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CENTRO DE CIÊNCIAS EXATAS E DE TECNOLOGIA
DEPARTAMENTO DE ENGENHARIA QUÍMICA

EMANOELA FERNANDA QUEIROZ PUCCI

SISTEMA MULTIENZIMÁTICO INOVADOR PARA A PRODUÇÃO DE ÁCIDO GLICÔNICO A PARTIR DE AMIDO USANDO CÉLULAS INTEIRAS DE *ASPERGILLUS NIGER*

SÃO CARLOS -SP
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Não viemos ao mundo para fazer o que os outros fazem. Não viemos para fazer um pouco melhor o que os outros fazem. Viemos para fazer o que só nós podemos fazer.

-Trigueirinho

RESUMO

O ácido glicônico ocorre naturalmente numa variedade de alimentos, incluindo frutas, mel, vinho, arroz, carne e produtos fermentados naturalmente. Devido à sua versatilidade e biodegradabilidade, esse ácido orgânico tem muitas aplicações nas indústrias de alimentos e bebidas, farmacêutica e de detergentes. Embora a fermentação tenha sido utilizada durante várias décadas, existem ainda inconvenientes e desafios associados a este processo. Os processos de fermentação resultam frequentemente em rendimentos inferiores aos dos métodos de síntese química e requerem nutrientes específicos para o crescimento e a atividade metabólica dos microrganismos. Além disso, a produção de ácido glicônico por fermentação pode produzir subprodutos, incluindo outros ácidos e compostos orgânicos, que têm de ser removidos na etapa de purificação do produto, aumentando o custo global e a complexidade do processo. Alternativamente ao processo de fermentação, o ácido glicônico pode ser produzido por biocatálise a partir de células inteiras. A biocatálise utilizando células inteiras envolve células microbianas intactas para realizar transformações químicas específicas ou produzir compostos desejados. Esta abordagem diferencia-se dos processos de fermentação porque as células do microrganismo são aplicadas sob condições de não crescimento como biocatalisadores. Esse estudo se iniciou com uma revisão da literatura cujo objetivo foi identificar e analisar estudos anteriores sobre a produção de ácido glicônico utilizando-se biocatálise de células inteiras, bem como os microrganismos, substratos e enzimas envolvidos neste processo. Esta revisão permitiu a seleção do amido, um dos polissacáridos naturais mais abundantes, para a produção de ácido glicônico. No presente estudo, foi proposto um sistema multienzimático inovador, numa única fase, para a produção de ácido glicônico a partir de amido, utilizando células inteiras de *Aspergillus niger* associadas a enzimas amilolíticas. Foram obtidos rendimentos elevados de ácido glicônico com elevada pureza do produto, por exemplo, concentração de ácido glicônico de 134,5 ± 4,3 g/L, rendimento de ácido glicônico de 98,2 ± 1,3%, rendimento do biocatalisador de 44,8 ± 1,4 $g_{\text{ácido glicônico}}/g_{\text{células inteiras}}$ e pureza do produto (96%) durante uma reação de 96 h. Embora o processo tenha sido desenvolvido utilizando o amido como matéria-prima, a abordagem tem potencial para outros substratos ou resíduos que possam ser hidrolisados em glicose. Portanto, o sistema multienzimático proposto abre novas perspectivas para produção de ácido glicônico por biocatálise de células inteiras.

Palavras-chave: Células inteiras; biocatalisador; *Aspergillus niger*; amido; sistema multienzimático; ácido glicônico; enzimas amilolíticas.

ABSTRACT

Gluconic acid occurs naturally in a wide variety of foods, including fruit, honey, wine, rice, meat and other naturally fermented products. Because of its versatility and biodegradability, it has many applications in the food and beverage, pharmaceutical, and detergent industries. Although fermentation has been used for several decades, there are still drawbacks and challenges associated with the process. Fermentation processes often result in lower yields than chemical synthesis methods and require specific nutrients for the growth and metabolic activity of microorganisms. In addition, gluconic acid produced by fermentation can produce by-products, including other acids and organic compounds, which must be removed in the product purification stage, increasing the overall cost and complexity of the process. As an alternative to the fermentation processes, gluconic acid can be produced by whole-cell biocatalysis. Whole-cell biocatalysis uses intact microbial cells to perform specific chemical transformations or produce desired compounds. This approach differs from fermentation because microbial cells are used as biocatalysts under non-growth conditions. This study began with a literature review aimed at identifying and analyzing previous studies on the production of gluconic acid by whole-cell biocatalysis, as well as the microorganisms, substrates, and enzymes involved in this process. This review enabled the selection of starch, one of the most abundant natural polysaccharides, to produce gluconic acid. In the present study, an innovative single-step multi-enzymatic system for gluconic acid production from starch using whole-cells of Aspergillus niger associated with amylolytic enzymes was proposed. High yields of gluconic acid with high product purity were obtained, e.g., a gluconic acid concentration of 134.5 ± 4.3 g/L, a gluconic acid yield of 98.2 ± 1.3%, a biocatalyst yield of 44.8 ± 1.4 $g_{gluconic\ acid}/g_{whole\text{-}cells}$ and a product purity (~96%) were achieved during a 96 h reaction. Although the process was developed using starch as the raw material, the approach has the potential to be applied to other substrates or residues that can be hydrolyzed to glucose. Therefore, the proposed multienzyme system opens new perspectives for gluconic acid production by whole-cell biocatalysis.

Keywords: Whole-cells; biocatalyst; *Aspergillus niger*; starch; multi-enzyme system; gluconic acid; amylolytic enzymes.

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ABBREVIATION AND ACRONYM LIST

GA – Gluconic Acid

SG – Sodium Gluconate

XA – Xylonic Acid

A. niger – Aspergillus niger

wc – whole-cell (s)

GOD – Glucose Oxidase

CAT – Catalase

GDH – Glucose Dehydrogenase

GFOR– Glucose-Fructose Oxidoreductase

AMG – Amyloglucosidase

GL – Gluconolactonase

CCRD – Composite Rotatable Design

MW_{GA} – Molecular Weight of Gluconic Acid

MW_{SG} – Molecular Weight of Sodium Gluconate

SLSO – Simultaneous Liquefaction, Saccharification, and Oxidation

SSF – Simultaneous Saccharification and Fermentation

SHF – Sequential Hydrolysis and Fermentation

HFCS – High-Fructose Corn Syrup

SR – Systematic Review

GRAS – Generally Recognized As Safe

FDA – Food and Drug Administration

PEI – Polyethyleneimine

GLU – Glutaraldehyde

NS – Nitrogen starvation

GICC – Growth Inhibition by Chemical Compound

GLI – Growth Limited by Immobilization

HT – High Temperature

SPC – sodium percarbonate

EDP – Entner-Doudoroff Pathway

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1 GENERAL INTRODUCTION

Gluconic acid (pentahydroxycaproic acid, $C_6H_{12}O_7$) is a naturally occurring organic compound found in various foods such as fruit, honey, wine, rice, meat, vinegar and other naturally fermented products. This hydroxycarboxylic acid's versatility, biodegradability, chelating properties, and low environmental impact make it suitable for various applications and generally recognized as safe (GRAS) by regulatory authorities (Anastassiadis; Morgunov, 2007; Ma *et al.*, 2022). In addition, gluconic acid can form various salts known as gluconates by reacting with metal cations such as sodium, potassium, calcium, and others. These salts are widely used in various industries such as the food industry as additives and acidity regulators to adjust the pH of meals and beverages and improve shelf life, in pharmaceutical formulations such as oral and intravenous drugs to control acidity, and in cleaning and detergent formulations to remove scale and rust from surfaces (Ramachandran *et al.*, 2006; Pal *et al.*, 2016).

The gluconic acid market has witnessed steady growth over the years, owing to its versatile applications in various industries. In 2017, the global gluconic acid market was valued at over US$ 50 million and is expected to exceed US$ 80 million by 2024, with more than 120 kilotons of gluconic acid consumed by industries (market price of 50% aqueous solution is approximately US$ 300-800/ton) (Ahuja; Singh, 2018). Gluconic acid is among the 30 chemicals selected by the US Department of Energy as the most valuable chemicals from biomass (Werpy; Petersen, 2004).

The international bibliography and patent literature of the last 100 years describe numerous production processes for gluconic acid from glucose, including chemical and electrochemical catalysis, enzymatic biocatalysis by free or immobilized enzymes, and fermentation processes (Anastassiadis; Morgunov, 2007; Pal *et al.*, 2016). Currently, gluconic acid is commonly produced on an industrial scale through fermentation processes by microorganisms such as *Aspergillus spp*. and *Gluconobacter spp*. using glucose as a substrate (Kornecki *et al*., 2020). Although gluconic acid fermentation has been used for several decades and has many advantages, there are some drawbacks and challenges associated with the process. The fermentation processes often result in lower yields of gluconic acid compared to chemical synthesis methods and require specific nutrients for the growth and metabolic activity of the microorganisms. Ensuring the availability of these nutrients and maintaining optimal conditions can be challenging and costly. In addition, gluconic acid fermentation can produce by-products, including other organic acids and compounds, which may need to be separated and purified, adding to the overall cost and complexity of the process. Therefore, the cost-effective and

environmentally friendly production of high-purity gluconic acid remains a challenge (Pal *et al.*, 2016; Ma *et al.*, 2022).

As an alternative to overcome these bottlenecks, gluconic acid can also be produced using whole-cell biocatalysis, a biotechnological approach that combines some of the advantages of both chemical synthesis and traditional fermentation. Whole-cell biocatalysis uses intact microbial cells under non-growing conditions as biocatalysts to perform various chemical reactions. Instead of extracting and using isolated enzymes, these cells are used as a complete biological system (Lin; Tao, 2017; de Carvalho, 2017).

Typical whole-cell biocatalysis involves two steps: the growth of the living "biocatalyst" and the subsequent bioconversion of the substrate(s) to the product(s) of interest. Typically, cells are harvested after cultivation, washed with water or a buffer solution, and suspended in the desired buffer for subsequent biocatalysis. When cells are washed, unconsumed growth substrates and nutrients, as well as unwanted metabolites produced during growth, are removed from the system, significantly improving product recovery rates and simplifying downstream processing (McAuliffe, 2012). Furthermore, the removal of essential nutrients arrests cell growth, allowing resting cells to produce higher yields from their carbon source, as the available carbon and energy are overwhelmingly used for product synthesis rather than biomass production (de Carvalho, 2017; Lin; Tao, 2017). Therefore, unlike traditional fermentation processes that focus on the production of biomass or specific metabolites, whole-cell biocatalysis harnesses the metabolic capabilities of living cells to perform specific chemical transformations (Woodley, 2006). This method is particularly valuable for the synthesis of products where traditional chemical methods may be less efficient or environmentally friendly, such as the production of pharmaceutical intermediates, amino acids, fine chemicals, foods, biosensors, and biofuels (Schrewe *et al.*, 2013; Song *et al.*, 2020; Madavi *et al.*, 2021; Zhong *et al.*, 2022).

Whole-cell biocatalysis offers several advantages, including versatility, high specificity and selectivity, the ability to perform complex and stereospecific reactions, and the potential for green and sustainable chemistry. The use of whole-cell biocatalysts eliminates the need for cell lysis and enzyme purification, significantly reducing costs. At the same time, residual cell wall components can protect the encapsulated enzyme from potentially harmful environments, enabling applications far from the natural enzyme environment. Its potential for sustainable and environmentally friendly processes has attracted attention, and ongoing research continues to expand its applications in various industries (Wachtmeister; Rother, 2016; de Carvalho, 2017; Lin; Tao, 2017).

The present study proposes an innovative multi-enzymatic system for gluconic acid production from starch, employing *Aspergillus niger* (*A. niger)* whole-cells and amylolytic enzymes. Starch, one of the most abundant natural polysaccharides, is a sustainable material, due to its biodegradability and renewability, and is widely used industrially (Zeng *et al.*, 2022). Enzymatic processing to transform raw starch into glucose is already widely applied on an industrial scale, where the main enzymes involved (α-amylase and amyloglucosidase) are extracellular enzymes commercialized at a cost that is not high when compared to intracellular enzymes or new enzymes (van der Maarel *et al.*, 2002; Cripwell *et al.*, 2020).

A. niger whole-cells are considered excellent enzyme bags and promising biocatalysts for GA production, because they contain the constitutive enzymes glucose oxidase (GOD) and catalase (CAT) (Witteveen; Veenhuis; Visser, 1992; Vassilev *et al.*, 1993; Ramachandran *et al.*, 2007, 2008). Briefly, the multi-enzymatic process proposed in this study for GA production from starch involves two key steps. First, starch hydrolysis takes place using commercial enzymes (α-amylase and amyloglucosidase). Following this, glucose oxidation is facilitated by the enzymes GOD and CAT within the *A. niger* whole-cell environment, as depicted in Figure 1.1. Notably, primary metabolic pathways essential for cell growth, development, and reproduction, such as glycolysis and the citric acid cycle (Cameselle *et al.*, 1998), are simplified and marked by the gray dotted area due to nitrogen starvation suppression.

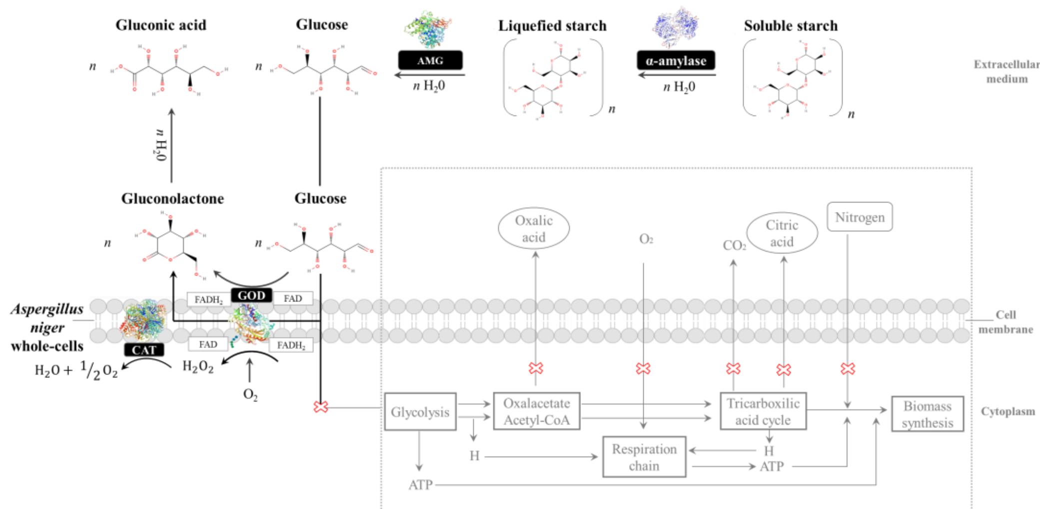


Figure 1.1. The innovative multi-enzymatic process for GA production from starch with commercial enzymes (α-amylase and amyloglucosidase) followed by the oxidation of glucose by the enzymes GOD and CAT in situ, in *A. niger* whole-cells. Primary metabolic pathways essential for cell growth, development, and reproduction, such as glycolysis and the citric acid cycle, are simplified and marked by the gray dotted area due to nitrogen starvation suppression. Alpha-amylase (4-α-D-glucan glucanohydrolase; EC 3.2.1.1); AMG (amyloglucosidase; glucoamylase; EC 3.2.1.3); GOD (FAD-dependent glucose oxidase; EC 1.1.3.4); CAT (catalase; EC 1.11.1.6). Adapted from (Cameselle *et al*., 1998).

This approach (Figure 1.1) can provide significant benefits to produce gluconic acid compared to fermentation: (1) economy with expensive nutrients for microorganism growth; (2) the possibility of maintaining operating conditions such as pH, temperature, and oxygen transfer at the optimum for enzymatic conversion, without precautions related to growth of the microorganism; (3) potential for greater conversion of substrate to the target product, since carbon from the substrate can be directed to acid gluconic production, avoiding other routes (growth, or production of another organic acid); (4) a higher purity product, reducing the costs of downstream steps; (5) low risk of contamination, due to the absence of a nitrogen source. Therefore, this study presents an innovative one-step multi-enzymatic system for gluconic acid production from starch using *A. niger* whole-cells in association with amylolytic enzymes.

Chapter 1 introduced the topic of the study and provided an overview of the thesis structure with brief descriptions of upcoming chapters.

Chapter 2 conduced a literature review to analyze existing research on the production of gluconic acid using whole-cell biocatalysis. The main goals of this chapter were to identify the microorganisms, substrates, and enzymes that have been employed in previous studies for this purpose. Additionally, it aimed to provide insights that guided the present study while also offering new perspectives for future research on whole-cell biocatalysis for gluconic acid production.

Chapter 3 focused on assessing how effectively *A. niger* whole cells associated with the

appropriate commercial enzyme produce gluconic acid from substrates: dextrin, sucrose and lactose. These substrates were chosen based on the literature gaps mapped in Chapter 2. The high GA yield (%) from dextrin and the similarity between its substrate and starch opens a new perspective for an innovative multi-enzymatic system to GA production from starch using *A. niger* whole-cells associated with amylolytic enzymes.

Chapter 4 presented the development of an innovative one-step multi-enzymatic system for gluconic acid production from starch using *A. niger* whole cells in association with amylolytic enzymes. This chapter has been published as an article in Enzyme and Microbial Technology 2023, Volume 171, 110309 (DOI: 10.1016/j.enzmictec.2023.110309). Although the process was developed for starch, the approach has the potential to be applied to other materials that can be hydrolyzed to glucose. The novel approach to produce gluconic acid from starch proposed in this chapter also resulted in a patent application to Instituto Nacional de Propriedade Industrial (INPI), under the process number BR 10 2022 026217-9, submitted on December 21, 2022. Patent title: "Produção de ácido glicônico e seus sais a partir de polissacarídeos de glicose utilizando biocatalisador de células inteiras e enzimas amilolíticas e/ou celulolíticas" ("Production of gluconic acid and its salts from glucose polysaccharides using wc biocatalyst and amylolytic and/or cellulolytic enzymes"). Inventors: Emanoela Fernanda Queiroz Pucci; Paulo Waldir Tardioli, and Alberto Colli Badino.

Chapter 5 highlighted the importance of oxygen as a substrate for the glucose oxidation reaction. Preliminary assays carried out in a bench scale column bubble bioreactor indicated that the proposed multi-enzymatic process most likely is not limited by external O_2 transfer (bulk). Most likely, the process is limited by enzymatic reactions or diffusion limitations in the whole cells, which could be overcome by increasing the whole cell concentration and/or decreasing the whole cell pellet size.

Chapter 6, entitled "Conclusions", summarizes the key findings and contributions of the present study, and provides ideas for future research.

REFERENCES

AHUJA, K., SINGH, S., 2018. Gluconic Acid Market size worth over $80 mn by 2024. https://www.gminsights.com/pressrelease/gluconic-acid-market. Accessed on June 19th, 2023.

ANASTASSIADIS, Savas; MORGUNOV, Igor. Gluconic Acid Production. **Recent Patents on Biotechnology**, vol. 1, n. 2, p. 167-180, 1 June 2007c. Available from: https://doi.org/10.2174/187220807780809472.

