which was not necessarily at the last day of the fermentation as IA was consumed at some assays.

C and N concentrations	$Y_{P/S}$ (g IA/g TRS)	$Y_{X/S}$ (g IA/g TRS)	Productivity (g IA/L/h)	Efficiency (%)
Low C, Low N	0.36	0.13	0.15	50.0
Low C, High N	0.53	0.13	0.15	74.6
Medium C, Medium N	0.45	0.09	0.18	63.2
High C, Low N	0.42	0.08	0.15	58.0
High C, High N	0.48	0.06	0.21	66.7

The results of IA production in media with different initial concentrations of VHP and NH_4NO_3 could have been applied to do an experimental design to propose a response surface and the respective equation to describe the behavior of the response with respect to the changes in media components concentrations. This approach may be very useful to estimate a fermentative medium that produces high concentration of the desired product. However, this type of empirical model is not very well effective if the goal is to scale up the production since the variables of the model are not physically explained. In this work, it was applied a mathematical model that combines empirical results with theoretical existing models, as explained in Chapter 4. This type of mathematical model proposes a reaction scheme that intends to macroscopically explain the bioprocess. Moreover, the estimation of kinetic parameters of the proposed reactions, the application of the model in a scaling up stage is more reliable compared to the models which are exclusively empirical. Therefore, the different kinetic profiles obtained for each combination of concentrations of VHP and NH_4^+ were applied to propose a dynamic mathematical model for describing IA production by *A. terreus* NRRL 1960.

7.8 Macroscopicmodeling and parameter estimation of IA production by *A. terreus* NRRL 1960 in agitated flask

A previous study has presented the use of the mathematical model by Luedeking – Piret to describe IA production by *A. tereus* (section 4.4). This study proposes a different approach, which IA production is estimated considering cell growth and IA production separately. Moreover, the consumption of IA by the microorganism during the last days of fermentation is also considered. The proposed macroscopic model describes the synthesis of IA at the expense of sugar and nitrogen consumption, besides the production of cells.

The experimental data presented and discussed on section 7.7 were used in this study to develop the macroscopic mathematical model that describes IA production by *A. terreus* NRRL 1960 in VHP medium and NH_4NO_3, as previously mentioned. Each assay was used as individual experiments, even the ones that represented the same initial concentration. Therefore, a total of 9 assays were used for building the model, with a total of 5 different conditions, presented on Figures 7.17 to 21. It should be mentioned that N represents ammonium ion NH_4^+, however, the medium was prepared with 2, 4 and 6 g/L NH_4NO_3, which should correspond to 0.45, 0.9 and 1.35 g/L NH_4^+ for respectively low, medium and high concentration of N. Considering the method used for quantifying NH_4^+ and the measurement errors for that input, it was chosen to estimate the initial values of NH_4^+, as well, i.e., N at time zero was also a parameter to be estimated.

The following descriptions detail some of the steps which directed to the definition of the macroscopic model. The concentrations of substrate (TRS - S), ammoniacal nitrogen (N), IA (I) and cell (or biomass, X) were applied as variables for building the model structures that describes the fermentation system of IA production by *A. terreus*. As mentioned previously (section 4.5.4), the design of the reaction schemes and the parameters values were achieved through numerous trials and errors, based on the validation of the experimental values.

This study proposes a reaction scheme composed by three distinct reactions for describing the system of IA production defined experimentally and presented on Figure 7.22. The first reaction (7.1) describes cell production (X) with regards substrate (S) and ammonium (N) consumptions. The second one (7.2) describes IA (I) production by substrate consumption,

which does not include cell production. IA consumption (7.3) occurs with the consumption of ammonium and the production of biomass.

$$S + \nu_1 N \xrightarrow{\varphi_1} \nu_2 X \quad (7.1)$$

$$S \xrightarrow{\varphi_2} \nu_3 I \quad (7.2)$$

$$I + \nu_4 N \xrightarrow{\varphi_3} \nu_5 X \quad (7.3)$$

S, X, I, N: Substrate, itaconic acid, dry cell, ammonium concentrations

$\varphi_1, \varphi_2, \varphi_3$: rate of the 1st, 2nd and 3rd reaction, respectively

$\nu_1, \nu_2, \nu_3, \nu_4, \nu_5$: pseudo-stoichiometric coefficient of each component for each reaction.

The two phenomena stated by the first two reactions (7.1 and 7.2) are presented separately, however, it does not indicate that they necessarily occur at different moments. Nevertheless, IA is only produced after cell concentration reaches a certain concentration, demonstrated by the lag phase for IA production observed on the assays a, c, d, e and i.

A drastic consumption of ammonium was observed during the accelerated cell growth in the beginning of the fermentation, which is well verified on the assays with low initial concentration of that nitrogen source (Figure 7.22a to d) and on Figure 7.22e and g. The phase that follows the stage of an accelerated rate of ammonium consumption and cell growth is a null or slow cell growth rate and the continuous production of IA. The reactions 7.1 and 7.2 continue to prevail until there is a limitation of substrate. When the sucrose concentration reaches low values, IA is the consumed carbon source, and cell is produced, together with the consumption of nitrogen (Figure 7.22 a, f and g).