CAMESELLE, C. *et al*. Oxalic acid production by *Aspergillus niger*. **Bioprocess Engineering**,

vol. 19, n. 4, p. 247-252, 20 Oct. 1998. Available from: https://doi.org/10.1007/pl00009017.

CRIPWELL, R. A. *et al*. Consolidated bioprocessing of raw starch to ethanol by *Saccharomyces cerevisiae*: Achievements and challenges. **Biotechnology Advances**, vol. 42, p. 107579, Sept. 2020. Available from: https://doi.org/10.1016/j.biotechadv.2020.107579.

DE CARVALHO, C. C. C. R. Whole cell biocatalysts: essential workers from Nature to the industry. **Microbial Biotechnology**, vol. 10, n. 2, p. 250-263, 3 May 2016. Available from: https://doi.org/10.1111/1751-7915.12363.

KORNECKI, J. F. *et al*. Enzyme production of d-gluconic acid and glucose oxidase: successful tales of cascade reactions. **Catalysis Science & Technology**, vol. 10, n. 17, p. 5740-5771, 2020. Available from: https://doi.org/10.1039/d0cy00819b.

LIN, B.; TAO, Y. Whole-cell biocatalysts by design. **Microbial Cell Factories**, vol. 16, n. 1, 13 June 2017. Available from: https://doi.org/10.1186/s12934-017-0724-7.

MA, Y. *et al.* Production of Gluconic Acid and Its Derivatives by Microbial Fermentation: Process Improvement Based on Integrated Routes. **Frontiers in Bioengineering and Biotechnology**, vol. 10, 16 May 2022. Available from: https://doi.org/10.3389/fbioe.2022.864787.

MADAVI, T. B. *et al*. Whole-cell biocatalysis: Advancements toward the biosynthesis of fuels. **Biofuels, Bioproducts and Biorefining**, 11 Dec. 2021. Available from: https://doi.org/10.1002/bbb.2331.

MCAULIFFE, J. C. Industrial Enzymes and Biocatalysis. In: MCAULIFFE, Joseph C. **Handbook of Industrial Chemistry and Biotechnology**. Boston, MA: Springer US, 2012. p. 1183-1227. ISBN 9781461442585. Available from: https://doi.org/10.1007/978-1-4614-4259-2_31.

PAL, P.; KUMAR, R.; BANERJEE, S. Manufacture of gluconic acid: A review towards process intensification for green production. **Chemical Engineering and Processing**: Process Intensification, vol. 104, p. 160-171, June 2016b. Available from: https://doi.org/10.1016/j.cep.2016.03.009.

RAMACHANDRAN, S. *et al*. Gluconic Acid: Properties, Applications and Microbial Production. **Food Technology and Biotechnology**, v. 44, n. 2, p. 185-95, Apr. 2006.

RAMACHANDRAN, S. *et al.* Permeabilization and inhibition of the germination of spores of *Aspergillus niger* for gluconic acid production from glucose. **Bioresource Technology**, vol. 99, n. 11, p. 4559-4565, July 2008. Available from: https://doi.org/10.1016/j.biortech.2007.06.055.

RAMACHANDRAN, S. *et al.* Spores of *Aspergillus niger* as reservoir of glucose oxidase synthesized during solid-state fermentation and their use as catalyst in gluconic acid production. **Letters in Applied Microbiology**, vol. 44, n. 2, p. 155-160, Feb. 2007. Available from: https://doi.org/10.1111/j.1472-765x.2006.02051.x.

SCHREWE, M. *et al*. Whole-cell biocatalysis for selective and productive C–O functional group introduction and modification. **Chemical Society Reviews**, vol. 42, n. 15, p. 6346, 2013. Available from: https://doi.org/10.1039/c3cs60011d.

SONG, J. W. *et al*. Design and engineering of whole-cell biocatalytic cascades for the valorization of fatty acids. **Catalysis Science & Technology**, vol. 10, n. 1, p. 46-64, 2020. Available from: https://doi.org/10.1039/c9cy01802f.

VAN DER MAAREL, M. J. E. C. *et al.* Properties and applications of starch-converting enzymes of the α-amylase family. **Journal of Biotechnology**, vol. 94, no. 2, p. 137-155, Mar. 2002. Available from: https://doi.org/10.1016/s0168-1656(01)00407-2.

VASSILEV, N. B; VASSILEVA, M. C.; SPASSOVA, D. Production of gluconic acid by *Aspergillus niger* immobilized on polyurethane foam. **Applied Microbiology and Biotechnology**, vol. 39, n. 3, June 1993. Available from: https://doi.org/10.1007/bf00192079.

WACHTMEISTER, J.; ROTHER, D. Recent advances in whole cell biocatalysis techniques bridging from investigative to industrial scale. **Current Opinion in Biotechnology**, vol. 42, p. 169-177, Dec. 2016. Available from: https://doi.org/10.1016/j.copbio.2016.05.005.

WERPY T.; PETERSEN G. **Top Value Added Chemicals from Biomass**: Volume I -- Results of Screening for Potential Candidates from Sugars and Synthesis Gas. 1 Aug. 2004. Available from: https://www.osti.gov/biblio/15008859.

WITTEVEEN, Cor F. B.; VEENHUIS, M.; VISSER, J. Localization of Glucose Oxidase and Catalase Activities in *Aspergillus niger*. **Applied and Environmental Microbiology**, vol. 58, no. 4, p. 1190-1194, 1992. Available from: https://doi.org/10.1128/aem.58.4.1190-1194.1992.

WOODLEY, J. M. Microbial Biocatalytic Processes and Their Development. **Advances in Applied Microbiology**. Elsevier, 2006. p. 1-15. ISBN 9780120026623. Available from: https://doi.org/10.1016/s0065-2164(06)60001-4.

ZENG, L. *et al*. Structure and functionality of cassava starch in different deep eutectic solvents/water mixtures: A comparative study. **Industrial Crops and Products**, vol. 177, p. 114453, Mar. 2022. Available from: https://doi.org/10.1016/j.indcrop.2021.114453.

ZHONG, P. *et al.* Piezoelectric sensing of glucose oxidase activity of *Aspergillus niger* spores pretreated by different methods. **Food Chemistry**, vol. 370, p. 130901, Feb. 2022. Available from: https://doi.org/10.1016/j.foodchem.2021.130901.

2 A REVIEW OF THE GLUCONIC ACID PRODUCTION BY WHOLE-CELL BIOCATALYSIS

RESUMO

O ácido glicônico é um ácido orgânico versátil usado em inúmeras aplicações, como alimentos e bebidas, produtos farmacêuticos e processos industriais. Embora o processo de fermentação tenha sido empregado há vários anos para produzir ácido glicônico, ele ainda apresenta algumas limitações como a demanda de nutrientes para o crescimento e manutenção celular, riscos de contaminação e processamento a jusante. Alternativamente, visando superar as desvantagens citadas, o ácido glicônico pode ser produzido eficientemente usando biocatalisadores de células inteiras. A biocatálise utilizando células inteiras envolve células microbianas intactas para realizar transformações químicas específicas ou produzir compostos desejados. Esta abordagem distingue-se dos processos de fermentação porque as células do microrganismo são aplicadas sob condições de não crescimento como biocatalisadores. A biocatálise utilizando células inteiras pode apresentar diversas vantagens sobre o processo fermentativo, incluindo altos rendimentos, custos mais baixos, maior seletividade, maior estabilidade e reutilização do biocatalisador e um processamento a jusante simplificado. Esta revisão aplicou princípios de mapeamento sistemático para fornecer uma visão abrangente do estado atual da arte na produção de ácido glicônico por biocatálise de células inteiras. O objetivo é identificar e analisar estudos anteriores sobre a produção de ácido glicônico utilizando biocatálise de células inteiras, bem como microrganismos, substratos e enzimas envolvidos nesse processo, para orientar estudos futuros nessa area. Os métodos de revisão sistemática são uma ferramenta para ajudar pesquisadores a identificar e organizar a literatura existente e fornecer uma visão geral do âmbito e da natureza das evidências disponíveis sobre um tópico de investigação específico.

Palavras-chave: Mapeamento sistemático; análise co-ocorrências; ácido glicônico; célula inteira; biocatálise; análise de rede; sistema multienzimático.

ABSTRACT

Gluconic acid is a versatile organic acid used in numerous applications, such as food and beverage, pharmaceuticals, and industrial processes. Although the fermentation process has been employed for several years to produce gluconic acid, it still has some limitations as nutrient demand for cell growth and maintenance, contamination risks, and downstream processing. Alternatively, to overcome the cited drawbacks, gluconic acid can be efficiently produced using whole-cell biocatalysts. Whole-cell biocatalysis involves intact microbial cells to perform specific chemical transformations or produce desired compounds. This approach is distinguished from fermentation processes because the microorganism cells are applied under non-growth conditions as biocatalysts. Whole-cell biocatalysis can present several advantages over the fermentative process, including high yields, lower costs, higher selectivity, higher stability and reusability of the biocatalyst, and simpler downstream processing. This review applied systematic mapping principles to provide a comprehensive overview of the current state of the art on gluconic acid production by whole-cell biocatalysis. The aim is to identify and analyze previous studies on gluconic acid production by whole cell-biocatalysis, as well as microorganisms, substrates, and enzymes involved in this process, to guide future studies toward expanding the use of whole-cells for gluconic acid production. Systematic review methods are a tool to help researchers identify and organize existing literature and provide a visual overview of the scope and nature of available evidence on a specific research topic.

Keywords: Systematic mapping; co-occurrence analysis; gluconic acid; whole-cell; biocatalysis; network analysis; multi-enzymatic system.

2.1 Introduction

Gluconic acid (GA) is a naturally occurring organic acid, a white crystalline powder, soluble in water, and with a slightly acidic taste. GA is generally recognized as safe (GRAS) by the US Food and Drug Administration (FDA), as a non-toxic, and environmentally friendly alternative to other acids, such as citric acid and phosphoric acid (Ma *et al.*, 2022). It makes GA a more sustainable choice for diverse applications in the food and beverage industries, such as an acidulant, flavor enhancer, and preservative. As GA is also biodegradable, it has been used in medicines and as a chelating agent in cleaning industrial applications (Cañete-Rodríguez *et al.*, 2016; Zhang *et al.*, 2021). From an economic perspective, the annual worldwide market of GA is between US$ 50 million and US$ 80 million, and its industrial consumption will probably exceed 1.2×10^5 tons by 2024 (Ahuja; Singh, 2018).

The traditional manufacturing processes for GA include chemical, biochemical, electrochemical, bioelectrochemical, enzymatic, and fermentative methods (Yan *et al.*, 2022). The fermentative processes by glucose oxidation using the microorganisms *Aspergillus niger* (*A. niger*) or *Gluconobacter oxydans* (*G. oxidans*) have been predominant for decades at an industrial scale (Kornecki *et al.*, 2020; Ma *et al.*, 2022). However, fermentative processes still present drawbacks: 1) the possibility of the formation of unwanted byproducts, such as other organic acids; 2) byproducts can reduce the purity of the final product and may require additional purification steps, increasing the overall cost and complexity of the process; 3) the additional supplementation costs for cell growth and maintenance of cell viability, and 4) risk of contamination (Ma *et al.*, 2022).

GA can alternatively be produced by whole-cell biocatalysts (Kornecki *et al.*, 2020) since this organic acid is not directly involved in primary metabolic pathways such as glycolysis or the citric acid cycle (Cameselle *et al.*, 1998). Thus, it can be produced during the stationary phase or under non-growth conditions due to nutrient starvation or stress (Znad; Markoš; Baleš, 2004).

The whole-cell biocatalysis uses intact living cells rather than purified enzymes or chemical catalysts. It involves using natural or engineered microorganisms, such as bacteria or fungi, containing enzymes and pathways to produce a specific product in biotechnology (de Carvalho, 2017; Jaishankar; Srivastava, 2017; Garzón-Posse *et al.*, 2018; Ng, 2020). However, it is distinguished from fermentation processes by the application of microorganism cells under non-growth conditions as biocatalysts (Woodley, 2006). In summary, whole-cell biocatalysis

consists of an enzymatic process, utilizing *in-situ* enzymes provided by the whole cells of the microorganisms instead of purified enzymes.

Typical whole-cell biocatalysis comprises two stages: growth of the living "biocatalyst" and subsequently bioconversion of the substrate(s) to the interest(s) product(s). In general, after cells are cultivated, they are harvested and washed with water or a buffer solution and suspended in the desired buffer for later biocatalysis. When the cells are washed, unconsumed growth substrates and nutrients, as well as undesired metabolites that were produced during growth, are removed from the system, allowing significantly better product recovery rates and simplifying downstream processing (McAuliffe, 2012). Furthermore, the removal of essential nutrients arrests cell growth, allowing resting cells to generate products from their carbon source in higher yields since the available carbon and energy are predominantly directed toward product synthesis rather than biomass production (de Carvalho, 2017; Lin; Tao, 2017).

Whole-cell biocatalysis allows the production of compounds through multi-step reactions, with cofactor regeneration, high regio- and stereo-selectivity, high yield, high selectivity, high stability, reusability of the biocatalyst under mild operational, environment-friendly conditions, and better cost-effectiveness (de Carvalho, 2017; Cheng *et al.*, 2020; Song *et al.*, 2020; Intasian *et al.*, 2021).

In the context of GA production using whole-cells biocatalysis, the general process to produce GA from different glucose-based substrates is summarized in Figure 2.1.

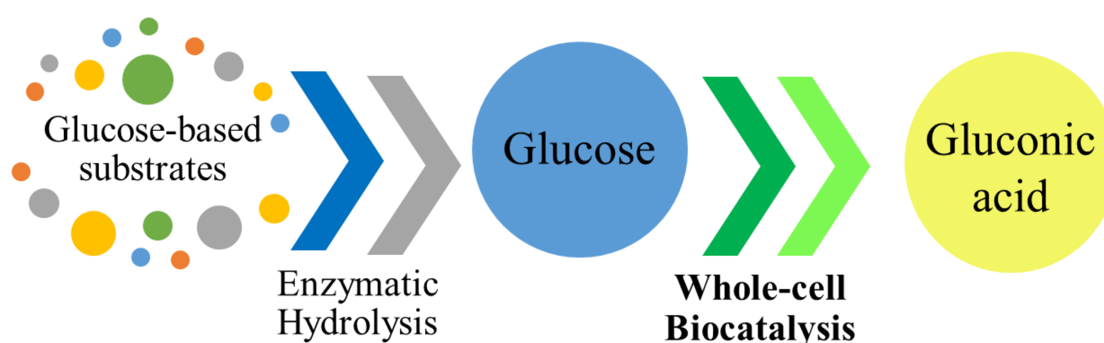


Figure 2.1. General process to produce GA from different substrates using whole-cell biocatalysis. Personal collection, 2023.

The methodology of this work incorporated systematic review (SR) principles to provide a comprehensive view of the current state of the art on GA production by whole-cell biocatalysis by elucidating relevant aspects of this bioprocess, such as microorganisms, substrates, enzymes, and immobilization methods applied.

Essentially, the advancement of knowledge must build upon existing studies. To push the knowledge frontier, it is crucial to understand its current position (Xiao; Watson, 2017). In this context, SR methods allow researchers to identify and organize existing literature on a particular topic and provide a visual overview of the scope and nature of available evidence on a specific research topic. SR ensures comprehensive searches across multiple bibliographic sources, transparent screening of retrieved articles, critical appraisal of the study quality, review for possible biases, well-documented extraction, data report, and appropriate quantitative or qualitative data synthesis (Dwan *et al.*, 2008; Petersen; Vakkalanka; Kuzniarz, 2015; Romanelli *et al.*, 2020).

Formal coordinating review bodies have been established to standardize SR methods across various disciplines, including Cochrane in healthcare, the Campbell Collaboration in social welfare, and the Collaboration for Environmental Evidence in conservation and environmental management (Haddaway *et al*., 2019). In the sphere of chemical engineering, there is no specialized organization dedicated to guiding the conduct of evidence synthesis. Consequently, the predominant reviews published in this field of knowledge could be classified as non-systematic by these standards since they did not follow guidelines specifically designed to avoid errors and biases in the review process (Higgins; White; Anzures-Cabrera, 2008; Haddaway *et al*., 2019)

Succinctly, this review aimed to identify and analyze previous studies on GA production by whole-cell biocatalysis by elucidating relevant aspects of this bioprocess, such as microorganisms, substrates, enzymes, and immobilization methods. In this manner, it becomes feasible to pinpoint gaps and trends within the examined literature, serving as a compass for future research endeavors seeking to enhance the application of whole-cell biocatalysis in gluconic acid production.

2.2 Methodology

This review was designed according to the criteria issued by the Collaboration for Environmental Evidence, 2022. This section summarizes a step-by-step guide on how this review was conducted.

2.2.1 Conducting the search

The first step in planning a literature search is to establish a strategy to maximize the probability of identifying relevant articles, as they are often linked to search terms (Livoreil *et*

al., 2017). First, to develop and test a search strategy, preliminary research was conducted to identify existing primary studies or SR and to determine the keywords related to the topic of research. A methodic and reproducible search strategy involving a combination of keywords and Boolean operators (e.g., AND, OR) designated as "search string" was established. The search string was formulated using known keywords of the research topic, followed by identification and association with alternative words, synonyms, or related terms using the Boolean operators "OR" and "AND".

The following search string was applied in different electronic databases to retrieve titles, abstracts, and keywords: ("biotransformation"OR"bioconversion"OR"microbial*"OR"whole*"OR"non-grow*"OR"resting" OR"biocatalyst*"OR"cascade*"OR"multienzymatic*"OR"enzyme*"OR"glucose oxidase" OR"GOx"OR"GOD"OR"glucose dehydrogenase"OR"catalase*"OR"CAT"OR"immobiliz*")AND("gluconic*"OR"gluconate*")AND"cell*" to identify correlated studies. A systematic search (Romanelli *et al.*, 2021a) of the peer-reviewed literature was performed from the Web of Science platform (bibliographic resources - core collection: Science Citation Index Expanded (SCI-E) and Emerging Sources Citation Index (ESCI)), besides Scopus, and CAB Direct platforms. The SCI-E and ESCI databases were selected within the Web of Science platform because the first one covers the majority of significant scientific results, as well as other online databases that also contain citation information such as Google Scholar and Science Direct, while the ESCI contains complete records of papers indexed by journals not yet covered by SCI-E. Journals indexed in ESCI reach the minimum standards of editorial quality, but as they are relatively new, they are still under evaluation to be indexed in SCI-E. Thus, relevant scientific results that can influence the bibliometric metrics can also be found in this database (Romanelli *et al.*, 2021b). (Complete searching procedures are in Figure S2.1 and Table S2.1 – Supplementary Material).

The publications identified through the search string used to conduct this study were screened based on the inclusion criteria, and relevant data were extracted for further analysis. The inclusion criteria included: (i) articles should be a primary study (Research Paper) about gluconic acid production (i.e., review articles were not eligible for inclusion); (ii) studies should contemplate the GA production by a microorganism; (iii) the microorganism applied in GA production should be in non-growth conditions. The extracted data included information on the microbial strain used as biocatalysts, strategies for non-growth conditions, substrates, and enzymes involved in GA production and immobilization methods.

At first, the search conducted in the three online bibliographic sources previously cited resulted in a total of 3677 published articles. After a coverage analysis followed by overlapping

to remove replicates from the dataset, 2687 publications remained in the retrieved database. The replicates (990 articles) were removed using the analysis of coverage and overlapping (revtools package) of the statistical software R (or RStudio) (R Core, 2019). This analysis is required after retrieving a list of references from different academic bibliographic sources (the fundamental stage of SR). The traditional approach to reviewing the retrieved evidence is to sort the information, locating and removing duplicate entries by viewing titles and DOI (Digital Object Identifier) numbers (Supplementary material).

2.2.2 Screening process

The screening process typically involves multiple stages, including title and abstract screening and full-text screening (Livoreil *et al.*, 2017). To ensure that unrelated publications were not included in the reference list, the screening process was conducted by first analyzing titles and abstracts of the articles retrieved from the investigated databases according to the inclusion criteria previously described.

After this first screening, a total of 159 records remained in our database and were subsequently subjected to a full-text analysis. Finally, 42 primary studies were selected to be included in this study. The methodology employed for the article's selection is summarized in Figure 2.2 and details of the articles eliminated after title/abstract screening and full-text analysis are shown in Table S2.2 (supplementary material).

2.2.3 Data synthesis and handling

The articles selected according to our inclusion criteria (n = 42) were submitted to data extraction addressing information on microbial strains used as biocatalysts, strategies for whole-cell application as biocatalysts (non-growth conditions), substrates and enzymes involved in gluconic acid production and whole-cell immobilization methods. Data extraction consists of systematically extracting relevant information from the articles included in the evidence synthesis (Collaboration for Environmental Evidence, 2022).

After data extraction, the articles meeting our inclusion criteria (n=42) were subjected to data synthesis and handling. Considering the limited literature existing on GA production by whole-cells, relevant aspects in this field remain to be clarified. This review aimed to elucidate some of these relevant aspects, including strategies for whole-cell application as biocatalysts on GA production, microorganisms applied, respective enzymatic pathways, as well as substrates and immobilization methods used for GA production by whole-cell biocatalysis. The extracted data were analyzed using MS Excel (v. 2016) to perform calculations. Graphs were plotted using MS Excel (v. 2016) and VOSviewer (v. 1.16.15). VOSviewer is a software tool

designed specifically for bibliometric data analysis (van Eck; Waltman, 2009; Gonçalves *et al.*, 2019). VOS viewer's text mining functions were used to construct and visualize co-occurrences of terms, enabling the identification of gaps and trends across the literature included.

A co-occurrence analysis of substrates, multi-enzymatic systems, and immobilization techniques present in our database was conducted through the construction of bibliometric maps (van Eck; Waltman, 2009).

The co-occurrence analysis can be understood as the counting of paired data within a 'collection unit'. For example, considering the term 'immobilization techniques' as a whole group of methodologies for immobilizing whole-cells, within this group there may be 'entrapment in alginate beads (EAB)' and 'cross-linking with glutaraldehyde (GLU)' as an example of co-occurrence if they are both cited in the same study. Here, the data are the 'EAB' and the 'GLU', and the 'collection unit' is the group 'immobilization techniques'. In this example, the paired data are {EAB and GLU} and they occur once. Of course, more pretreatment methods can be cited at the same time in a primary study, so the pairings become more numerous as each item (e.g., EAB) is paired with each other item. For instance, if in addition to the two immobilization techniques, a third is also cited, say, pretreatment with polyethyleneimine (PEI), then there are three pairings ({EAB and GLU}, {EAB and PEI}, {GLU and PEI}), each with a count of one. The 'collection unit' and the data therein can be varied (e.g., a questionnaire with the responses to the questions as the elements or a paper's bibliography with each cited author as the focus of the analysis). The combination of all the paired data within a "collection unit" forms the support for the analysis.

In the present study, data were analyzed from three different 'collection units': substrates, multi-enzymatic systems, and immobilization techniques. The Excel spreadsheet with the categorization of our data into 'collection units' is provided as supplementary material to this review (co-occurrence analysis).

The analysis of co-occurrence aims to investigate the level of association among items of a 'collection unit'. If a pairing is done only once in the support, the association is tenuous or spurious. If the pairing is done many times, then the association is made stronger with each additional pairing. In the previous example, both pretreatment methods {EAB and GLU} were cited within the same study. If only one or two papers performed this scope of research and cited both methods, we would have little association. On the other hand, following the previously described example, if many studies cited the combination {EAB and PEI} to perform their analysis, then we would have an extremely strong relationship between these two components for that certain scope of study.

2.2.4 Critical appraisal of study validity

Recognizing the possibility of subjective decisions in the screening stage (inclusion-exclusions) (Collaboration for Environmental Evidence, 2022), a sample of retrieved papers (n = 40) was also analyzed by a second assessor. To achieve a high level of repeatability, the assessors' decisions were compared using Cohen's kappa test of agreement. Scoring decisions (between assessors 1 and 2) were analyzed considering the percentage of agreement between assessors. Kappa values may range from 0 to 1, and higher values indicate greater agreement. Scores lower than 0.6 indicate inconsistencies among assessors so the inclusion criteria must be redefined (Cohen, 1960). Further information on the kappa test is provided in the supplementary material (Table S2.3). As a result, a Kappa score of 0.826 (95% Lower/Upper Confidence Limit) was obtained, indicating good agreement between the reviewer's decisions (Table S2.3 – Supplementary Material). Figure 2.2 summarizes the flow diagram for primary studies selection.

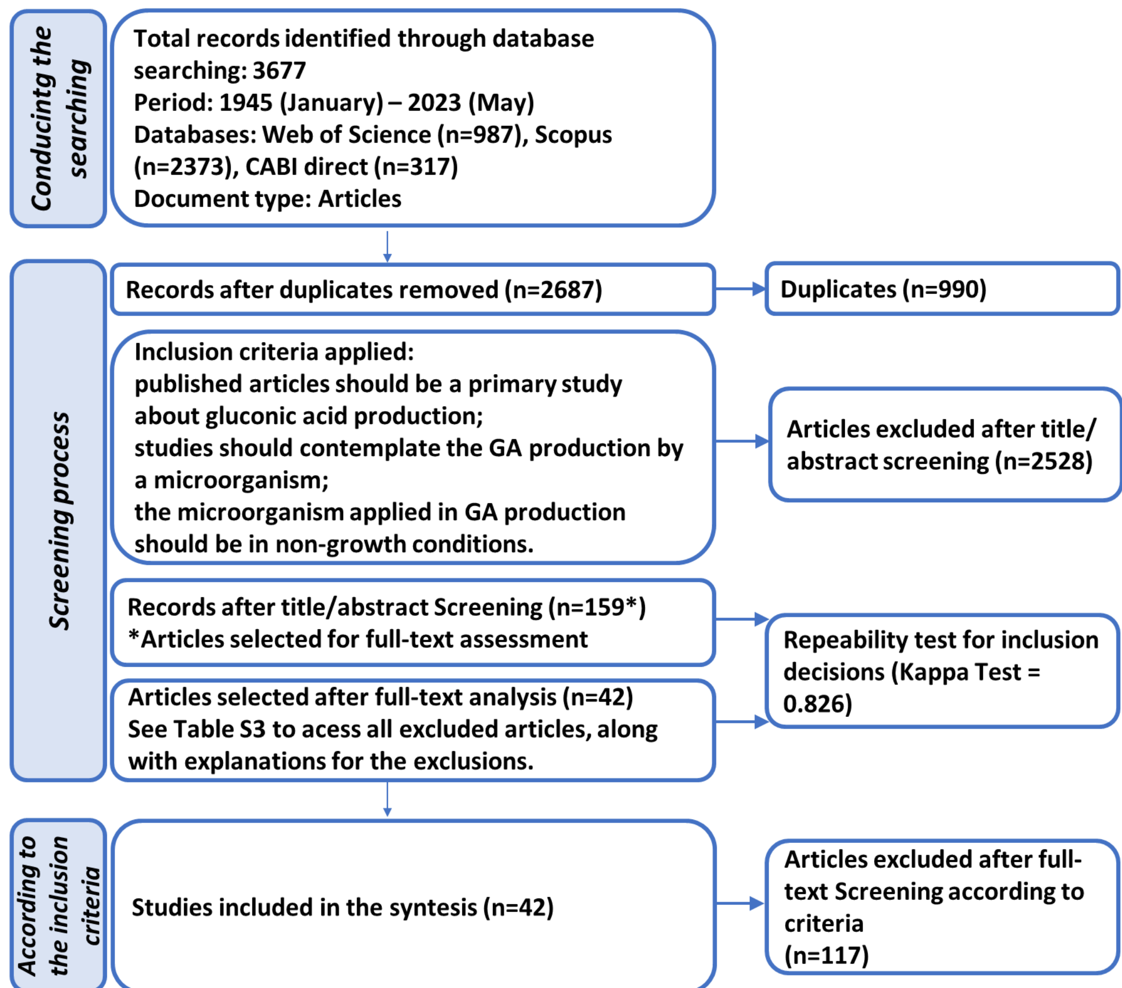


Figure 2.2. Flow diagram for primary studies selection. (Adapted from ROSES Flow Diagram for Systematic Maps. Version 1.0).

Additionally, to promote transparency in the SR principles application, an Excel spreadsheet containing all articles excluded after the full-text analysis is provided along with the respective reasons for the exclusion (Supplementary material).

2.3 Results and discussion (interpreting findings)

2.3.1 Operational strategies for GA production by whole-cell biocatalysis

As previously mentioned, whole-cell biocatalysis requires environmental conditions in which cells are maintained in a state of non-growth, but cells remain metabolically active to produce a target biotechnological compound (Lin; Tao, 2017). Non-growth conditions can be induced through various approaches, including stationary phase, nutrient starvation, osmotic pressure, pH, temperature, and the presence of growth-inhibiting or toxic compounds (Werner-Washburne *et al.*, 1996; Gasch; Werner-Washburne, 2002; Rajpurohit; Eiteman, 2022).

Different strategies for whole-cell biocatalysis on GA production were identified in selected studies. The most applied was nitrogen-starvation (NS); followed by growth inhibition by a chemical compound (GICC); growth limited by immobilization (GLI), and high temperature (HT), according to Figure 2.3.

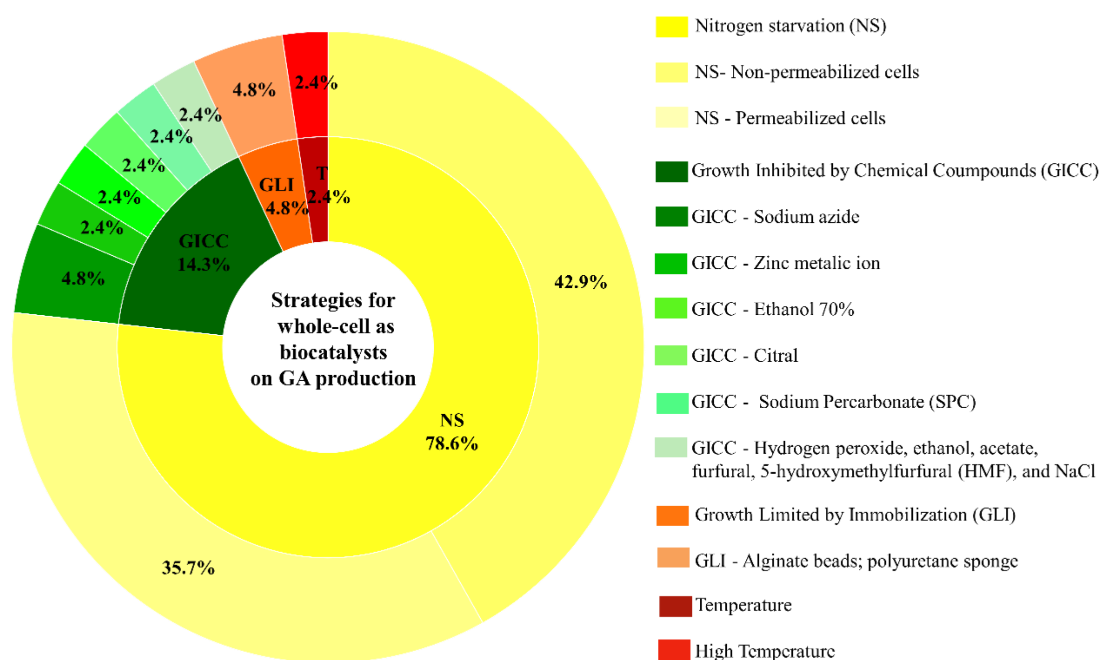


Figure 2.3. Proportions of the main strategies for non-growth conditions on GA production by whole-cell biocatalysis in the selected articles.

From the cells' perspective, nutrient limitation is quite different from nutrient starvation, the latter of which is often called a resting cell or whole-cell (Förberg; Häggström, 1987). Nutrient limitation permits continued, possibly steady-state growth, whereas starvation in one or more essential nutrients induces dynamic stress responses and ultimately prevents further growth (Gasch; Werner-Washburne, 2002; Rajpurohit; Eiteman, 2022).

Nitrogen starvation was present in most of the selected articles (78.6%). By depriving microorganisms of nitrogen, their metabolism is suppressed, typically undergoing metabolic changes to adapt to the lack of nitrogen to use available resources (Ng, 2020; Lin; Tao, 2017). Therefore, the carbon source can be directed solely toward a desired product rather than biomass production, enhancing the production of target compounds such as GA. This can lead to higher yields and improved productivity (Woodley, 2006).

Even under the operational strategy of nitrogen starvation, *Zymomonas mobilis* (*Z. mobilis*) can use glucose and fructose as a substrate for ethanol production (it will be discussed in more detail in section 2.3.2). Because of this, 87.5% of the selected studies that used this microorganism as a whole-cell biocatalyst for GA production, used the cell permeabilization technique mainly by toluene for the inactivation of the fermentative metabolism of *Z. mobilis*, since metallic ions and cofactors diffuse out of the cells inactivating the pathway from GA to ethanol (Chun; Rogers, 1988); Rehr; Wilhelm; Sahm, 1991).

One additional advantage of employing nitrogen starvation conditions is the cost savings associated with the elimination of nitrogen supplementation salts. In conventional fermentation processes, nitrogen sources such as ammonium salts are added to provide the necessary nitrogen for microorganism growth and cell maintenance. Thus, by eliminating the need for these supplementation salts, the overall cost of the process is reduced.

Growth inhibition by a chemical compound was the strategy for whole-cell biocatalysis in 14.3% of selected studies. Chemical compounds that are toxic to microbial cells can also be used to inhibit growth. These compounds can be added to the growth medium at concentrations that do not kill the cells, but significantly slow down their growth rate. Examples of such compounds include heavy metals, organic solvents, or other toxic substances (Barreiro; Pratt, 1992). For the growth inhibition of *Gluconobacter oxydans*, the chemical compounds applied were zinc metallic (Zhou *et al.*, 2017), ethanol 70% (v/v) (Mehmetoglu; Ateş; Berber, 1996), sodium percarbonate (SPC) (Han *et al.*, 2023). *A. niger* growth was inhibited by sodium azide; and citral (Ramachandran *et al.*, 2008). *Z. mobilis* growth was inhibited by hydrogen peroxide, ethanol, acetate, furfural, 5-hydroxymethylfurfural (HMF), and NaCl (Alvin *et al.*, 2017).

The impact of immobilization on cellular growth depends on several factors, including the specific cell type, immobilization method, support matrix, and microenvironmental conditions (Wessel *et al.*, 2013). Depending on the immobilization method and support matrix used, cells may experience mechanical constraints that can affect their growth. For example, encapsulating cells in a rigid gel or attaching them to a solid surface can restrict their ability to expand and proliferate (Hol; Dekker, 2014; Liu *et al.*, 2022). Thus, the immobilization matrix or carrier material provides a suitable environment for the cells to function without the need for continuous growth or proliferation for the conversion of substrates into valuable products.

Although 61.9% of the selected studies applied immobilization techniques, here were considered only 4.8% of the studies that explicitly cited microrganism growth limited by cell immobilization. These studies exploited only the one entrapment immobilization method on two types of supports, alginate beads and polyurethane sponge (Petruccioli *et al.*, 1994; Singh, 2008). However, entrapment in calcium alginate is not considered an appropriate immobilization method for GA production processes due to its excellent chelator property, which ends up complexing calcium from calcium alginate, resulting in the rupture of the spheres (Ferraz; Borges; Alves, 2000; An; Hu; Bao, 2013). Numerous well-established cell immobilization techniques, such as entrapment in various solid supports, adsorption, covalent attachment, membrane encapsulation and surface modification, have been applied in diverse bioprocesses (Lou *et al.*, 2021). However, as revealed by this review, there is a scarcity of available studies exploring diverse immobilization techniques for the application of microorganisms as whole-cell biocatalysts in GA production. This observation sheds light on the considerable research opportunities within this field.

At last, by shifting the temperature of a culture to a non-permissive temperature for growth, cells can be rendered non-dividing while still being viable. Han *et al.* (2018) reported the utilization of a higher temperature, inhibiting the growth of *P. oxalicum*. However, cellulases, GOD, and CAT activities were retained as the operational strategy for GA production by whole-cell biocatalysis, corresponding to 2.4% of the selected studies.

2.3.2 Microrganisms applied as whole-cells biocatalysts to produce gluconic acid

The whole-cell approach recognizes that the behavior and function of a cell emerge from the complex interplay of numerous molecular components, pathways, and regulatory networks (Lin; Tao, 2017). By studying cells as holistic systems, researchers aim for a more comprehensive understanding of their functioning, behavior, and responses to various stimuli or perturbations.

From GA's production perspective, any microorganisms able to produce GA from the oxidation of glucose as a non-growth-associated product have the potential to be applied as whole-cell biocatalysts for GA production. This section 2.3.2 aimed for a more comprehensive understanding of how the main strains of bacteria or fungi utilize different sets of enzymes or alternative pathways to produce GA.

The naturally occurring (not engineered) microorganisms reported as whole-cell biocatalysts by selected studies were *Zymomonas mobilis* (*Z. mobilis*), *Aspergillus niger* (*A. niger*) *and Glucanobacter oxydans* (*G. oxydans*). Additionally, engineered microrganisms reported were *Saccharomyces cerevisiae* (*S. cerevisiae*) *Penicillium species* (*Penicillium spp.*) and *Escherichia coli* (*E. coli*), as summarized in Figure 2.4.