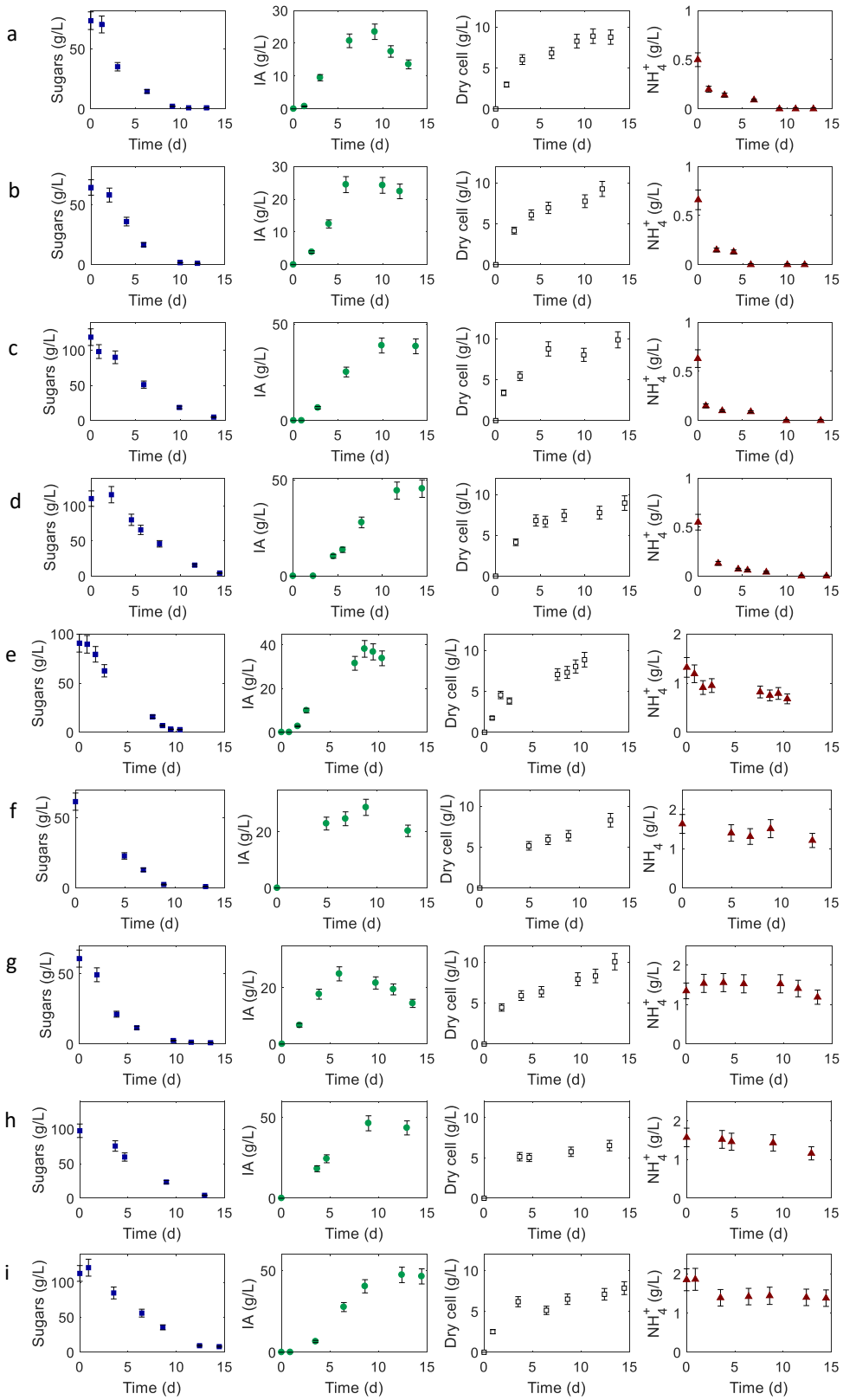

Figure 7.22 Experimental data of IA production in VHP medium by *A. terreus* NRRL 1960. Low C + low N (a and b); high C + low N (c and d); medium C + medium N (e); low C + high N (f and g); high C + high N (h and i). High, medium and low values of C respectively: 114, 95 and 76 g/L VHP. High, medium and low values of N: 6, 4 and 2 g/L of NH4NO3. TRS (■), IA (●), dry cell (□), and NH_4^+ (▲).

The representation of the dynamic process of consumption and production of reactants and products consist of mass balances on the equations 7.5 to 7.8. The consumed reactants are represented with a negative term regarding its reaction rate, whereas the variables that are produced have a positive term. Since each reaction is with respect to the specific reaction rates, cell concentration is considered on the mass balance equations (7.4).

$$\varphi_i = \mu_i * X \tag{7.4}$$

φ_i: each i^{th} reaction rate
μ_i: specific reaction rate

$$\frac{dS}{dt} = -\mu_1 * X - \mu_2 * X \tag{7.5}$$

$$\frac{dX}{dt} = \nu_2 * \mu_1 * X + \nu_5 * \mu_3 * X \tag{7.6}$$

$$\frac{dI}{dt} = \nu_3 * \mu_2 * X - \mu_3 * X \tag{7.7}$$

$$\frac{dN}{dt} = -\nu_1 * \mu_1 * X - \nu_4 * \mu_3 * X \tag{7.8}$$

The kinetic rates could be represented by different mathematical models that have been previously proposed in the literature. In this study, it was chosen to apply the extended Monod Laws to express the specific reaction rates (μ_1, μ_2 and μ_3). Those theoretical models have been effective to describe activation an inhibition effects in previous macroscopic models (Sonnleitner and Käppeli 1986; Richelle et al. 2015). The activation factors described by the Monod law are applied to the reactants, which are necessarily activators of the reaction, or to components that are catalysts of the reaction. The inhibition factors are identified when a component of the medium induces the inhibition of a reaction. Thus, those kinetic expressions are represented by activation (k) or inhibition (k_i) factors of the state variables (S, X, I, N) of the model variables (input and output) and by the maximum specific rate of that reaction (μ_{max}).

$$\mu_1 = \mu_{1\,max} * \frac{S}{k_{S1} + S} * \frac{N}{k_{N1} + N} * \frac{k_{iI1}}{k_{iI1} + I} \quad (7.9)$$

$$\mu_2 = \mu_{2\,max} * \frac{S}{k_{S2} + S} * \frac{X}{k_{X2} + X} \quad (7.10)$$

$$\mu_3 = \mu_{3\,max} * \frac{I}{k_{I3} + I} * \frac{N}{k_{N3} + N} * \frac{k_{iS3}}{k_{iS3} + S} \quad (7.11)$$

The kinetic rate of the first reaction (7.1) contains the activation parameters (k_{S1} and k_{N1}) regarding the reactants (S and N) and an inhibition parameter (k_{iI1}) concerning IA. In this representation of the system, cell production is restricted by the presence of IA in the medium. The second reaction (7.2) is activated by the biomass ($X/(k_{X2} + X)$) produced in the first reaction (7.1).