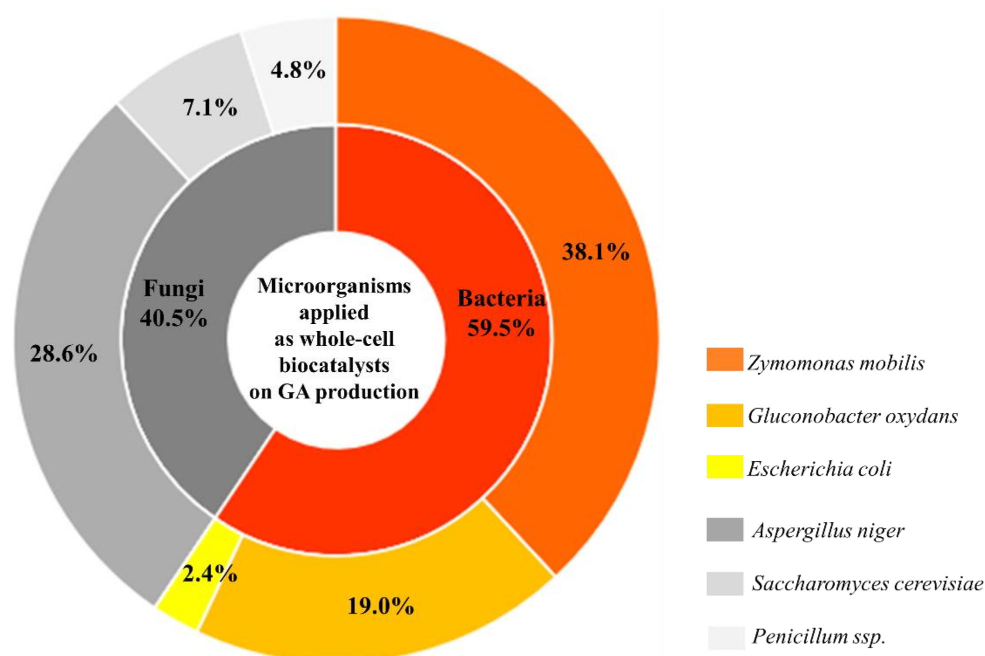


Figure 2.4. Microorganisms were applied as whole-cell biocatalysts to produce GA in selected studies.

Z. mobilis was the microrganism most applied as whole-cell biocatalysts in the selected studies (38.1%). This bacterium is known for its ability to efficiently ferment various sugars into ethanol through the Entner-Doudoroff pathway (Rogers *et al.*, 2007; He *et al.*, 2014).

However, under non-growth conditions, *Z. mobilis* can be applied as a biocatalyst for the biotechnological production of sorbitol and GA through two key enzymes: glucose-fructose oxidoreductase (GFOR) and gluconolactonase (GL) (Erzinger; Vitolo, 1996). The action of GFOR and GL enzymes in *Z. mobilis* whole-cells leads to the conversion of glucose to GA and fructose to sorbitol is highlighted in green in Figure 2.5.

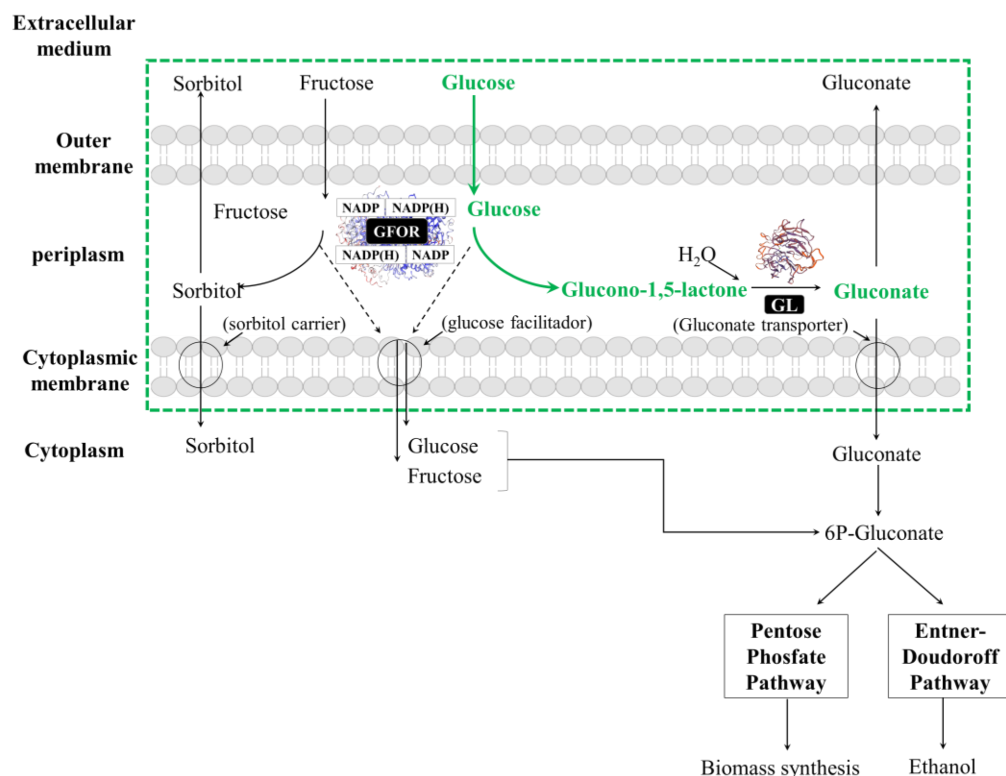


Figure 2.5. *Z. mobilis* production and utilization of gluconate and sorbitol from glucose and fructose. The periplasmic enzyme glucose-fructose oxidoreductase (GFOR) with a tightly bound NADP (EC 1.1.99.28) converts the two sugars to D-gluconolactone and D-sorbitol. Gluconolactone is hydrolyzed to gluconate (gluconic acid) by a periplasmic gluconolactonase, GL (EC 3.1.1.17). During normal metabolism, gluconate is utilized for carbon and energy via the Entner-Doudoroff pathway. Some glucose and fructose also are utilized. (Redrawn from Rogers *et al.* (2013), with permission).

During normal *Z. mobilis* metabolism, gluconate is utilized for carbon and energy via the Entner-Doudoroff pathway to produce ethanol. When normal metabolism is blocked, gluconate and sorbitol are manufactured and exit the cell's periplasm in equimolar amounts. One of the most common approaches used to block *Z. mobilis* metabolism is the use of permeabilized cells. This methodology allowed the inactivation of the fermentative metabolism of *Z. mobilis* since metallic ions and cofactors diffuse out of the cells inactivating the pathway to ethanol (Chun; Rogers, 1988); Rehr; Wilhelm; Sahm, 1991).

The mechanism of GA and sorbitol production by GFOR was first described by Zachariout & Scopes (1986). This reaction involves the transfer of electrons from glucose to a cofactor, usually NAD(P)H, resulting in the production of gluconic acid and sorbitol. Glucono-1,5-lactone, the product of the GFOR reaction, is subsequently hydrolyzed by GL to form GA. GL is an enzyme that catalyzes the hydrolysis of glucono-1,5-lactone, which spontaneously opens to form GA (Intasian *et al*., 2021) (Figure 2.6).

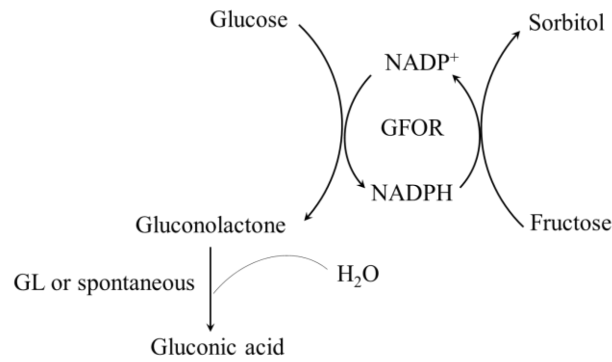


Figure 2.6. The mechanism for GA production by *Z. mobilis* whole-cell biocatalysis. Adapted from (Intasian *et al.*, 2021). GFOR, NADP-glucose-fructose oxidoreductase. GL, gluconolactonase.

A. niger was the second microrganism most applied for whole-cell biocatalysis for GA production in selected studies (28.6%). The properties of *A. niger* allow GA production under both growth and non-growth conditions (Znad; Markoš; Baleš, 2004). Under growth conditions, as in traditional fermentative processes, in addition to producing GA, the *A. niger* also uses the carbon source for biomass growth, citric acid, and oxalic acid production (Cameselle *et al.*, 1998). In the case of non-growth conditions (whole-cell biocatalysis), the concentration of biomass in the liquid phase is constant, and oxygen and glucose are consumed only for the GA production through glucose oxidase (GOD) and catalase (CAT) membrane-associated enzymes, according to the mechanism highlighted in green in Figure 2.7.

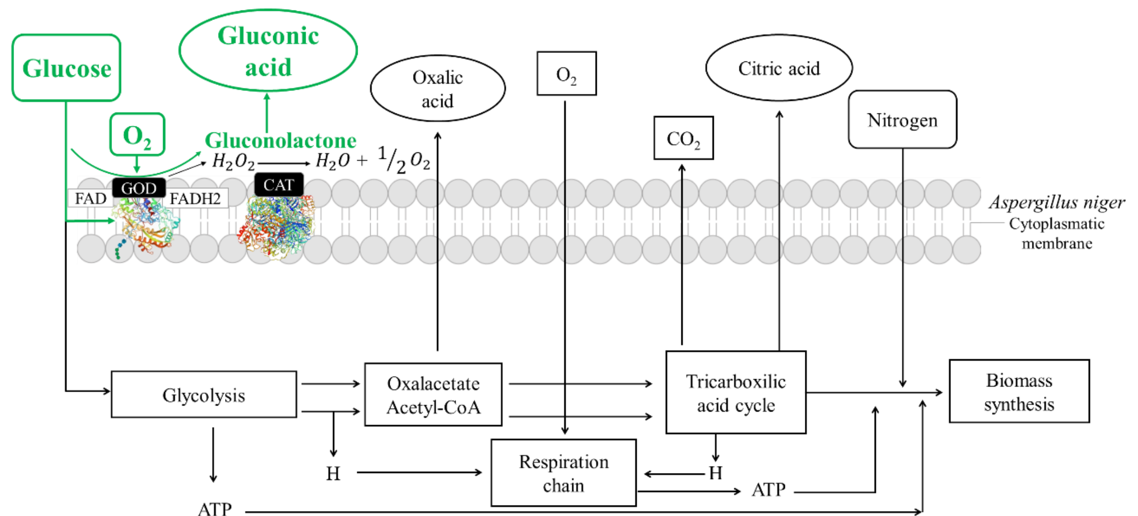


Figure 2.7. Glucose metabolism in *A. niger*. GA production pathway by *A. niger* whole-cells biocatalysis is highlighted in green. GOD, FAD-dependent glucose oxidase (EC 1.1.3.4); CAT, catalase (EC 1.11.1.6). Adapted from (Cameselle *et al.*, 1998), with permission.

A. niger cells have glucose oxidase (GOD) and catalase (CAT) membrane-associated enzymes (Witteveen; Veenhuis; Visser, 1992). This allows even metabolically inert cells after storage at -20°C for 3 months can convert glucose to GA without any loss of enzyme activity (Moksia; Larroche; Gros, 1996; Ramachandran *et al.*, 2008).

GOD catalyzes the oxidation of glucose to produce GA. This reaction involves the transfer of electrons from glucose to molecular oxygen, resulting in the production of GA and the release of hydrogen peroxide as a byproduct. The hydrogen peroxide generated during the glucose oxidation step is potentially inactivating the GOD enzymes. However, CAT rapidly breaks down hydrogen peroxide into water and molecular oxygen, preventing its accumulation and maintaining a favorable environment to produce GA (Ramachandran *et al.*, 2006) (Figure 2.8).

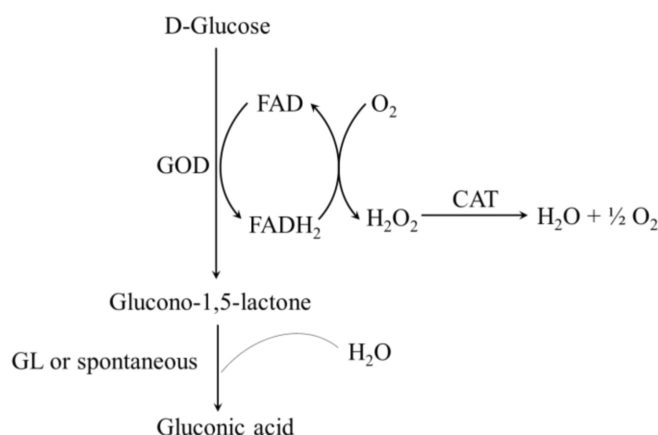


Figure 2.8. The mechanism for GA production by *A. niger* whole-cells biocatalysis. Adapted from (Ramachandran *et al.*, 2006). GOD, FAD-dependent glucose oxidase (EC 1.1.3.4); CAT, catalase (EC 1.11.1.6). GL, gluconolactonase (EC 3.1.1.17).

In addition to cost-effectiveness, a relevant advantage in the use of *A. niger* whole cells as biocatalysts compared to commercial enzymes in this process is to avoid the risks of glucose oxidase inactivation by hydrogen peroxide, as the whole metabolic machinery may eliminate the hydrogen peroxide produced (Kornecki *et al.*, 2020).

G. oxydans has a variety of membrane-bound dehydrogenases on cell membranes with unique oxidation capacities. Sugars can be oxidized directly into corresponding acids (Zhang *et al.*, 2016a, 2016b). Moreover, since these dehydrogenases are located on the cell membrane, the products are released directly into the extracellular matrix without passing through the cell, which greatly increases the efficiency and yield of biocatalysis (Hua *et al.*, 2020). *G. oxydans* thus has a wide range of industrial applications for the production of biobased platform compounds such as xylonic acid (XA) (Hahn *et al.*, 2020), gluconic acid (GA) (Zhou *et al.*, 2018), and 1, 3-dihydroxyacetone (DHA) (Zhou *et al.*, 2016). Therefore, *G. oxydans* is a gram-

negative bacterium known for its ability to oxidize glucose mainly to GA. *G. oxydans* uptakes glucose from the surrounding environment through its cell membrane as the substrate for GA production. Inside the cell, glucose is oxidized by the enzyme glucose dehydrogenase (GDH). The oxidation catalyzed by membrane-bound GDH is a nongrowth-associated process (Macauley; McNeil; Harvey, 2001; Zhou *et al.*, 2018). The *G. oxydans* whole-cells pathway for GA production is highlighted in green in Figure 2.9.

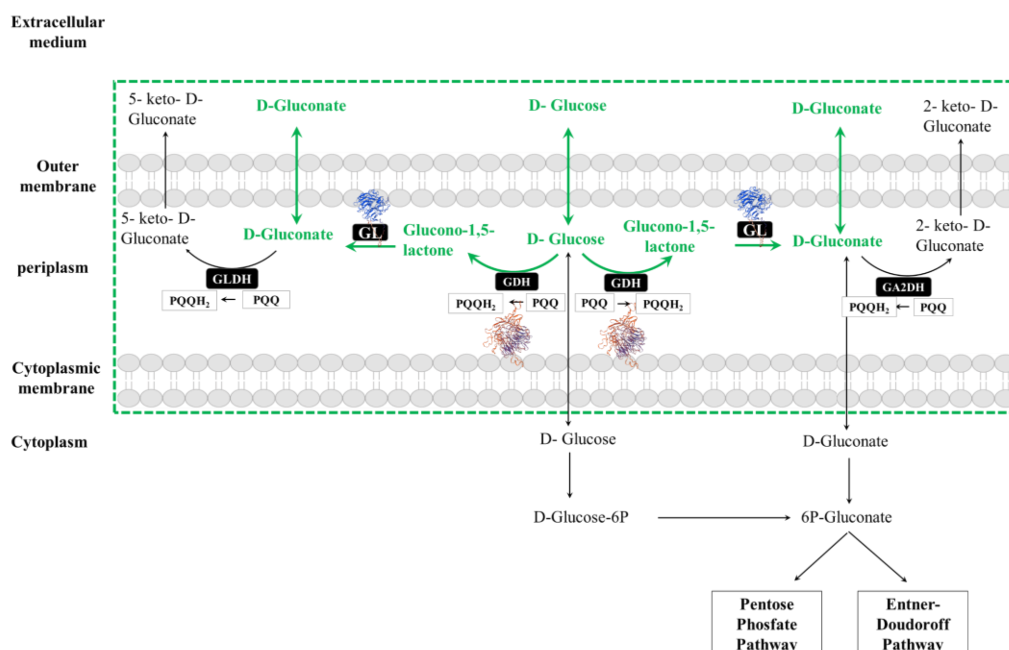


Figure 2.9. *G. oxydans* production and utilization of gluconate from glucose. GA production pathway by *G. oxydans* whole-cells biocatalysis highlighted in green. GDH, PQQ-dependent glucose dehydrogenase (EC 1.1.99.17); GLDH, PQQ-dependent glycerol dehydrogenase (EC 1.1.99.22); GA2DH, FAD-dependent gluconate-2-dehydrogenase (EC 1.1.1.215) are located on the outer surface of the cytoplasmic membrane (Adapted from Silva *et al.* (2022), with permission). GL, gluconolactonase (EC 3.1.1.17).

G. oxydans can rapidly and incompletely oxidize glucose to GA. Once glucose is depleted, the gluconic acid will be further bio-oxidized to the unwanted by-products 2-keto gluconic acid and 5-keto gluconic acid. The endpoint is difficult to be controlled, especially in an industrial fermentation process (Dai *et al.*, 2023)

GDH catalyzes the conversion of glucose to glucono-1,5-lactone while simultaneously reducing the coenzyme pyrroloquinoline quinone (PQQ) to its reduced form ($PQQH_2$). This reaction also generates two protons (H^+) as by-products. After glucose oxidation, the reduced form of PQQ ($PQQH_2$) needs to be regenerated to its oxidized form (PQQ) for the glucose oxidation process to continue. *G. oxydans* accomplishes this regeneration by transferring electrons from $PQQH_2$ to molecular oxygen (O_2) in a process known as oxidative phosphorylation. This electron transfer generates water (H_2O) as a byproduct and restores PQQ to its oxidized

form, allowing GDH to continue oxidizing glucose. The glucono-1,5-lactone produced in the previous step is inherently unstable and tends to hydrolyze spontaneously to gluconic acid. Furthermore+, *G. oxydans* possesses GL enzymes that accelerate the hydrolysis of glucono-1,5-lactone, resulting in the formation of gluconic acid (Hölscher *et al.*, 2009) (Figure 2.10).

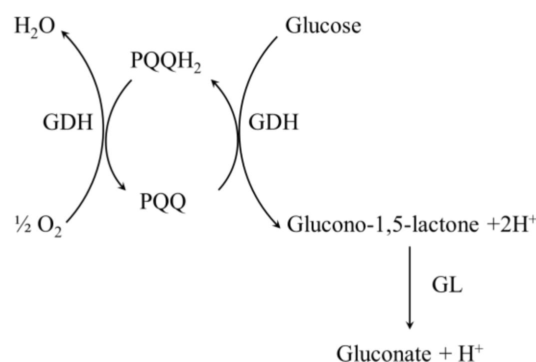


Figure 2.10. The mechanism for GA production by *G. oxydans* whole-cells biocatalysis. GDH, PQQ-dependent glucose dehydrogenase (EC 1.1.99.17). GL, gluconolactonase (EC 3.1.1.17).

By repeating these steps, *G. oxydans* whole-cells efficiently convert glucose to GA. In non-growth conditions (metabolism suppressed), the produced gluconic acid tends to be excreted into the surrounding medium instead of being consumed by the metabolism.