The third kinetic rate (7.3) states that IA consumption is inhibited by the presence of sugar in the medium ($k_{iS3}/(k_{iS3} + S)$), which decreases throughout the fermentation period. When the concentration of that easily fermentable carbon source is at sufficiently low value, IA is consumed by the microorganism and cells are produced at the expense of ammonium consumption as well.

The estimation of the parameters for the proposed model was done considering the range of initialization presented on Table 7.6, which were the same values used throughout this study. The units of each parameter are presented on Table 7.7. It is important to mention that the range of initialization also included 100 uniformly distributed pseudorandom numbers random values between 0 and 1 (function rand on MatLab® R2016b for student use) over the used range (Table 7.6). That factor results in different initial values each time the parameter estimation calculations are performed.

Table 7.6 Range of initialization of the parameter identification.

Reaction 1		Reaction 2		Reaction 3		Initial values of NH_4^+	
ν_1	0.02 – 2	ν_3	0.1 – 10	ν_4	0.001 – 0.1	N_{01}	0.01 – 1
ν_2	0.1 – 10	$\mu_{2\,max}$	0.05 – 5	ν_5	0.03 – 3	N_{02}	0.01 – 1
$\mu_{1\,max}$	0.8 – 80	k_{S2}	0.05 – 5	$\mu_{3\,max}$	0.1 – 10	N_{03}	0.01 – 1
k_{S1}	0.05 – 5	k_{X2}	0.05 – 5	k_{I3}	0.001 – 0.01	N_{04}	0.01 – 1
k_{iI1}	0.03 – 3			k_{iS3}	0.8 – 80	N_{05}	0.02 – 2
k_{N1}	0.0001 – 0.01			k_{N3}	0.001 – 0.01	N_{06}	0.03 – 3
						N_{07}	0.03 – 3
						N_{08}	0.03 – 3
						N_{09}	0.03 – 3

Table 7.7 Units of the parameters of the proposed model.

Reaction 1		**Reaction 2**		**Reaction 3**		**Initial values of NH_4^+**	
ν_1	g N/ g S	ν_3	g I/g S	ν_4	g N/g I	N_{01}	g N/L
ν_2	g X/ g S	$\mu_{2\,max}$	h^{-1}	ν_5	g X/g I	N_{02}	g N/L
$\mu_{1\,max}$	h^{-1}	k_{S2}	g S/L	$\mu_{3\,max}$	h^{-1}	N_{03}	g N/L
k_{S1}	g S/L	k_{X2}	g X/L	k_{I3}	g I/L	N_{04}	g N/L
k_{iI1}	g I/L			k_{iS3}	g S/L	N_{05}	g N/L
k_{N1}	g N/L			k_{N3}	g N/L	N_{06}	g N/L
						N_{07}	g N/L
						N_{08}	g N/L
						N_{09}	g N/L

The kinetic profiles obtained with the model proposed and its estimated parameters values are presented respectively on Table 7.8 and Figure 7.23. The initial identification of the parameters shows that the model (*curves*) and the experimental data (*points*) are visually well adjusted, and the obtained parameters values represented an appropriate fit of the results obtained experimentally. However, that result is not sufficient to consider the proposed model as a good representation of the phenomenon studied. Further steps were performed to identify the parameters more accurately.

Table 7.8 Parameter values obtained from the parameter identification of the proposed model. Cost function value obtained: 3.85.

Reaction 1		**Reaction 2**		**Reaction 3**		**Initial values of NH_4^+**	
ν_1	0.04	ν_3	0.39	ν_4	0.03	N_{01}	0.27
ν_2	0.71	$\mu_{2\,max}$	1.57	ν_5	0.83	N_{02}	0.27
$\mu_{1\,max}$	14.12	k_{S2}	0.99	$\mu_{3\,max}$	0.05	N_{03}	0.29
k_{S1}	1.59	k_{X2}	0.54	k_{I3}	5.51×10^{-5}	N_{04}	0.27
k_{iI1}	0.05			k_{iS3}	15.96	N_{05}	1.02
k_{N1}	1.21×10^{-4}			k_{N3}	8.64×10^{-5}	N_{06}	1.01
						N_{07}	1.70
						N_{08}	1.63
						N_{09}	1.60

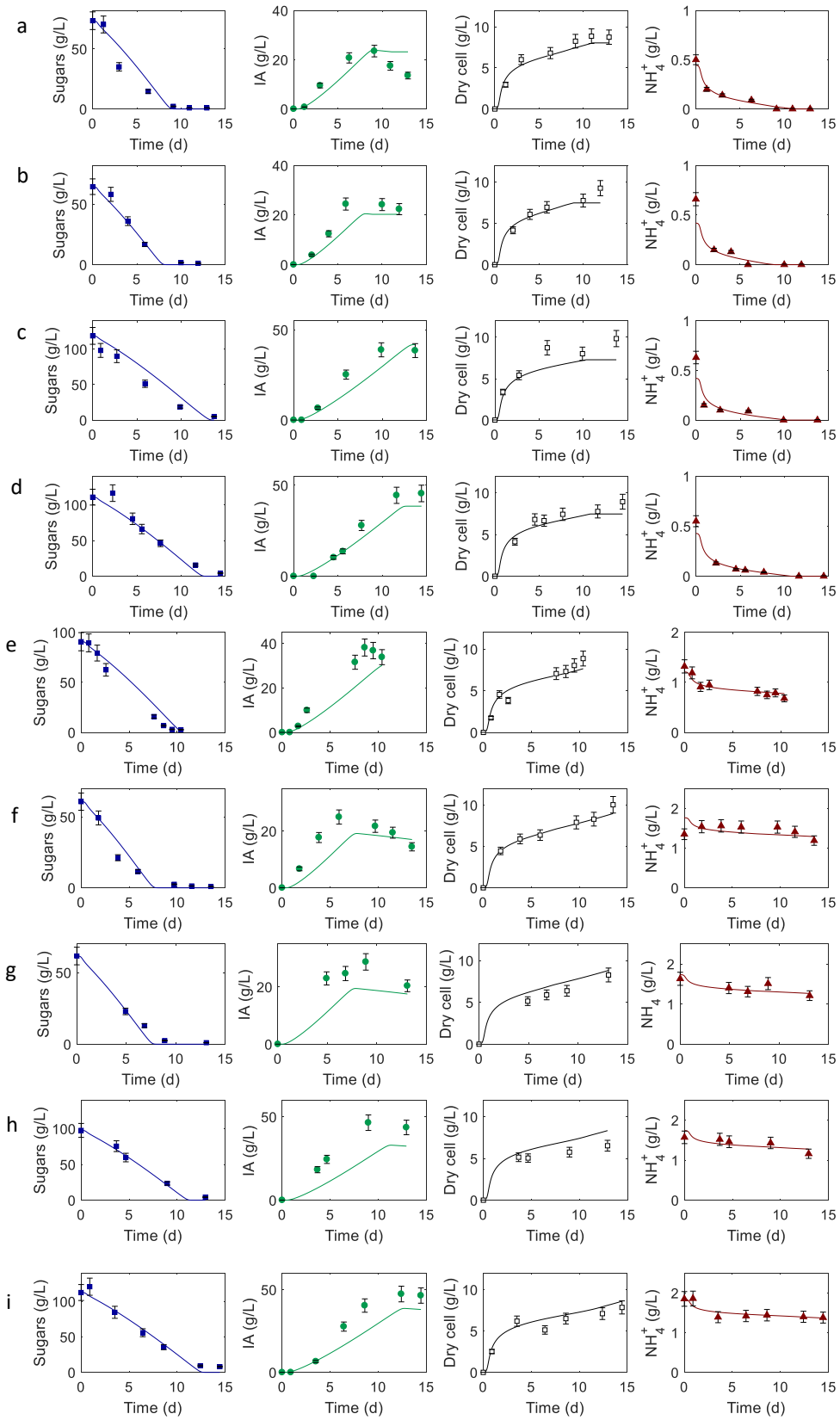

Figure 7.23 Dynamic model (lines) and experimental data (points) of IA production by *A. terreus* NRRL 1960 in VHP medium. Low C + low N (a and b); high C + low N (c and d); medium C + medium N (e); low C + high N (f and g); high C + high N (h and i). High, medium and low values of C respectively: 114, 95 and 76 g/L VHP. High, medium and low values of N: 6, 4 and 2 g/L of NH4NO3. TRS (■), IA (●), dry cell (□), and NH_4^+ (▲).

It is observed that some parameters from the first and third reactions, which concern the activation factors ($k_{N1}\ and\ k_{N3}$) and the activation term for IA in the third reaction (k_{iI3}) are negligible, considering the other values of the kinetic rate reactions (Table 7.2). Those values were negligible in relation to Despite the negligibility, those parameters were not withdrawn from the model because the pseudo-stoichiometric values (they represent that N and I are constituents of the model) for mass balance of NH_4^+ (ν_1 and ν_4) and IA (k_{I3}) were not negligible. In that case, arbitrary sufficiently low value were fixed as each parameter (0.0001), and they were no longer stated as variables to be identified (Richelle et al. 2015). Moreover, the consideration of ammonium as an important reactant for cell production is relevant, as proteins and enzymes synthesis require nitrogen source (Marzluf 1981).

The estimation of the parameter values considering the parameters mentioned as fixed values resulted in similar estimation, as presented on Table 7.9. The kinetic profiles with those new considerations are illustrated on Figure 7.24. In this step, a sensitivity analysis of the parameters was done regarding the values obtained considering the fixed values.

Table 7.9 Parameter estimation for dynamic model of IA production by *A. terreus* NRRL 1960 in different initial concentrations of VHP and NH_4NO_3. Values obtained from all the assays (9 assays, 5 different initial conditions) and fixed values of k_{N1}, k_{I3} and k_{N3} (0.0001). Cost function value obtained: 3.77.

Reaction 1		**Reaction 2**		**Reaction 3**		**Initial values of NH_4^+**	
ν_1	0.04 ± 0.10	ν_3	0.40 ± 0.02	ν_4	0.03 ± 0.20	$N_{0\,1}$	0.42 ± 0.01
ν_2	0.77 ± 0.10	$\mu_{2\,max}$	1.67 ± 0.03	ν_5	0.77 ± 0.23	$N_{0\,2}$	0.43 ± 0.01
$\mu_{1\,max}$	14.83± 0.11	k_{S2}	1.08 ± 0.30	$\mu_{3\,max}$	0.06 ± 0.21	$N_{0\,3}$	0.44 ± 0.01
k_{S1}	1.87 ± 0.49	k_{X2}	0.54 ± 0.15	k_{I3}	0.0001	$N_{0\,4}$	0.42 ± 0.01
k_{iI1}	0.05 ± 0.03			k_{iS3}	5.44 ± 0.63	$N_{0\,5}$	1.20 ± 0.01
k_{N1}	0.0001			k_{N3}	0.0001	$N_{0\,6}$	1.73 ± 0.03
						$N_{0\,7}$	1.84 ± 0.03
						$N_{0\,8}$	1.77 ± 0.04
						$N_{0\,9}$	1.73 ± 0.06