With recent advances in genomic and genetic engineering, interest in the use of whole-cell biocatalysis for industrial synthetic chemistry is growing rapidly (Liu *et al.*, 2017). Whole-cell biocatalysis using engineered microorganisms uses microorganisms that have been genetically modified to possess specific enzymatic capabilities for use in various biotechnological applications. These microorganisms are designed to express enzymes or metabolic pathways that can efficiently catalyze specific chemical reactions (Lin; Tao, 2017). Through genetic engineering techniques, the metabolic pathways of microorganisms can be modified by introducing or overexpressing genes encoding enzymes involved in GA synthesis or manipulating the expression of existing genes to redirect metabolic flux toward GA production. Although this approach allows for the development of more efficient whole-cell biocatalysts, only 11.9% of the selected studies applied engineered microorganisms as whole-cell biocatalysts.

E. coli is a commonly used organism in synthetic biology and genetic engineering due to its well-characterized genetics, rapid growth, and ease of manipulation. It is primarily known as a bacterial host for recombinant protein production and is often used as a chassis organism for various biotechnological applications. Although the primary pathway involved in gluconate metabolism in *E. coli* is the Entner-Doudoroff (EDP) pathway, it also can be engineered to

produce GA. In a recent study (Feng *et al.*, 2023), the enzymes D-psicose-3-epimerase (DPEase), glucose dehydrogenase (GDH), and ribitol dehydrogenase (RDH) were heterologously expressed in whole-cells of *E. coli*, allowing for the co-production of both allitol and GA (Figure 2.11).

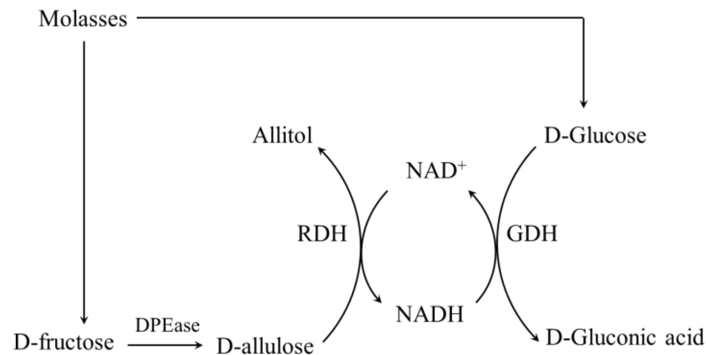


Figure 2.11. The mechanism for GA and allitol co-production by engineered *E. coli* whole-cells. Adapted from (Feng *et al.*, 2023). DPEase, D-psicose-3-epimerase. GDH, glucose dehydrogenase. RDH, ribitol dehydrogenase.

Saccharomyces cerevisiae, known as bakers' yeast, is one of the most utilized yeasts in industry, mainly for ethanol fermentation. However, several enzymes that are naturally produced by yeast, such as invertase and catalase, combined with heterologously expressed GOD, represent the enzyme machinery for fructose and GA production (Kovačević *et al.*, 2022) (Figure 2.12).

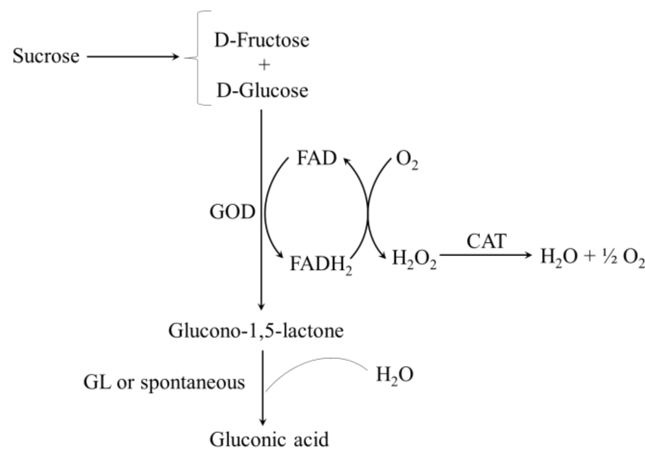


Figure 2.12. The mechanism for GA and fructose co-production by engineered *S. cerevisiae* whole-cells. GOD, FAD-dependent glucose oxidase (EC 1.1.3.4); CAT, catalase (EC 1.11.1.6).

Therefore, *Saccharomyces cerevisiae* can be engineered to heterologously express GOD or also associated with GOD through immobilization techniques (D'Souza; Nadkarni, 1980; D'Souza; Melo, 1991) to produce GA and fructose from sucrose using whole-cell biocatalysis.

Although several *Penicillium sp.* (at least twenty-five) are known for producing gluconic acid through the fermentative process long since (Elnaghy; Megalia, 1975), a more recent study has evaluated the use of *Penicillium* engineered to produce GA using whole-cells as biocatalysts for simultaneous expression of glucose oxidase and catalase from *A. niger* (Han *et al.*, 2018). The mechanism for GA production by engineered *Penicillium ssp.* whole-cells is illustrated in Figure 2.13.

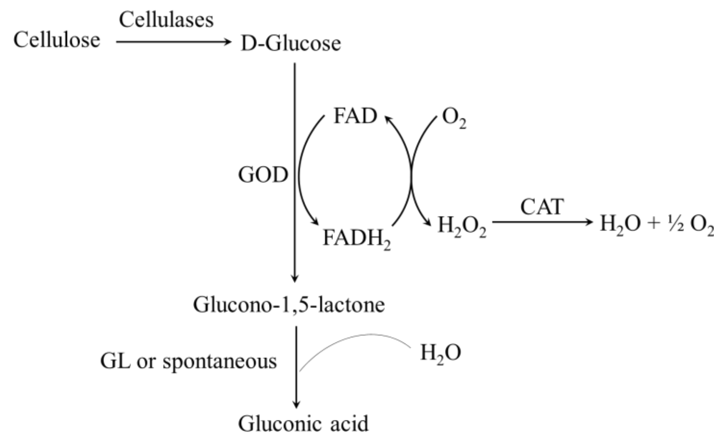


Figure 2.13. The mechanism for GA production by engineered *Penicillium ssp*. whole-cells. Adapted from (Han *et al.*, 2018). GOD, FAD-dependent glucose oxidase (EC 1.1.3.4); CAT, catalase (EC 1.11.1.6).

Han *et al.* (2018) reported that after 120 h of fermentation when *P. oxalicum* had already grown and produced cellulase, the temperature of the culture was raised to 45 °C. At this temperature, *P. oxalicum* could not grow; however, cellulases, GOD, and CAT activities were retained. In this way, this study highlights that, in addition to combining commercial enzymes and whole-cell biocatalysis, the latter can also be integrated with traditional fermentation processes to obtain the desired multi-enzyme complex at a low cost.

These were the microorganisms applied as whole-cell biocatalysts for GA production in the selected articles according to the methodology described in Section 2.2. However, there are several microorganisms able to produce GA such as *Pseudomonas*, *Acetobacter*, *Candida*, *Fusarium*, *Gliocadium species*, and yeasts such as *Aureobasidium pullulans* (Van Schie *et al.*, 1987; Anastassiadis *et al.*, 2005; Shindia *et al.*, 2006). The choice of the microorganism depends on factors such as substrate availability, production efficiency, and downstream processing considerations.

2.3.3 Feedstoks for gluconic acid production by whole-cells

Section 2.3.2 showed that although microorganisms have different enzyme sets and alternative pathways to produce GA, all microorganisms uptake glucose as substrate. However,

GA production from pure glucose has been deemed costly and so research has been conducted to find more economically friendly feedstock (Crain-Zamora, 2017). Alternatively, several sources including corn starch, sugarcane, other carbohydrate-rich materials, and even agri-food industry wastes (residues after processing of cereals, corn, oilseeds, sugar beet bagasse, molasses, whey, starchy, lignocellulosic biomass) that can be hydrolyzed to glucose, are potential substrates for GA production (Cañete-Rodríguez *et al.*, 2016) (Figure 2.14).

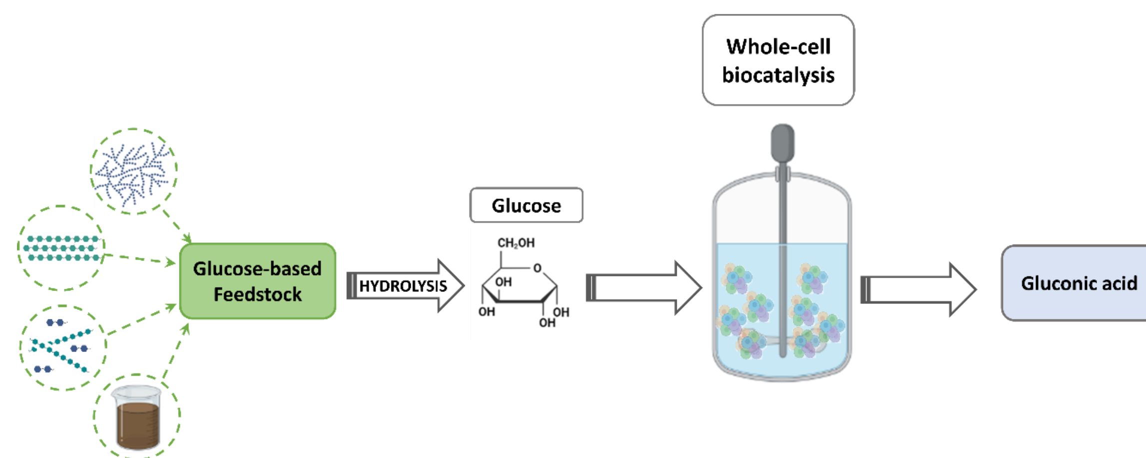


Figure 2.14. General scheme of the process to produce GA by whole-cell biocatalysis from glucose-based feedstocks. Personal collection, 2023.

Hydrolysis refers to the cleavage of chemical bonds in the presence of a water molecule. It can be acid hydrolysis and enzymatic hydrolysis. The main difference between both is that acid hydrolysis is a chemical process, whereas enzymatic hydrolysis is a biochemical process (Amezcua-Allieri; Sánchez Durán; Aburto, 2017).

Although several glucose-based feedstocks can be hydrolyzed to glucose, including wastes from the agri-food industry, glucose was the most applied substrate to GA production by whole-cells in selected studies (66.7%).

Network maps allowed us to elucidate the structure, network distribution, and frequency of the co-occurrence of related terms, to clarify the research hotspots in the field of GA production by whole-cells (Gao; Huang; Zhang, 2019). Our analysis revealed that 11 substrates were cited by the studies here investigated. The highest number of occurrences was shown by glucose (66.7%), sucrose (11.9%), lignocellulosic hydrolysate (4.8%), cane molasses (4.8%), and (2.4%) of each of the other substrates (waste of office paper, cellulose, Jerusalem-artichoke, potato waste, hydrol, and grape must) (Figure 2.15).

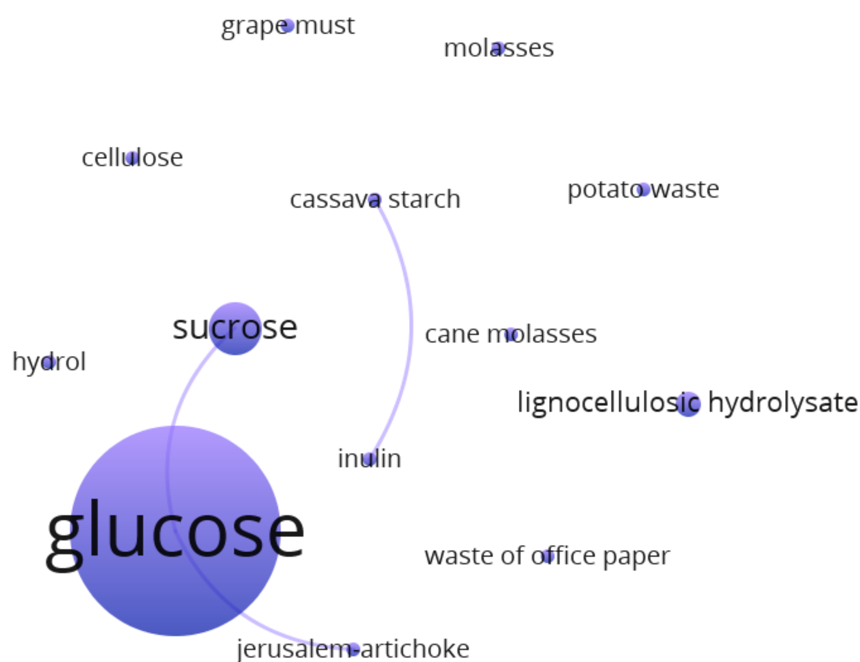


Figure 2.15. Network analysis of substrates used for GA production by whole-cell biocatalysis in selected studies.

Some of the selected studies cited fructose as a substrate due to the equimolar feeding of glucose and fructose for the simultaneous production of GA and sorbitol, respectively, according to the mechanism illustrated by Figure 2.5, when *Z. mobilis* was applied as whole-cells. However, fructose was not included in the network map since it results in sorbitol. Therefore, the few available that applied alternative glucose sources for GA production by whole-cell biocatalysis make clear the opportunities in this research field.

The association of intracellular enzymes (whole-cells) with added commercial enzymes can provide a suitable multi-enzymatic system for the conversion of a huge variety of glucose-based feedstocks to GA. Different combinations of intracellular enzymes (whole-cells) with commercial enzymes were applied to convert the substrates to GA as shown by the network map following (Figure 2.16).

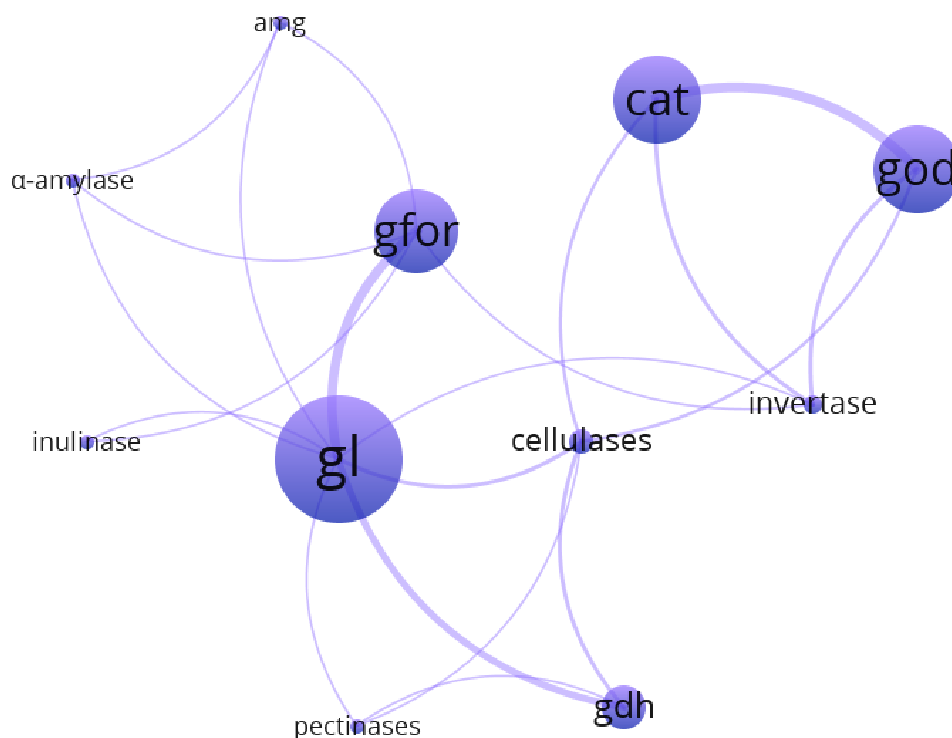


Figure 2.16. Network analysis of intracellular enzymes (whole-cells) associated with extracellular enzymes for GA production in selected studies. GOD, FAD-dependent glucose oxidase (EC 1.1.3.4); GL, gluconolactonase (EC 3.1.1.17); and CAT, catalase (EC 1.11.1.6). GFOR, NADP-dependent glucose-fructose oxidoreductase (GFOR) and gluconolactonase (GL). GDH, PQQ-dependent glucose dehydrogenase. AMG, amyloglucosidase.

The strongest interactions occurred between GOD and CAT; GFOR and GL; and GDH and GL, corresponding mainly to the different intracellular enzyme sets of the microorganisms *A. niger, Z. mobilis*, *and G. oxydans*, respectively, as shown in Section 2.3.2. Invertase was found as an intracellular and extracellular enzyme to convert sucrose into glucose and fructose. As an intracellular enzyme when *S. cerevisiae* was applied as a whole-cell biocatalyst (D'Souza; Nadkarni, 1980; D'Souza; Melo, 1991; Kovačević *et al.*, 2022), and as an extracellular enzyme trough an addition of commercial invertase when *Z. mobilis* was applied as a whole-cell biocatalyst (Ro; Kim, 1991). The other enzymes such as α-amylases and AMG (An *et al.*, 2013); cellulases (Hou; Zhang; Bao, 2018; Ikeda; Park; Okuda, 2006; Jiang *et al.*, 2017); pectinases (Jiang *et al.*, 2017), and inulinases (Kim; Kim, 1994) were commercial enzymes added for the substrate hydrolysis to glucose.

Although entirely enzymatic processes can overcome the drawbacks of traditional fermentation processes (Kornecki *et al.*, 2020), the high cost of commercial enzymes still makes the use of these biocatalysts on an industrial scale unfeasible. Considering the higher cost of intracellular enzymes compared to extracellular enzymes, due to their more complex

purification process (Sakhuja *et al.*, 2021), the association of commercial enzymes and whole-cells could be a more economically viable alternative to entirely enzymatic processes.

From this perspective, the association of whole-cells and commercial enzymes brings new perspectives for the development of a complete, robust, and reusable whole-cell biocatalyst able to convert several glucose-based feedstocks in GA. The functioning of biocatalysts could be optimized through recombinant biocatalysts (engineered microorganisms) or immobilization techniques (Efremenko *et al.*, 2022).

2.3.4 Immobilization methods for whole-cell biocatalysts to produce gluconic acid

The stability and performance of whole-cell biocatalysts can be preserved and improved by controlled immobilization techniques (Garzón-Posse *et al.*, 2018). Aiming for the development of a stable and reusable whole-cell biocatalyst to produce GA, 61.9% of the studies selected applied immobilization techniques. Table 2.1 shows the percentage of occurrence of immobilization techniques applied in whole-cell biocatalysis for GA production in selected studies.

Table 2.1. Percentage of occurrence of immobilization techniques applied in whole-cell biocatalysis for GA production in selected studies.