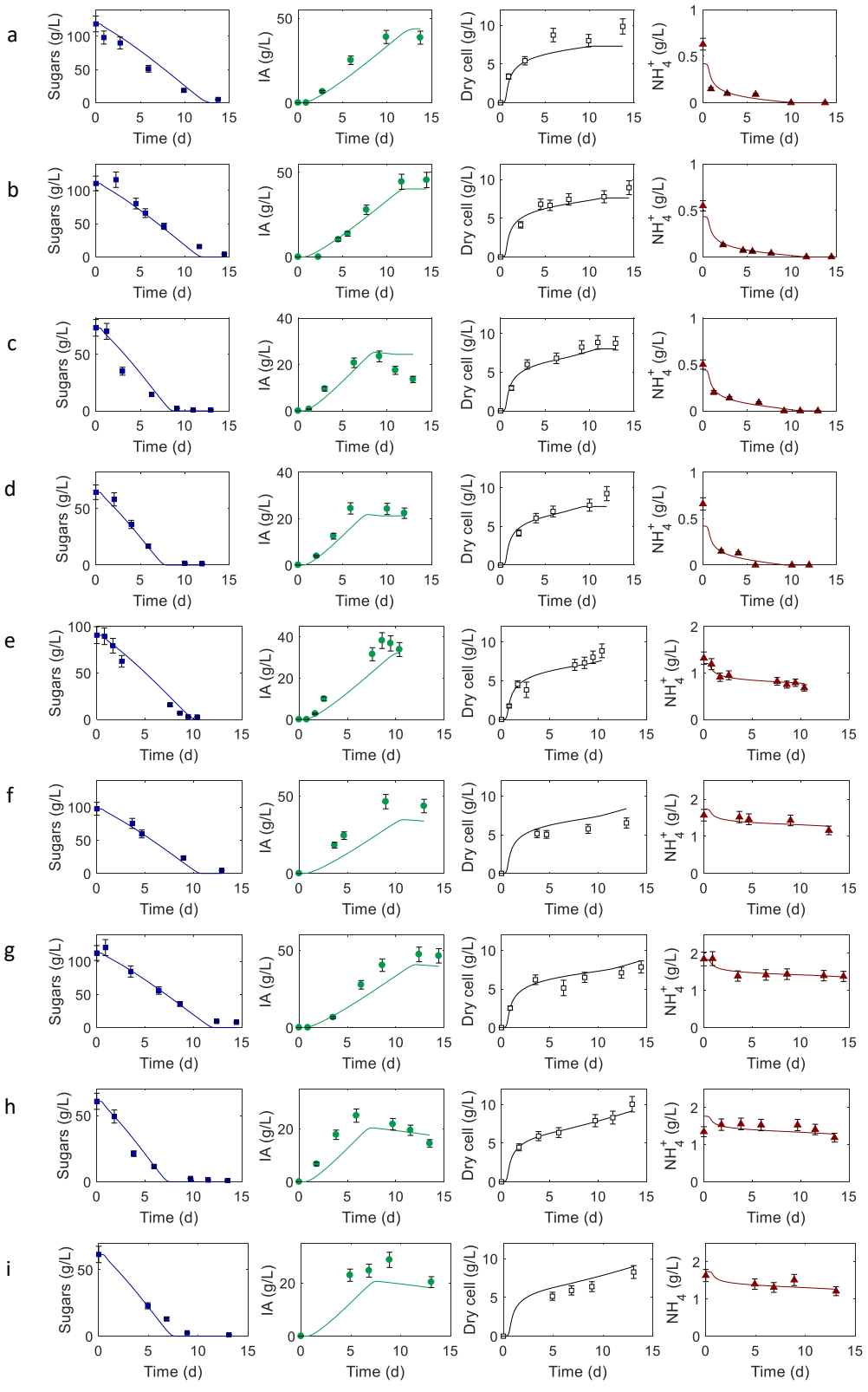

Figure 7.24 Parameter identification of the macroscopic model (lines) with k_{N1}, k_{I3} and k_{N3} at fixed value (0.0001) and experimental data (points) of IA production by *A. terreus* NRRL 1960 in VHP medium. Low C + low N (a and b); high C + low N (c and d); medium C + medium N (e); low C + high N (f and g); high C + high N (h and i). High, medium and low values of C respectively: 114, 95 and 76 g/L VHP. High, medium and low values of N: 6, 4 and 2 g/L of NH4NO3. TRS (■), IA (●), dry cell (□), and NH_4^+ (▲).

The cross validation step is a should verify if the parameters estimated may describe a part of the total set of assays, as presented previously (section 4.5.4.2). It was chosen to consider the two extreme conditions of all the assays to estimate the parameters values at this stage – low C + low N (Figure 7.22a and b) and high C + high N (Figure 7.22h and i). The values identified with those assays were applied on the other set of experiments to verify how well those values fit the intermediate assays: high C + low N (Figure 7.22c and d), medium C + medium N (Figure 7.22e), low C + high N (Figure 7.22f and g). Those results are presented on Table 7.10 and Figure 7.25.

Table 7.10 Parameters values for model definition with cross validation of IA production by *A. terreus* NRRL 1960 in different initial concentrations of VHP and NH_4NO_3. Values obtained from all the assays (9 assays, 5 different initial conditions) and fixed values of k_{N1}, k_{I3} and k_{N3} (0.0001). Cost function value obtained: 0.97.