Immobilization technique	Occurrence (%)
1) Entrapment in calcium alginate beads	33.3%
2) Cross-linking with glutaraldehyde (GLU)	19.0%
3) Pretreatment with polietilenoimina (PEI)	9.5%
4) Entrapment in polyurethane sponge	9.5%
5) Entrapment in k-carrageenan	7.1%
6) Adsorption on chitin	7.1%
7) Adsorption on hollow fiber membranes	4.8%
8) Adsorption on cellulosic fabric	4.8%
9) Entrapment in chitosan	2.4%
10) Adsorption on active carbon pieces	2.4%
11) Entrapment in pearlite	2.4%
12) Adhesion in cotton thread	2.4%
13) Adsorption on glass wool	2.4%
14) Entrapment in acrylate membrane	2.4%
15) Covalent binding in concanavalin A	2.4%
16) Entrapment in polyacrylamide gel	2.4%
17) Entrapment in polymeric membrane	2.4%
18) Flocculation with polyelectrolytes	2.4%
19) Adsorption in fibrous nylon	2.4%
20) Entrapment in PVA-alginate beads	2.4%
21) Entrapment in agar	2.4%

Network maps of immobilization techniques (Figure 2.18) allowed us to visualize the network distribution and frequency of the co-occurrence of related terms, to clarify the research hotspots in the field of GA production by whole-cell biocatalysis (Gao; Huang; Zhang, 2019). Figure 2.17 presents the network of frequency of the co-occurrence of immobilization techniques applied to whole-cells for the 42 selected studies.

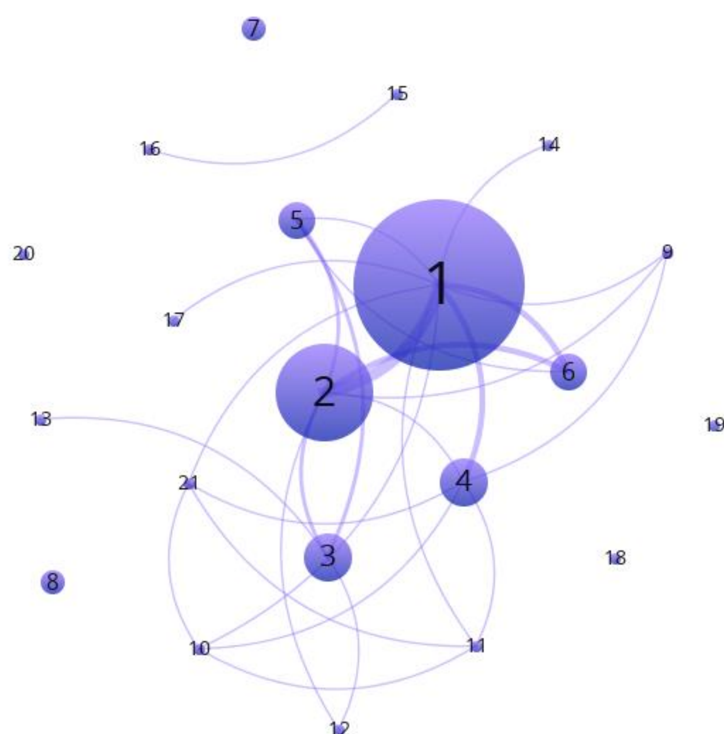


Figure 2.17. Network analysis of immobilization techniques (Table 2.1) used for GA production in selected studies.

Our analysis revealed the application of 19 immobilization techniques in the scrutinized studies (Figure 2.17). The highest number of occurrences was shown by entrapment in calcium alginate (33.3%) and cross-linking with glutaraldehyde (GLU) (19.0%). Although entrapment in calcium alginate beads was the most applied immobilization technique in whole-cell biocatalysts to produce GA, previous works revealed that calcium alginate is not a suitable material for cell immobilization for GA production, as GA acts as a Ca^{2+} chelating agent (Milson; Meers, 1985). Cell leakage from alginate entrapment and crackdown of the structure of the bead occurs when GA is produced. Furthermore, a diffusional barrier generated by the calcium alginate granules hinders the transport of GA from the internal space of the granules to the external environment, resulting in enzymatic inactivation (Malvessi *et al.*, 2010; An; Hu; Bao, 2013).

Ro & Kim (1991) immobilized invertase on chitin by crosslinking with glutaraldehyde. Chitin-immobilized invertase was then co-immobilized with the permeabilized cells of *Z. mobilis* in calcium alginate beads. This approach made it possible to use sucrose, a much cheaper substrate, instead of a glucose solution. However, during continuous production in a CSTR, abrasion of the calcium alginate beads was observed after 60 h of operation, leading to a rapid decrease in the activity of the co-immobilized enzymes. The co-immobilized enzymes remained

stable for 250 h without any loss of activity in the Recirculating Packed Bed Reactor (RPBR), a less shear stress device (Ro; Kim, 1991).

A similar system was applied to the continuous production of GA and sorbitol from Jerusalem-artichoke and glucose using *Z. mobilis* and inulinase. Inulinase was immobilized on chitin by cross-linking with glutaraldehyde. Whole-cells of *Z. mobilis* permeabilized with toluene were co-immobilized with chitin-immobilized inulinase in alginate beads. The co-immobilized system was stable for 250 h in a recycle packed-bed reactor (RPBR) without any loss of activity, while half-life was observed to be about 150 h in a continuous stirred tank reactor (CSTR) (Kim; Kim, 1992).

Ro & Kim (1991) and Kim & Kim (1994) reached promising results in terms of GA production through the utilization of entrapment in alginate beads as an immobilization method, deemed inappropriate. These studies showed the potential for the development of an efficient biocatalyst by immobilization techniques for GA production from cheaper substrates than glucose. An additional advantage is the possibility of applying a continuous process for GA production using a co-immobilized biocatalyst instead of the fed-batch mode currently employed in traditional fermentative processes (Kornecki *et al.*, 2020).

The second most applied technique for whole-cells immobilization is cross-linking with GLU (19.0%), followed by pretreatment with PEI (9.5%). Whole-cells containing enzymes have been successfully immobilized in aqueous media using polyethyleneimine (PEI) and glutaraldehyde (GLU) since the 1980s (Lantero, 1982). Jang *et al.* (1992) reported an improvement in GFOR stability of permeabilized cells of *Zymomonas* mobilis to GA production through glutaraldehyde crosslinking (Jang *et. al*, 1992).

D'souza & Melo (1991) reported a successful method for obtaining co-immobilizates by the simultaneous binding of GOD to the cell wall of *S. cerevisiae*, and the enzyme-bound cell to cotton thread through adhesion using polyethyleneimine (PEI) and cross-linking with 2% glutaraldehyde. The co-immobilizate could be reused for over 10 batches without appreciable loss in activity. The same authors also reconstituted a multienzyme complex consisting of invertase, glucose oxidase, and catalase by binding glucose oxidase to the cell wall of *S. cerevisiae* using concanavalin A (Con A), previously induced for maximal activities of GOD and CAT. The reactor system did not show any loss in efficiency in a continuous operation over 20 days (D'Souza; Nadkarni, 1980)

Although there are few available studies about immobilized whole-cell biocatalysts for GA production, promising results were achieved, making clear the opportunities in this research field.

2.4 Limitations of the work

Reliable evidence reviews must include comprehensive search strategies among the main principles of their approaches so that they can capture as much relevant scientific information as possible (Abdulla *et al.*, 2015). In this work, publications were retrieved for the period from 1945 to 23rd June 2023, using a search string composed of 20 terms, including whole-cell synonyms such as resting or non-growth cells and microbial biocatalyst; and biocatalysis synonyms such as biotransformation, bioconversion, and multi-enzymatic cascade. Therefore, it is acknowledged that some articles related to this theme may not have been found, given the existence of an immense variety of terms to describe biocatalysis with cells under non-growth conditions. Overall, non-growing cells represent an important and complex aspect of biotechnology that is still being studied and understood. However, it is believed that the findings of this study should make an important contribution to the field of GA production by whole-cell biocatalysis since the procedure adopted was according to the principles of a rigorous review methodology that was recently introduced in the field of chemical engineering.

2.5 Final remarks and future perspective

Principles of SR were applied to studies of GA production by whole-cell biocatalysis, reported during the last almost 80 years, aiming to summarize the state-of-the-art and identify research opportunities in this subject area. This work was committed to systematically reviewing, developing, and promoting the evidence base for improving the current knowledge on this topic. The following contributions can be highlighted.

- The results evidenced that the last almost 80 years, were related to a few studies on the production of GA using whole-cell biocatalysis. It can be due to the well-established fermentation process producing GA predominant for decades. Nonetheless, progress in whole-cell biocatalysis, facilitated by genetic engineering and immobilization techniques, introduces novel perspectives for the creation of an efficient, robust, and reusable biocatalyst. This approach could simplify GA production and its downstream processes.
- With recent advances in genomic and genetic engineering, the interest in the use of whole-cell biocatalysis for industrial synthetic chemistry is growing rapidly. However,

few studies were identified in this regard for the development of an engineered whole-cell biocatalyst to produce GA, emphasizing the research opportunities in this field.

- An immobilized or co-immobilized whole-cell biocatalyst allows a continuous process instead of the fed-batch mode currently employed in the traditional fermentative process for GA production.
- The co-occurrence analysis of terms assigned to the articles showed that the immobilization technique most applied for the development of a whole-cell biocatalyst was the entrapment in calcium alginate. However, it is considered an inappropriate immobilization method for GA production processes due to its excellent chelator property, which ends up complexing calcium from calcium alginate, resulting in the rupture of the spheres and enzyme inactivation.
- The association of commercial enzymes with whole-cells can provide different multi-enzymatic systems with better cost-effectiveness than an entirely enzymatic process. These multi-enzymatic systems can convert a huge variety of feedstock glucose-based to GA through simultaneous hydrolysis and biocatalysis.
- Although glucose was most widely used, a large variety of substrates or even residues can be applied to produce GA. The use of agri-food wastes as a variety of abundant sources of glucose is a low-cost and renewable feedstock.
- There was no co-occurrence network analysis between the microorganisms because co-cultures applied to whole-cell biocatalysis for GA production were not identified. However, by combining different microorganisms with complementary intracellular enzymes, a multi-enzymatic system can be established. For example, the combination between *S. cerevisiae* and *A. niger* provides the multi-enzymatic system invertase-GOD-CAT that can convert sucrose in GA.
- The naturally occurring (not engineered) microorganisms reported as whole-cell biocatalysts by selected studies were *A. niger*, *Z. mobilis*, and *G. oxydans*. Additionally, engineered microorganisms reported were *S. cerevisiae*, *Penicillium spp.*, and *E. coli*. However, several other microorganisms known to produce GA by fermentative processes such as *Pseudomonas species*, *Acetobacter species*, *Candida species*, *Fusarium*, *Gliocadium*, and *Aureobasidium pullulans*, among others, present potential to be applied in whole-cell biocatalysis.

These gaps indicate the necessity for research and development to obtain a whole-cell biocatalyst suited to the production of GA, alongside the bioprocess optimization. Overall, this

literature review highlights the main opportunities regarding the application of whole-cell biocatalysis to produce GA and encourages further studies on the development of robust and reusable whole-cell biocatalysts. Such studies will certainly enable the large-scale production of GA by whole-cell biocatalysis and promote novel potential applications.

Supplementary material – Tables

Table S2.1. Search methodology for locating articles addressing to produce gluconic acid by whole-cell biocatalysis. All searches were performed on 23rd June 2023.

Bibliographic sources	**Search String**
Searched terms (1945-2023, Articles)	*"biotransformation" OR "bioconversion" OR "microbial*" OR "whole*" OR "non-grow*" OR "resting" OR "biocatalyst*" OR "cascade*" OR "multienzymatic*" OR "enzyme*" OR "glucose oxidase" OR "GOx" OR "GOD" OR "glucose dehydrogenase" OR "catalase*" OR "CAT" OR "immobiliz*") AND ("gluconic*" OR "gluconate*") AND "cell*"*
Web of Science	*Basic search:* TOPIC (Core collection: SCI-E, ESCI) ***987 records***
Scopus	*Basic search: TITLE-ABS-KEYWORDS* ***2373 records***
CAB Direct	*Basic search: ALL FIELDS* ***317 records***
Total records	**3677**
Total record after removed replicates	**2687**
Records eliminated by title/abstract screening	**2528**
Records selected for full-text screening	**159**

Table S2.2. Records were eliminated after the full-text screening.

Categories	Number of publications
Production of other organic acids	42
GA production by fermentation processes	43
GA production by enzymatic processes	25
GA production by other processes	5
Review	1
Patent	1
	Total: 117

Table S2.3. Interpretation of Kappa statistic.

Kappa	**Interpretation**
<0.0	No agreement
0.0 - 0.20	Slight agreement
0.21 – 0.40	Fair agreement
0.41 – 0.60	Moderate agreement
0.61 – 0.80	Substantial agreement
0.81 – 1.00	Almost perfect agreement

Fonte. Okwuashi *et al*., 2012.

Table S2.4. Data extracted from the selected studies on GA production by whole-cells.

Substrate	**Microorganism**	**Operational strategies for GA production by whole-cell biocatalysis**	**Multi-enzymatic process**	**Immobilization techniques**	**References**
Sucrose; fructose	*Zymomonas mobilis*	Nitrogen absence	GFOR; GL	PEI; Cross-linking (GLU); Entrapment in κ-carrageenan	(Rehr; Wilhelm; Sahm, 1991)
Sucrose	*Saccharomyces cerevisiae*	Nitrogen absence	invertase; GOD; CAT	PEI; Cross-linking (GLU); adhesion in cotton thread	(D'Souza; Melo, 1991)
Glucose	*Aspergillus niger*	Nitrogen absence	GOD; CAT	flocculation with polyelectrolytes	(Mizunuma; Kokufuta; Sato, 2007)
Glucose; fructose	*Zymomonas mobilis*	Nitrogen absence	GFOR; GL	-	(Erzinger *et al.*, 2003)
Sucrose; Glucose	*Aspergillus niger*	Nitrogen absence	GOD; CAT	Adsorption on cellulosic fabric	(Sankpal *et al.*, 2001)
Glucose; fructose	*Zymomonas mobilis*	Nitrogen absence	GFOR; GL	-	(Silveira *et al.*, 1999)
Waste of office paper	*Aspergillus niger*	Nitrogen absence	Cellulases; GOD; CAT	-	(Ikeda; Park; Okuda, 2006)
Glucose	*Gluconobacter oxydans*	Nitrogen absence	GDH; GL	Entrapment in acrylate membrane; Entrapment in alginate beads	(Hartmeier, 1990)
Cane molasses	*Aspergillus niger*	Nitrogen absence	GOD; CAT	Cross-linking (GLU); Entrapment in alginate beads	(Rao; Panda, 1994)
Cellulose	*Penicillium oxalicum*	High Temperature	cellulases; GOD; CAT	-	(Han *et al.*, 2018)
Sucrose	*Saccharomyces cerevisiae*	Nitrogen absence	invertase; GOD; CAT	covalent binding to concanavalin A; Entrapment in polyacrylamide gel	(D'Souza; Nadkarni, 1980)
Jerusalem-artichoke; glucose	*Zymomonas mobilis*	Nitrogen absence	Inulinase; GFOR; GL	Cross-linking (GLU); Adsorption in chitin; Entrapment in alginate beads	(Kim D. M.; Kim H. S., 1992)

Sucrose	*Zymomonas mobilis*	Nitrogen absence	Invertase; GFOR; GL	Cross-linking (GLU); Adsorption in chitin; Entrapment in alginate beads	(Ro; Kim, 1991)
Glucose	*Aspergillus niger*	Nitrogen absence	GOD; CAT	Adsorption on cellulosic fabric	(Sankpal *et al.*, 1999)
Glucose	*Gluconobacter oxydans*	Nitrogen absence	GDH; GL	Adsorption on fibrous nylon	(Seiskari; Linko; Linko, 1985)
Glucose; fructose	*Zymomonas mobilis*	Nitrogen absence	GFOR; GL	Cross-linking (GLU); Entrapment in alginate beads	(Malvessi *et al.*, 2010)
lignocellulosic hydrolysate	*Gluconobacter oxydans*	no (Zn inhibition)	cellulases; GDH; GL	-	(Zhou *et al.*, 2017)
Glucose; fructose	*Zymomonas mobilis*	Nitrogen absence	GFOR; GL	-	(Wilberg; Alves; Nobrega, 1997)
Glucose; fructose	*Zymomonas mobilis*	Nitrogen absence	GFOR; GL	-	(Wisbeck *et al.*, 1997)
Potato waste	*Gluconobacter oxydans*	Nitrogen absence	cellulases; Pectinases; GDH; GL	-	(Jiang *et al.*, 2017)
Glucose	*Penicillium spp.*	Growth limited by immobilization	GOD; CAT	Entrapment in alginate beads; Entrapment in agar; Entrapment in polyurethane sponge; Entrapment in pearlite; Adsorption in active carbon pieces	(Petruccioli *et al.*, 1994)
Glucose; fructose	*Zymomonas mobilis*	Nitrogen absence	GFOR; GL	Cross-linking (GLU); Entrapment in κ-carrageenan; Entrapment in alginate beads; Adsorption on chitin	(Jang; Park; Chun, 1992)
lignocellulosic hydrolysate	*Gluconobacter oxydans*	Growth inhibited by sodium percarbonate (SPC)	GFOR; GL	-	(Han, J. *et al.*, 2023)

Glucose; fructose	*Zymomonas mobilis*	Growth inhibited by hydrogen peroxide, ethanol, acetate, furfural, 5-hydroxymethylfurfural (HMF), and NaCl	GFOR; GL	-	(Alvin *et al.*, 2017)
Glucose	*Gluconobacter oxydans*	Nitrogen absence	GDH; GL	Entrapment in alginate beads	(Tramper; Luyben; Tweel, 1983)
Glucose	*Gluconobacter oxydans*	Nitrogen absence	GDH; GL	Entrapment in polymeric membrane; Entrapment in alginate beads	(Hartmeier; Heinrichs, 1986)
Grape must	*Aspergillus niger*	Growth limited by immobilization	GOD; CAT	Entrapment in alginate beads; Entrapment in polyurethane sponge	(Singh, 2008)
Glucose	*Gluconobacter Suboxydans*	Microrganism inactivated by ethanol 70%	GOD; CAT	Entrapment in alginate beads	(Mehmetoglu; Ateş; Berber, 1996)
Glucose	*Aspergillus niger*	Growth is limited by sodium azide; Growth is limited by citral	GOD; CAT	-	(Ramachandran *et al.*, 2008)
Glucose	*Aspergillus niger*	Nitrogen absence	GOD; CAT	-	(Zhong *et al.*, 2022)
Sucrose	*Saccharomyces cerevisiae*	Nitrogen absence	invertase; GOD; CAT	PEI; Cross-linking (GLU); entrapment in alginate beads; entrapment em chitosan	(Kovačević *et al.*, 2022)
Hydrol	*Aspergillus niger*	Nitrogen absence	GOD; CAT	Entrapment in polyurethane sponge	(Vassilev; Vassileva; Spassova, 1993)
Glucose	*Aspergillus niger*	Nitrogen absence	GFOR; GL	PEI; Adsorption on glass wool	El-Enshasy, 2003
Glucose; fructose	*Zymomonas mobilis*	Nitrogen absence	GFOR; GL	PEI; Entrapment in κ-carrageenan	(Rehr; Wilhelm; Sahm, 1991)
Inulin; Cassava starch	*Zymomonas mobilis*	Nitrogen absence	α-amylase; AMG; GFOR; GL	Entrapment in PVA-alginate beads	(An; Hu; Bao, 2013)

Glucose; fructose	*Zymomonas mobilis*	Nitrogen absence	GFOR; GL	Adsorption on hollow fiber membranes	(Paterson et al., 1988)
Glucose; fructose	*Zymomonas mobilis*	Nitrogen absence	GOD; CAT	Adsorption in hollow fiber membranes	(Ferraz; Borges; Alves, 2000)
Glucose	*Aspergillus niger*	Growth limited by sodium azide	GOD; CAT	-	(Ramachandran, S. et al., 2007)
Glucose	*Aspergillus niger*	Nitrogen absence	GOD; CAT	-	(Liu *et al*., 1999)
Glucose; fructose	*Zymomonas mobilis*	Nitrogen absence	GFOR; GL	Entrapment in alginate beads	(Chun; Rogers, 1988)
Molasses	*Escherichia coli*	Nitrogen absence	GDH	-	(Feng et al., 2023)
Glucose; fructose	*Zymomonas mobilis*	Nitrogen absence	GFOR; GL	Entrapment in alginate beads	(Erzinger; Vitolo, 1996)

GOD, FAD-dependent glucose oxidase (EC 1.1.3.4); GNL, gluconolactonase (EC 3.1.1.17); and CAT, catalase (EC 1.11.1.6). GFOR, NADP-dependent glucose-fructose oxidoreductase (GFOR) and gluconolactonase (GL). GDH, PQQ-dependent glucose dehydrogenase. AMG, amyloglucosidase.

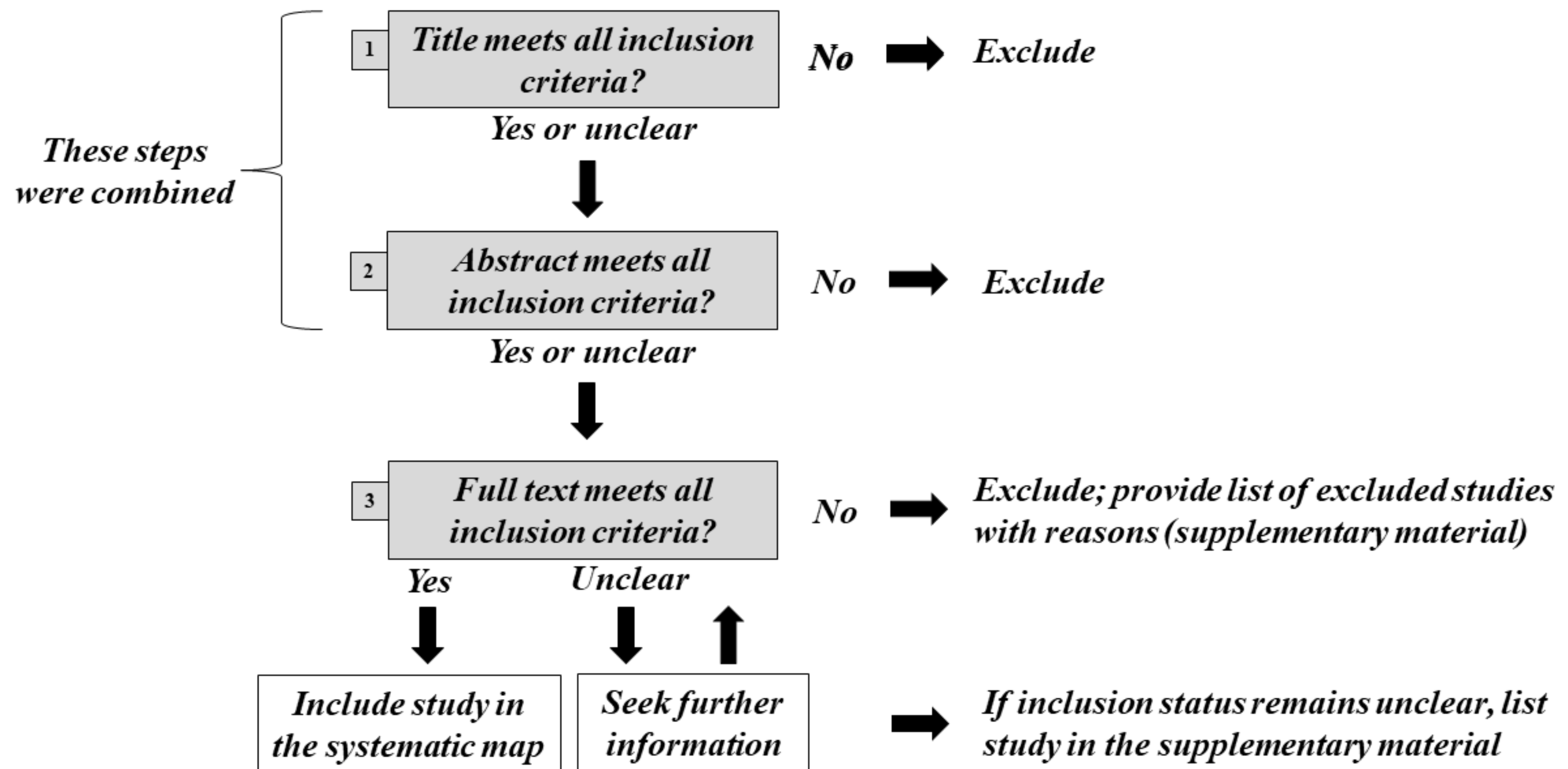


Figure S2.1. Stages of the literature screening process (Adapted from CEE, 2022; Available at: http://www.environmentalevidence.org/guidelines/section-6).

REFERENCES

ABDULLA, H. *et al.* Role of insulin in the regulation of human skeletal muscle protein synthesis and breakdown: a systematic review and meta-analysis. **Diabetologia**, vol. 59, no. 1, p. 44-55, 24 Sept. 2015. Available from: https://doi.org/10.1007/s00125-015-3751-0.

AHUJA, K., SINGH, S., 2018. Gluconic Acid Market size worth over $80 mn by 2024. https://www.gminsights.com/pressrelease/gluconic-acid-market. Accessed on June 19th, 2023.

ALVIN, A. *et al*. Industrial robustness linked to the gluconolactonase from *Zymomonas mobilis*. **Applied Microbiology and Biotechnology**, vol. 101, n. 12, p. 5089-5099, 24 Mar. 2017. Available from: https://doi.org/10.1007/s00253-017-8248-y.

AMEZCUA-ALLIERI, M. A.; SÁNCHEZ DURÁN, T.; ABURTO, J. Study of Chemical and Enzymatic Hydrolysis of Cellulosic Material to Obtain Fermentable Sugars. **Journal of Chemistry**, vol. 2017, p. 1-9, 2017. Available from: https://doi.org/10.1155/2017/5680105.

AN, K.; HU, F.; BAO, J. Simultaneous Saccharification of Inulin and Starch Using Commercial Glucoamylase and the Subsequent Bioconversion to High Titer Sorbitol and Gluconic Acid. **Applied Biochemistry and Biotechnology**, vol. 171, n. 8, p. 2093-2104, 12 Sept. 2013. Available from: https://doi.org/10.1007/s12010-013-0278-z.

ANASTASSIADIS, S. *et al*. Process optimization of continuous gluconic acid fermentation by isolated yeast-like strains of *Aureobasidium pullulans*. **Biotechnology and Bioengineering**, vol. 91, n. 4, p. 494-501, 2005. Available from: https://doi.org/10.1002/bit.20533.

ANASTASSIADIS, S.; MORGUNOV, Igor. Gluconic Acid Production. **Recent Patents on Biotechnology**, vol. 1, n. 2, p. 167-180, 1 June 2007. Available from: https://doi.org/10.2174/187220807780809472.

BARREIRO, R.; PRATT, J. R. Toxic effects of chemicals on microorganisms. **Water Environment Research**, vol. 64, n. 4, p. 632-641, June 1992. Available from: https://doi.org/10.1002/j.1554-7531.1992.tb00045.x.

CAMESELLE, C. *et al*. Oxalic acid production by *Aspergillus niger*. **Bioprocess Engineering**, vol. 19, n. 4, p. 247-252, 20 Oct. 1998. Available from: https://doi.org/10.1007/pl00009017.

CAÑETE-RODRÍGUEZ, A. M. *et al*. Gluconic acid: Properties, production methods and applications—An excellent opportunity for agro-industrial by-products and waste bio-valorization. **Process Biochemistry**, vol. 51, n. 12, p. 1891-1903, Dec. 2016. Available from: https://doi.org/10.1016/j.procbio.2016.08.028.

CHUN, U. H.; ROGERS, P. L. The simultaneous production of sorbitol from fructose and gluconic acid from glucose using an oxidoreductase of *Zymomonas mobilis*. Applied Microbiology and Biotechnology, vol. 29, n. 1, p. 19-24, Aug. 1988. Available from: https://doi.org/10.1007/bf00258345.

COHEN, J. A Coefficient of Agreement for Nominal Scales. **Educational and Psychological Measurement**, vol. 20, n. 1, p. 37-46, Apr. 1960. Available from: https://doi.org/10.1177/001316446002000104.

Collaboration for Environmental Evidence, 2022. **Guidelines and Standards for Evidence synthesis in Environmental Management**, Version 5.1 ed.

CRAIN-ZAMORA, M. (2017). Organic acid production from food wastes using **Gluconobacter oxydans**: A possible source of cheaper lixiviants for leaching REE from end-of-life products Ida ho National Laboratory. Available from: http://www.inl.gov. Accessed: 17 June 2023.

DAI, Lin, *et al.* Low pH Stress Enhances Gluconic Acid Accumulation with Enzymatic Hydrolysate as Feedstock Using *Gluconobacter oxydans*. **Fermentation**, vol. 9, n. 3, p. 278, 12 Mar. 2023. Available from: https://doi.org/10.3390/fermentation9030278.

DE CARVALHO, C. C. C. R. Whole cell biocatalysts: essential workers from Nature to the industry. **Microbial Biotechnology**, vol. 10, n. 2, p. 250-263, 3 May 2016. Available from: https://doi.org/10.1111/1751-7915.12363.

D'SOUZA, S. F.; MELO, J. S. A method for the preparation of coimmobilizates by adhesion using polyethyleneimine. **Enzyme and Microbial Technology**, vol. 13, n. 6, p. 508-511, June 1991. Available from: https://doi.org/10.1016/0141-0229(91)90011-x.

D'SOUZA, S. F.; NADKARNI, G. B. Continuous conversion of sucrose to fructose and gluconic acid by immobilized yeast cell multienzyme complex. **Biotechnology and Bioengineering**, vol. 22, n. 10, p. 2179-2189, Oct. 1980. Available from: https://doi.org/10.1002/bit.260221014.

DWAN, K *et al*. Systematic Review of the Empirical Evidence of Study Publication Bias and Outcome Reporting Bias. **PLoS ONE**, vol. 3, n. 8, p. e3081, 28 Aug. 2008. Available from: https://doi.org/10.1371/journal.pone.0003081.

EFREMENKO, E. *et al*. Biocatalysts in Synthesis of Microbial Polysaccharides: Properties and Development Trends. **Catalysts**, vol. 12, n. 11, p. 1377, 7 Nov. 2022. Available from: https://doi.org/10.3390/catal12111377

El ENSHASY, H. Production of Gluconic Acid by Free and Immobilized Cells of Recombinant *Aspergillus niger* in Batch and Repeated Batch Cultures. Deutsche Lebensmittel-Rundschau: Zeitschrift für Lebensmittelkunde und Lebensmittelrecht. 99. 409-415 (2003).

ELNAGHY, M. A.; MEGALIA, S. E. Gluconic acid production by *Penicillium puberulum*. **Folia Microbiologica**, vol. 20, n. 6, p. 504-508, Nov. 1975. Available from: https://doi.org/10.1007/bf02891710.

ERZINGER, G. S. *et al*. Activity of glucose-fructose oxidoreductase in fresh and permeabilised cells of *Zymomonas mobilis* grown in different glucose concentrations. **Brazilian Journal of Microbiology**, vol. 34, n. 4, p. 329-333, Dec. 2003. Available from: https://doi.org/10.1590/s1517-83822003000400008.

ERZINGER, G. S.; VITOLO, M. *Zymomonas mobilis* as catalyst for the biotechnological production of sorbitol and gluconic acid. **Applied Biochemistry and Biotechnology**, vol. 131, n. 1-3, p. 787-794, Mar. 1996. Available from: https://doi.org/10.1385/abab:131:1:787.

FENG, T. *et al*. Whole-cell biotransformation for simultaneous synthesis of allitol and d-gluconic acid in recombinant *Escherichia coli*. **Journal of Bioscience and Bioengineering**,

Mar. 2023. Available from: https://doi.org/10.1016/j.jbiosc.2023.03.004.

FERRAZ, H. C.; BORGES, C. P.; ALVES, T. L. M. Sorbitol and Gluconic Acid Production Using Permeabilized *Zymomonas mobilis* Cells Confined by Hollow-Fiber Membranes. **Applied Biochemistry and Biotechnology**, vol. 89, n. 1, p. 43-54, 2000. Available from: https://doi.org/10.1385/abab:89:1:43.

FÖRBERG, C.; HÄGGSTRÖM, L. Effects of cultural conditions on the production of phenylalanine from a plasmid-harboring E. coli strain. Applied Microbiology and Biotechnology, vol. 26, no. 2, p. 136-140, May 1987. Available from: https://doi.org/10.1007/bf00253897.

GAO; HUANG; ZHANG. Comparative Analysis between International Research Hotspots and National-Level Policy Keywords on Artificial Intelligence in China from 2009 to 2018. **Sustainability**, vol. 11, n. 23, p. 6574, 21 Nov. 2019. Available from: https://doi.org/10.3390/su11236574.

GARZÓN-POSSE, Fabián *et al*. Whole Cells as Biocatalysts in Organic Transformations. **Molecules**, vol. 23, n. 6, p. 1265, 25 May 2018. Available from: https://doi.org/10.3390/molecules23061265.

GASCH, A. P.; WERNER-WASHBURNE, M. The genomics of yeast responses to environmental stress and starvation. **Functional & Integrative Genomics**, vol. 2, n. 4-5, p. 181-192, Sept. 2002. Available from: https://doi.org/10.1007/s10142-002-0058-2.

GONÇALVES, M. C. P. *et al*. Trends on enzyme immobilization researches based on bibliometric analysis. **Process Biochemistry**, vol. 76, p. 95-110, Jan. 2019. Available from: https://doi.org/10.1016/j.procbio.2018.09.016

HADDAWAY, N. *et al*. ROSES Flow Diagram for Systematic Reviews. Version 1.0. figshare. **Online resource**, Apr. 2018. Available from: https://doi.org/10.6084/m9.figshare.5897389.v3. Accessed: 16 June 2023.

HADDAWAY, N. R. *et al.* EviAtlas: a tool for visualizing evidence synthesis databases. **Environmental Evidence**, vol. 8, n. 1, 4 June 2019. Available from: https://doi.org/10.1186/s13750-019-0167-1.

HAHN, T. *et al*. Determining different impact factors on the xylonic acid production using *Gluconobacter oxydans* DSM 2343. Process Biochemistry, vol. 94, p. 172-179, July 2020. Available from: https://doi.org/10.1016/j.procbio.2020.04.011.

HAN, J. *et al*. In-situ sodium percarbonate assisting and intensifying the aerobic whole-cell catalysis and bio-oxidation of lignocellulosic xylose, glucose and glycerol. **Industrial Crops and Products**, vol. 195, p. 116482, May 2023. Available from: https://doi.org/10.1016/j.indcrop.2023.116482.

HAN, X. *et al*. Consolidated bioprocessing for sodium gluconate production from cellulose using *Penicillium oxalicum*. **Bioresource Technology**, vol. 251, p. 407-410, Mar. 2018. Available from: https://doi.org/10.1016/j.biortech.2017.12.028.

HARTMEIER, W. Coimmobilization of enzymes and whole cells. **Food Biotechnology**, vol. 4, n. 1, p. 399-407, Jan. 1990. Available from: https://doi.org/10.1080/08905439009549751.

HARTMEIER, W.; HEINRICHS, A. Membrane enclosed alginate beads containing *Gluconobacter cells* and molecular dispersed catalase. **Biotechnology Letters**, vol. 8, n. 8, p. 565-570, Aug. 1986. Available from: https://doi.org/10.1007/bf01028084.

HE, M. *et al*. *Zymomonas mobilis*: a novel platform for future biorefineries. **Biotechnology for Biofuels**, vol. 7, n. 1, p. 101, 2014. Available from: https://doi.org/10.1186/1754-6834-7-101.

HIGGINS, Julian P. T.; WHITE, Ian R.; ANZURES-CABRERA, Judith. Meta-analysis of skewed data: Combining results reported on log-transformed or raw scales. **Statistics in Medicine**, vol. 27, n. 29, p. 6072-6092, 17 Sept. 2008. Available from: https://doi.org/10.1002/sim.3427.

HOL, F. J. H.; DEKKER, C. Zooming in to see the bigger picture: Microfluidic and nanofabrication tools to study bacteria. **Science**, vol. 346, n. 6208, p. 1251821, 23 Oct. 2014. Available from: https://doi.org/10.1126/science.1251821.

HÖLSCHER, T. *et al*. Glucose Oxidation and PQQ-Dependent Dehydrogenases in *Gluconobacter oxydans*. **Journal of Molecular Microbiology and Biotechnology**, vol. 16, n. 1-2, p. 6-13, 2009. Available from: https://doi.org/10.1159/000142890.

HOU, W.; ZHANG, M.; BAO, J. Cascade hydrolysis and fermentation of corn stover for production of high titer gluconic and xylonic acids. **Bioresource Technology**, vol. 264, p. 395-399, Sept. 2018. Available from: https://doi.org/10.1016/j.biortech.2018.06.025.

HUA, X. *et al*. Bioprocess Intensification for Whole-Cell Catalysis of Catabolized Chemicals with 2,4-Dinitrophenol Uncoupling. **ACS Sustainable Chemistry & Engineering**, vol. 8, n. 41, p. 15782-15790, 6 Oct. 2020. Available from: https://doi.org/10.1021/acssuschemeng.0c06466.

IKEDA, Y.; PARK, E.; OKUDA, N. Bioconversion of waste office paper to gluconic acid in a turbine blade reactor by the filamentous fungus *Aspergillus niger*. **Bioresource Technology**, vol. 97, n. 8, p. 1030-1035, May 2006. Available from: https://doi.org/10.1016/j.biortech.2005.04.040.

INTASIAN, Pattarawan *et al*. Enzymes, In Vivo Biocatalysis, and Metabolic Engineering for Enabling a Circular Economy and Sustainability. **Chemical Reviews**, vol. 121, n. 17, p. 10367-10451, 6 July 2021. Available from: https://doi.org/10.1021/acs.chemrev.1c00121.

JAISHANKAR, J.; SRIVASTAVA, P. Molecular Basis of Stationary Phase Survival and Applications. **Frontiers in Microbiology**, vol. 8, 16 Oct. 2017. Available from: https://doi.org/10.3389/fmicb.2017.02000.