Reaction 1		**Reaction 2**		**Reaction 3**		**Initial values of NH_4^+**	
ν_1	0.05 ± 0.22	ν_3	0.44 ± 0.04	ν_4	0.01 ± 0.99	$N_{0\,1}$	0.57 [a]
ν_2	0.69 ± 0.21	$\mu_{2\,max}$	2.11 ± 0.12	ν_5	0.47 ± 0.31	$N_{0\,2}$	0.65 [a]
$\mu_{1\,max}$	18.74 ± 0.21	k_{S2}	1.00 ± 0.88	$\mu_{3\,max}$	0.10 ± 0.34	$N_{0\,3}$	0.51 ± 0.02
k_{S1}	0.82 ± 2.73	k_{X2}	0.96 ± 0.57	k_{I3}	0.0001	$N_{0\,4}$	0.51 ± 0.02
k_{iI1}	0.07 ± 0.13			k_{iS3}	18.36 ± 1.57	$N_{0\,5}$	1.42 [a]
k_{N1}	0.0001			k_{N3}	0.0001	$N_{0\,6}$	1.79 ± 0.06
						$N_{0\,7}$	1.91 ± 0.08
						$N_{0\,8}$	1.97 [a]
						$N_{0\,9}$	1.94 [a]

[a]Values estimated with the application of the kinetic parameters.

The parameter estimation done with the variables calculated with the extreme conditions (low C + low N and high C + high N) resulted in a satisfactory value of coefficient of determination (R^2) of the overall model (Table 7.11) and the description of each variable. Moreover, the same analysis of the model regarding the direct validation showed that a very good description of the model was obtained, with a R^2 of 0.93.

Table 7.11 Coefficients of determination of the model proposed regarding the experimental data, calculated for the direct and cross validations. The estimation of the fitting values was done considering the experimental data from all the variables (sugar - S, cell - X, IA - I and ammonium - N) and considering each variable separately.

Variable	**Coefficient of determination of direct validation** (R^2)	**Coefficient of determination of cross validation** (R^2)
All	0.93	0.87
Sugar	0.97	0.93
Cell (X)	0.93	0.83
IA (I)	0.90	0.86
Ammonium (N)	0.97	0.94

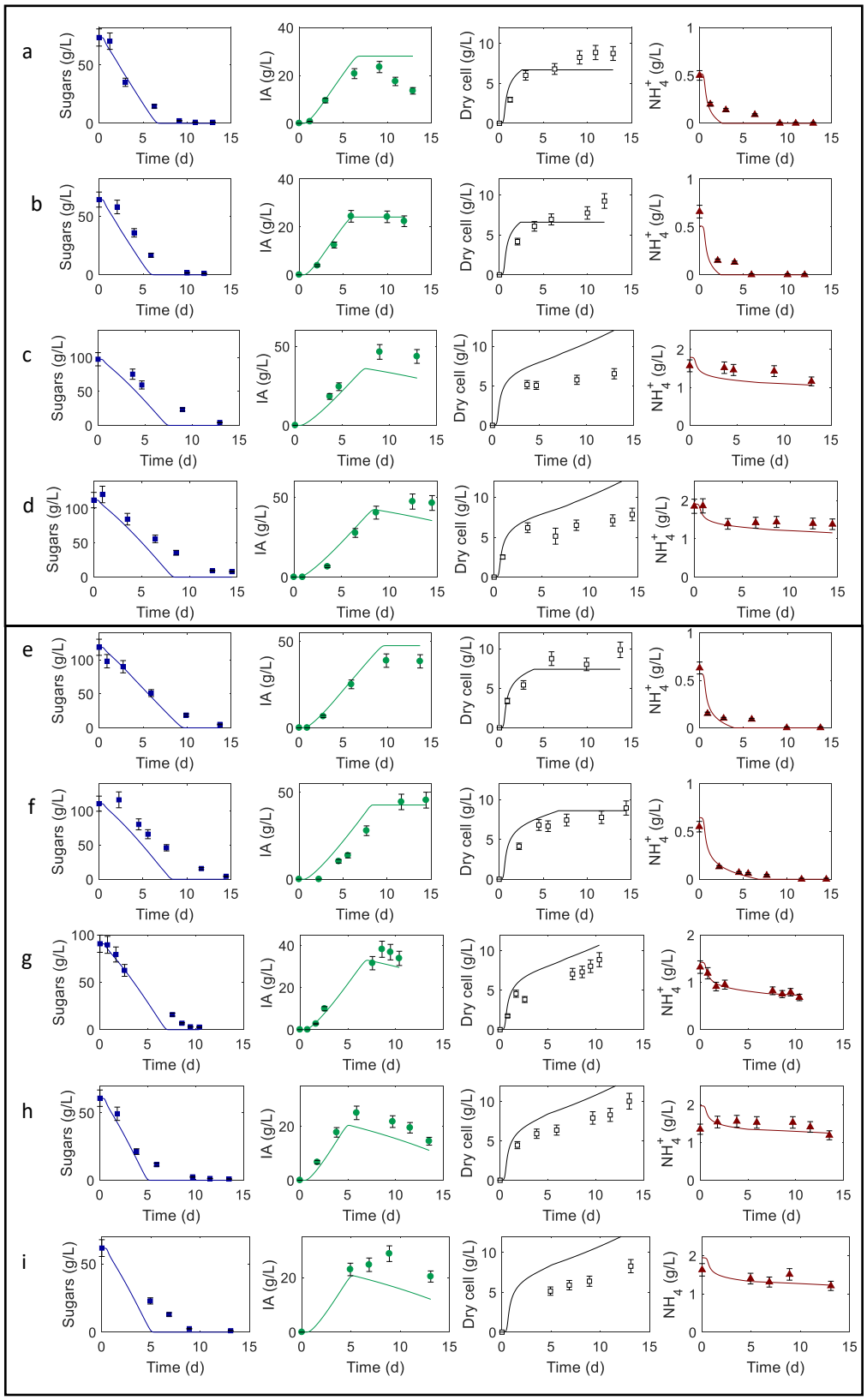

Figure 7.25 Cross validation of the macroscopic model (lines) with k_{N1}, k_{I3} and k_{N3} at fixed value (0.0001) and experimental data (points) of IA prodcution by A. terreus NRRL 1960 in VHP medium. Experiments set used to estimate the parameters (a-d) and parameters validation with a different set of experiments (e-i). Low C + low N (a and b); high C + high N (c and d); low C + high N (e and f); medium C + medium N (g); high C + low N (h and i). High, medium and low values of C respectively: 114, 95 and 76 g/L VHP. High, medium and low values of N: 6, 4 and 2 g/L of NH4NO3. TRS (■), IA (●), dry cell (□), and NH_4^+ (▲).

As it may be observed on Table 7.9, the model proposed in this study predicts the production of IA in VHP medium by *A. terreus*, even in the cross validation step. The obtained value indicates that the parameters estimated describe well the experimental data. Moreover, the predicted parameters describe very well the variables profiles separately (S, X, I or N), with better prediction of ammonium and sugar profiles.

Many of the parameters identified present small confidence interval, which indicates a good estimation of those values. However, some values showed large confidence intervals, as observed for k_1, k_4 and μ_{3max}. Despite the several attempts to obtain values with a narrower confidence interval, those were the closest values obtained.

Two of the parameters with high confidence intervals are related to nitrogen concentration: v_1 and v_4. Regarding that, it is important to mention that nitrate is also available in the medium, but it is less preferable than ammonium by microorganisms, as mentioned in the previous section. However, precious studies have shown the uptake of nitrate from the fermentative medium of IA production by *A. terreus* from the beginning of the fermentation (Kuenz et al. 2012). Possibly that is the reason why it was not possible to obtain small confidence intervals of the mentioned parameters, including μ_{3max}, since it also concerns N reaction.

Regarding the second specific reaction rate identified (7.10) shows an activation parameter concerning cell concentration (k_{X2}), representing a catalyst factor by cells. In fact, it does not exactly represent an activation term by cell itself, but rather a contribution of enzymatic systems from IA metabolic pathway inside the cells. The phenomenon could be related to the microorganism production of CAD enzymes and MTT transporter, which are essential for high IA production, as mentioned previously (section 2.4). Moreover, the deviation of *cis*-aconitate from the TCA cycle through the MTT to produce IA, which is the reason for the slow or null cell growth rate when high IA is produced, could explain the inhibition effect caused by IA on the cell growth (reaction 7.1).

The macroscopic model presented in this study is a good representation of IA production by *A. terreus* NRRL 1960 in VHP medium with NH_4NO_3 as nitrogen source. This is a more complete description of the system compared with the only study that describes mathematically IA production by *A. terreus*, to the best of the author's knowledge.

7.9 Final considerations

The investigations accomplished in this study enabled the analysis of different aspects of IA production by *A. terreus*. Some characteristics of the fermentative process are discussed in this section as a final consideration of this work.

The initial stage of this study proposed the selection of an *A. terreus* strain that produces high concentrations of IA, based on the IA concentrations presented in other studies. From that part, it was observed that the high IA producer strains also produced lower cell growth compared with the other strains. For instance, strains such as *A. terreus* DSM 826 (Figure 7.4a), NRRL 260 and 265 (Figure 7.9 and 7.10) produced 1 to 30 g/L IA, whereas dry cell concentrations were about 20 g/L dry cell for all the strains by the 10^{th} day of fermentation in glucose medium. On the other hand, the conditions which enabled the production of over 30g/L IA resulted in less than 10 g/L of cells for most assays (Figure 7.6, 7.7, 7.9, 7.10).

The observation regarding cell production in contrast to IA synthesis is consistence with the knowledge about the mechanism of overproduction of certain metabolites. The literature states that IA is a primary metabolite (Miall 1978), produced semi-associated to cell growth (Kuenz et al. 2012; Krull et al. 2017). The overproduction of primary metabolites is related to the deviation of those metabolites, or their intermediate, from the TCA cycle, thus, there is a partial interruption of the biochemical reactions of that cycle. In the case of IA production, *cis*-aconitate is deviated to be transported to the cytosol which is, further, transformed in IA by CAD enzyme. The regulation of that deviation of metabolites may occur by nutritional regulation (carbon or nitrogen source regulation) or others, such as feedback regulation (Sanchez and Demain 2008).

The reaction schemes proposed by the macroscopic model presented in this study is corroborated by some of the knowledge about IA production. It was proposed that an inhibition effect by the product affects cell growth. Moreover, it was demonstrated a catalyst effect of cells to the production of IA, which has been proposed to be the production of CAD and MTT by high IA-producers strains. It was also presented that the consumption of IA by the cells when carbohydrates are no longer present in the medium induces cell growth at the expense of ammonium consumption as well.

The model description of cell production does not include the different stages of cell growth and the correspondent cell morphologies. The cycle of cell growth from spores to mycelium for filamentous fungi involves a vast heterogeneity in morphology and physiology factors. The unknow number of parameters that would describe such phenomena is a bottleneck for the definition of a detailed mathematical model contemplating the different states of biomass growth and metabolite production (Papagianni 2004). The macroscopic representation of cell production regarding substrate and nitrogen consumption in this study are satisfactory for a simple, but a sufficient representation of the system.

The proposed model may be applied in fermentative processes of IA production by *A. terreus* NRRL 1960 in larger scale bioprocess. This is one of the advantages of developing a mathematical model that has theoretical basis, which presents the model's parameters with physical meaning. Despite the more time consumption compared to static models that are exclusively empirical and are represented by a surface response, the dynamic model proposed would probably have similar fermentation profiles within the range of the model at a different scale, for example, at a bioreactor. This reinforces the importance of the novelty of the mathematical model proposed in this study.

The other novelty of this study, regarding the use of VHP sugar as carbon source, showed that high IA yields may be obtained with that sugary feedstock of lower cost than the pure substrate sources. The possibility of decreasing the end-product cost by applying VHP as feedstock is even more promising if it is considered introducing the bioprocess to the biorefinery concept, where IA would be produced at sugar/ethanol mill, where VHP is abundantly produced. A biorefinery plant with IA is proposed on Figure 7.26.

Considering the requirement for operating a sugar and bioethanol industrial plant, the implementation of IA to the portfolio of the current 3-platform biorefinery (C6 sugars, bagasse, electricity & heat) would have the advantage of the knowledge of fermentative processes. The experience concerning bioethanol production would decrease the learning curve time that a new process requires. Besides, the sugar processing facilities should be easily adaptable to a different end-product as they already alternate from one end-product to another – sugar or ethanol – depending on market price of those materials. This would decrease the end-product cost, thus, increase the economic feasibility of IA products.

Moreover, the dynamic macroscopic model proposed in this study could be applied for scaling up the production and for optimizing the performance of IA synthesis with VHP.

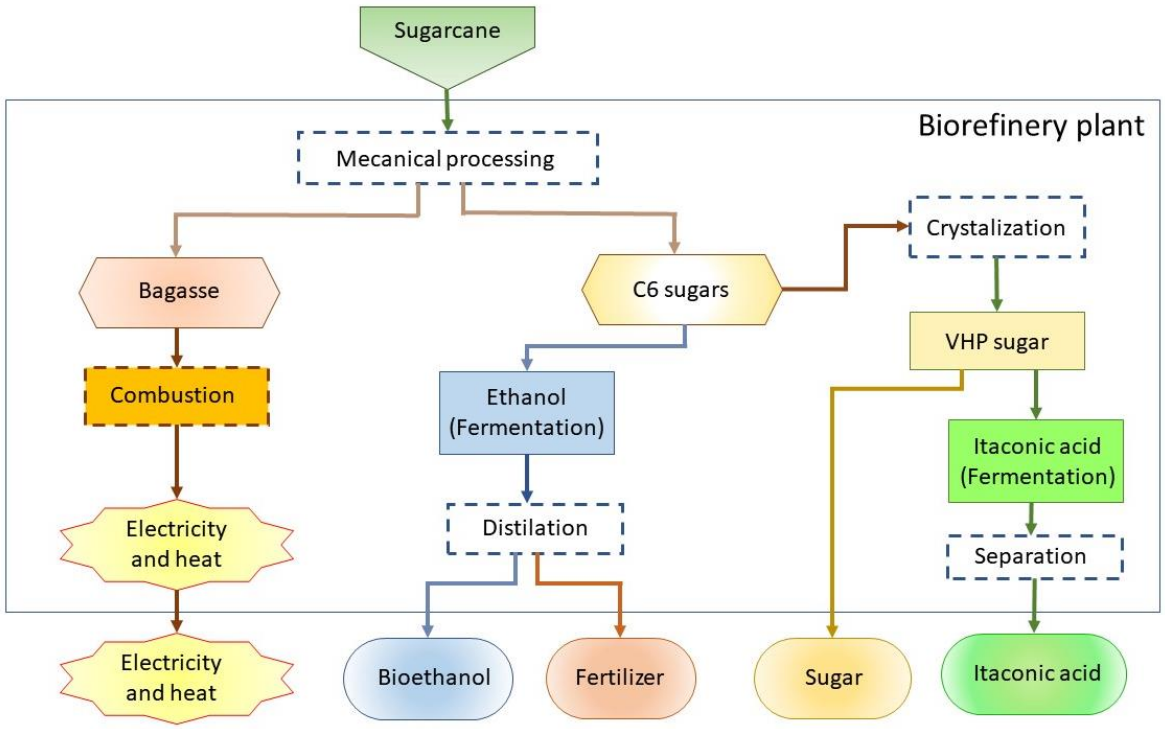

Figure 7.26 Proposal of a 4-platform biorefinery (C6 sugars, bagasse, electricity & heat, itaconic acid) biorefinery using sugarcane for bioethanol, electricity, heat, sugar, fertilizer and itaconic acid (adapted from IEA Bioenergy (2013)).

Chapter 8

CONCLUSIONS

This study showed that the strain *Aspergillus terreus* NRRL 1960 produced the highest concentrations of IA in pure glucose or sucrose media, among a total of 7 strains evaluated.

The evaluation of four combinations between two carbon and two nitrogen sources showed that granulated sugar and NH_4NO_3 produced the highest IA concentration at the conditions evaluated. Despite of that, VHP and NH_4NO_3 were the selected combinations of carbon and nitrogen sources to compose the IA production medium for further investigations.

The study of different initial concentration of VHP and NH_4NO_3 showed that the use of 114 g/L of the carbon source and 6 g/L of the nitrogen source resulted in the best condition for IA production among the conditions analyzed. Moreover, it was observed that a limitation of ammonium in the medium is not mandatory for obtaining high IA concentrations.

The dynamic macroscopic model proposed in this study describes the IA production by *A. terreus* NRRL 1960 in VHP medium. The reaction scheme developed in this study consists in three reactions, which describes the production of cell and IA, and the consumption of IA when substrate is no longer available in the medium.

This study showed that IA was produced in high yield in VHP sugar medium, a low-cost feedstock. The best initial condition of VHP and NH_4NO_3 concentrations resulted in 67% of the theoretical yield by *A. terreus* NRRL 1960.

SUGESTION OF FUTURE WORK

The author suggests further investigation regarding the possible inhibition of IA production by *trans*-aconitate in the absence of dissolved oxygen in the fermentative medium. Moreover, it is suggested to apply the dynamic mathematical model developed in this study in bioreactor.

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