JANG, K. *et al*. Production of Sorbitol with Cetyltrimethylammonium bromide *Permeabilized Cells of Zymomonas Mobilis*. **Biochemical Engineering**. Tokyo: Springer Japan, 1992. p. 389-392. ISBN 9784431681823. Available from: https://doi.org/10.1007/978-4-431-68180-9_103.

JANG, K. H.; PARK, C. J.; CHUN, U. H. Improvement of oxidoreductase stability of cethyltrimethylammoniumbromide permeabilized cells of *Zymomonas mobilis* through glutaraldehyde crosslinking. **Biotechnology Letters**, vol. 14, n. 4, p. 311-316, Apr. 1992. Available from: https://doi.org/10.1007/bf01022330.

JIANG, Y. *et al*. Gluconic Acid Production from Potato Waste by *Gluconobacter oxidans* Using Sequential Hydrolysis and Fermentation. **ACS Sustainable Chemistry & Engineering**, vol. 5, n. 7, p. 6116-6123, 2 June 2017. Available from: https://doi.org/10.1021/acssuschemeng.7b00992.

KIM, D. M; KIM, H. S. Continuous production of gluconic acid and sorbitol from Jerusalem artichoke and glucose using an oxidoreductase of *Zymomonas mobilis* and inulinase. **Biotechnology and Bioengineering**, vol. 39, n. 3, p. 336-342, 5 Feb. 1992. Available from: https://doi.org/10.1002/bit.260390312.

KORNECKI, J. F. *et al*. Enzyme production of d-gluconic acid and glucose oxidase: successful tales of cascade reactions. **Catalysis Science & Technology**, vol. 10, n. 17, p. 5740-5771, 2020. Available from: https://doi.org/10.1039/d0cy00819b.

KOVAČEVIĆ, G. *et al*. Production of fructose and gluconic acid from sucrose with cross-linked yeast cell walls expressing glucose oxidase on the surface. **Molecular Catalysis**, vol. 522, p. 112215, Apr. 2022. Available from: https://doi.org/10.1016/j.mcat.2022.112215.

LANTERO, J. Glutaraldehyde/polyethyleneimine immobilization of whole microbial cells. US4355105. 19 Oct. 1982.

LIN, B.; TAO, Y. Whole-cell biocatalysts by design. **Microbial Cell Factories**, vol. 16, n. 1, 13 June 2017. Available from: https://doi.org/10.1186/s12934-017-0724-7.

LIU, J. Z. *et al*. Synthesis of glucose oxidase and catalase by *Aspergillus niger* in resting cell culture system. **Letters in Applied Microbiology**, vol. 29, n. 5, p. 337-341, Nov. 1999. Available from: https://doi.org/10.1046/j.1472-765x.1999.00637.x.

LIU, X. *et al*. Engineered Living Hydrogels. **Advanced Materials**, p. 2201326, 3 Mar. 2022. Available from: https://doi.org/10.1002/adma.202201326.

LIU, X; DING, W.; JIANG, H. Engineering microbial cell factories for the production of plant natural products: from design principles to industrial-scale production. **Microbial Cell Factories**, vol. 16, n. 1, 19 July 2017. Available from: https://doi.org/10.1186/s12934-017-0732-7.

LIVOREIL, B. *et al*. Systematic searching for environmental evidence using multiple tools and sources. **Environmental Evidence**, vol. 6, n. 1, 21 Aug. 2017. Available from: https://doi.org/10.1186/s13750-017-0099-6.

LOU, W. *et al*. Editorial: Enzyme or Whole Cell Immobilization for Efficient Biocatalysis: Focusing on Novel Supporting Platforms and Immobilization Techniques. **Frontiers in Bioengineering and Biotechnology**, vol. 9, 1 Mar. 2021. Available from: https://doi.org/10.3389/fbioe.2021.620292.

MA, Y. *et al.* Production of Gluconic Acid and Its Derivatives by Microbial Fermentation: Process Improvement Based on Integrated Routes. **Frontiers in Bioengineering and Biotechnology**, vol. 10, 16 May 2022. Available from: https://doi.org/10.3389/fbioe.2022.864787.

MACAULEY, S.; MCNEIL, B.; HARVEY, L. M. The Genus *Gluconobacter* and Its Applications in Biotechnology. **Critical Reviews in Biotechnology**, vol. 21, n. 1, p. 1-25, Jan. 2001. Available from: https://doi.org/10.1080/20013891081665 54.

MALVESSI, E. *et al*. Effect of substrate concentration, pH, and temperature on the activity of the complex glucose–fructose oxidoreductase/glucono-δ-lactonase present in calcium alginate-immobilized *Zymomonas mobilis* cells. **Biochemical Engineering Journal**, vol. 51, n. 1-2, p. 1-6, Aug. 2010. Available from: https://doi.org/10.1016/j.bej.2010.04.003.

MCAULIFFE, J. C. Industrial Enzymes and Biocatalysis. In: MCAULIFFE, Joseph C. **Handbook of Industrial Chemistry and Biotechnology**. Boston, MA: Springer US, 2012. p. 1183-1227. ISBN 9781461442585. Available from: https://doi.org/10.1007/978-1-4614-4259-2_31.

MEHMETOGLU, Ü.; ATEŞ, S.; BERBER, R. Oxygen Diffusivity in Calcium Alginate Gel Beads Containing *Gluconobacter Suboxydans*. **Artificial Cells, Blood Substitutes, and Biotechnology**, vol. 24, n. 2, p. 91-106, Jan. 1996. Available from: https://doi.org/10.3109/10731199609118877.

MILSOM, P.E.; MEERS, J.L. Gluconic and itaconic acids. **Comprehensive Biotechnology**, vol. 3, pp. 681–700, 1985. Oxford: Pergamon Press.

MIZUNUMA, T.; KOKUFUTA, E.; SATO, S. A mycelium with polyelectrolyte complex-bunched hyphae: Preparation and fermentation performance. **Colloids and Surfaces B: Biointerfaces**, vol. 56, n. 1-2, p. 155-160, Apr. 2007. Available from: https://doi.org/10.1016/j.colsurfb.2006.11.011.

MOKSIA, J.; LARROCHE, C.; GROS, J. B. Gluconate production by spores of *Aspergillus niger*. **Biotechnology Letters**, vol. 18, n. 9, p. 1025-1030, Sept. 1996. Available from: https://doi.org/10.1007/bf00129725.

NG, W. Metabolism of Non-growing Cells and Their Application in Biotransformation. *Preprints* **2020**, 2020070402. https://doi.org/10.20944/preprints202007.0402.v1.

OKWUASHI, O. *et al*. GIS Cellular Automata Using Artificial Neural Network for Land Use Change Simulation of Lagos, Nigeria. **Journal of Geography and Geology**, vol. 4, n. 2, 18 May 2012. Available from: https://doi.org/10.5539/jgg.v4n2p94.

PATERSON, S. L. *et al*. Sorbitol and Gluconate Production in a Hollow Fiber Membrane Reactor by Immobilized *Zymomonas Mobilis*. **Biocatalysis**, vol. 1, n. 3, p. 217-229, Jan. 1988. Available from: https://doi.org/10.3109/10242428809014848.

PETERSEN, K.; VAKKALANKA, S.; KUZNIARZ, L. Guidelines for conducting systematic mapping studies in software engineering: An update. **Information and Software Technology**, vol. 64, p. 1-18, Aug. 2015. Available from: https://doi.org/10.1016/j.infsof.2015.03.007.

PETRUCCIOLI, M. *et al*. Glucose oxidase, catalase and gluconic acid production by immobilized mycelium of *Penicillium variable P16*. **Biotechnology Letters**, vol. 16, n. 9, p. 939-942, Sept. 1994. Available from: https://doi.org/10.1007/bf00128629.

RAJPUROHIT, H.; EITEMAN, M. A. Nutrient-Limited Operational Strategies for the Microbial Production of Biochemicals. **Microorganisms**, vol. 10, n. 11, p. 2226, 10 Nov. 2022. Available from: https://doi.org/10.3390/microorganisms10112226.

RAMACHANDRAN, S. *et al*. Gluconic Acid: Properties, Applications and Microbial

Production. **Food Technology and Biotechnology**, v. 44, n. 2, p. 185-95, Apr. 2006.

RAMACHANDRAN, S. *et al.* Permeabilization and inhibition of the germination of spores of *Aspergillus niger* for gluconic acid production from glucose. **Bioresource Technology**, vol. 99, n. 11, p. 4559-4565, July 2008. Available from: https://doi.org/10.1016/j.biortech.2007.06.055.

RAMACHANDRAN, S. *et al.* Spores of *Aspergillus niger* as reservoir of glucose oxidase synthesized during solid-state fermentation and their use as catalyst in gluconic acid production. **Letters in Applied Microbiology**, vol. 44, n. 2, p. 155-160, Feb. 2007. Available from: https://doi.org/10.1111/j.1472-765x.2006.02051.x.

RAO, D. S.; PANDA, T. Comparative analysis of different whole cell immobilized *aspergillus niger* catalysts for gluconic acid fermentation using pretreated cane molasses. **Bioprocess Engineering**, vol. 11, n. 5, p. 209-212, Oct. 1994. Available from: https://doi.org/10.1007/bf00369632.

REHR, B.; WILHELM, C.; SAHM, H. Production of sorbitol and gluconic acid by permeabilized cells of *Zymomonas mobilis*. **Applied Microbiology and Biotechnology**, vol. 35, n. 2, May 1991. Available from: https://doi.org/10.1007/bf00184677.

RO, H. S.; KIM, H. S. Continuous production of gluconic acid and sorbitol from sucrose using invertase and an oxidoreductase of *Zymomonas mobilis*. **Enzyme and Microbial Technology**, vol. 13, n. 11, p. 920-924, Nov. 1991. Available from: https://doi.org/10.1016/0141-0229(91)90109-n.

ROGERS, P. L. *et al*. *Zymomonas mobilis* for Fuel Ethanol and Higher Value Products. **Biofuels**. Berlin, Heidelberg: Springer Berlin Heidelberg. p. 263-288, 2007. ISBN 9783540736509. Available from: https://doi.org/10.1007/10_2007_060.

ROGERS, P.; C., J.; ZIDWICK, M. J. Organic Acid and Solvent Production: Acetic, Lactic, Gluconic, Succinic, and Polyhydroxyalkanoic Acids. In: ROGERS, Palmer; CHEN, Jiann-Shin; ZIDWICK, Mary Jo. **The Prokaryotes**. Berlin, Heidelberg: Springer Berlin Heidelberg, 2013. p. 3-75. ISBN 9783642313301. Available from: https://doi.org/10.1007/978-3-642-31331-8_23.

ROMANELLI, João P. *et al*. Reliability of evidence-review methods in restoration ecology. **Conservation Biology**, 21 Dec. 2020. Available from: https://doi.org/10.1111/cobi.13661.

ROMANELLI, João Paulo *et al*. Four challenges when conducting bibliometric reviews and how to deal with them. **Environmental Science and Pollution Research**, 20 Sept. 2021b. Available from: https://doi.org/10.1007/s11356-021-16420-x.

ROMANELLI, João Paulo *et al*. Repeatability of the searching process in reviews of restoration outcomes. **Restoration Ecology**, 12 Aug. 2021a. Available from: https://doi.org/10.1111/rec.13496.

SAKHUJA, D. *et al*. Cost-effective production of biocatalysts using inexpensive plant biomass: a review. 3 **Biotech**, vol. 11, n. 6, 20 May 2021. Available from: https://doi.org/10.1007/s13205-021-02847-z.

SANKPAL, N. V. *et al*. An artificial intelligence tool for bioprocess monitoring: application to

continuous production of gluconic acid by immobilized *Aspergillus niger*. Biotechnology Letters, vol. 23, n. 11, p. 911-916, 2001. Available from: https://doi.org/10.1023/a:1010551719536.

SANKPAL, N. V. *et al*. Continuous production of gluconic acid by *Aspergillus niger* immobilized on a cellulosic support: study of low pH fermentative behavior of Aspergillus niger. **Process Biochemistry**, vol. 35, n. 3-4, p. 317-325, Nov. 1999. Available from: https://doi.org/10.1016/s0032-9592(99)00074-6.

SEISKARI, P.; LINKO, Y. Y.; LINKO, P. Continuous production of gluconic acid by immobilized *Gluconobacter oxydans* cell bioreactor. **Applied Microbiology and Biotechnology**, vol. 21, n. 6, Apr. 1985. Available from: https://doi.org/10.1007/bf00249979.

SHINDIA, A. A. *et al*. Production of Gluconic Acid by Some Local Fungi. **Mycobiology**, vol. 34, n. 1, p. 22, 2006. Available from: https://doi.org/10.4489/myco.2006.34.1.022.

SILVA, G. A. R. *et al.* The industrial versatility of *Gluconobacter oxydans*: current applications and future perspectives. **World Journal of Microbiology and Biotechnology**, vol. 38, n. 8, 11 June 2022. Available from: https://doi.org/10.1007/s11274-022-03310-8.

SILVEIRA, M. M. *et al*. Bioconversion of glucose and fructose to sorbitol and gluconic acid by untreated cells of *Zymomonas mobilis*. **Journal of Biotechnology**, vol. 75, n. 2-3, p. 99-103, Oct. 1999. Available from: https://doi.org/10.1016/s0168-1656(99)00149-2.

SINGH, O. V. Modulated gluconic acid production from immobilized cells of *Aspergillus niger ORS-4.410* utilizing grape must. **Journal of Chemical Technology & Biotechnology**, vol. 83, n. 6, p. 780-787, 2008. Available from: https://doi.org/10.1002/jctb.1866.

TRAMPER, J.; LUYBEN, K. C. A. M.; TWEEL, W. J. J. Kinetic aspects of glucose oxidation by *Gluconobacter oxydans* cells immobilized in calcium alginate. **European Journal of Applied Microbiology and Biotechnology**, vol. 17, n. 1, p. 13-18, 1983. Available from: https://doi.org/10.1007/bf00510565.

VAN ECK, N. J.; WALTMAN, L. Software survey: VOSviewer, a computer program for bibliometric mapping. **Scientometrics**, vol. 84, n. 2, p. 523-538, 31 Dec. 2009. Available from: https://doi.org/10.1007/s11192-009-0146-3.

VASSILEV, N. B; VASSILEVA, M. C; SPASSOVA, D. Production of gluconic acid by *Aspergillus niger* immobilized on polyurethane foam. **Applied Microbiology and Biotechnology**, vol. 39, n. 3, June 1993. Available from: https://doi.org/10.1007/bf00192079.

WERNER-WASHBURNE, M. *et al*. Stationary phase in *Saccharomyces cerevisiae*. Molecular Microbiology, vol. 19, n. 6, p. 1159-1166, Mar. 1996. Available from: https://doi.org/10.1111/j.1365-2958.1996.tb02461.x.

WESSEL, A. K. *et al*. Going local: technologies for exploring bacterial microenvironments. **Nature Reviews Microbiology**, vol. 11, n. 5, p. 337-348, 16 Apr. 2013. Available from: https://doi.org/10.1038/nrmicro3010.

WILBERG, K. Q.; ALVES, T. L. M.; NOBREGA, R. Enzymatic catalysis by permeabilized cells. **Brazilian Journal of Chemical Engineering**, vol. 14, n. 4, p. 347-352, Dec. 1997.

Available from: https://doi.org/10.1590/s0104-66321997000400008.

WISBECK, E. *et al*. Evaluation of the flocculent strain *Zymomonas mobilis Z1-81* for the production of sorbitol and gluconic acid. **Journal of Basic Microbiology**, vol. 37, n. 6, p. 445-449, 1997. Available from: https://doi.org/10.1002/jobm.3620370613.

WITTEVEEN, C. F. B.; VEENHUIS, M.; VISSER, J. Localization of Glucose Oxidase and Catalase Activities in *Aspergillus niger*. **Applied and Environmental Microbiology**, vol. 58, no. 4, p. 1190-1194, 1992. Available from: https://doi.org/10.1128/aem.58.4.1190-1194.1992

WOODLEY, J. M. Microbial Biocatalytic Processes and Their Development. **Advances in Applied Microbiology**. Elsevier, 2006. p. 1-15. ISBN 9780120026623. Available from: https://doi.org/10.1016/s0065-2164(06)60001-4.

XIAO, Y.; WATSON, M. Guidance on Conducting a Systematic Literature Review. **Journal of Planning Education and Research**, vol. 39, n. 1, p. 93-112, 28 Aug. 2017. Available from: https://doi.org/10.1177/0739456x17723971.

YAN, Yaru, *et al*. Surface charge modifications modulate glucose oxidase pH-activity profiles for efficient gluconic acid production. **Journal of Cleaner Production**, vol. 372, p. 133817, Oct. 2022. Available from: https://doi.org/10.1016/j.jclepro.2022.133817.

YU, X. *et al*. Co-immobilization of multi-enzyme on reversibly soluble polymers in cascade catalysis for the one-pot conversion of gluconic acid from corn straw. **Bioresource Technology**, vol. 321, p. 124509, Feb. 2021. Available from: https://doi.org/10.1016/j.biortech.2020.124509.

ZACHARIOU, M.; SCOPES, R. K. Glucose-fructose oxidoreductase, a new enzyme isolated from *Zymomonas mobilis* that is responsible for sorbitol production. **Journal of Bacteriology**, vol. 167, n. 3, p. 863-869, 1986. Available from: https://doi.org/10.1128/jb.167.3.863-869.1986.

ZHANG, H. *et al*. Fermentative production of high titer gluconic and xylonic acids from corn stover feedstock by *Gluconobacter oxydans* and techno-economic analysis. **Bioresource Technology**, vol. 219, p. 123-131, Nov. 2016a. Available from: https://doi.org/10.1016/j.biortech.2016.07.068.

ZHANG, H. *et al*. Oxidative conversion of glucose to gluconic acid by iron(iii) chloride in water under mild conditions. **Green Chemistry**, vol. 18, n. 8, p. 2308-2312, 2016b. Available from: https://doi.org/10.1039/c5gc02614h.

ZHANG, Q. *et al*. Sustainable production of high-value gluconic acid and glucaric acid through oxidation of biomass-derived glucose: A critical review. **Journal of Cleaner Production**, vol. 312, p. 127745, Aug. 2021. Available from: https://doi.org/10.1016/j.jclepro.2021.127745.

ZHONG, P. *et al*. Piezoelectric sensing of glucose oxidase activity of *Aspergillus niger* spores pretreated by different methods. **Food Chemistry**, vol. 370, p. 130901, Feb. 2022. Available from: https://doi.org/10.1016/j.foodchem.2021.130901.

ZHOU, X. *et al*. Efficient coproduction of gluconic acid and xylonic acid from lignocellulosic hydrolysate by Zn (II)-selective inhibition on whole-cell catalysis by **Gluconobacter oxydans**. Bioresource Technology, vol. 243, p. 855-859, Nov. 2017. Available from: https://doi.org/10.1016/j.biortech.2017.07.023.

ZHOU, X. *et al*. Improving the production yield and productivity of 1,3-dihydroxyacetone from glycerol fermentation using *Gluconobacter oxydans* NL71 in a compressed oxygen supply-sealed and stirred tank reactor (COS-SSTR). **Bioprocess and Biosystems Engineering**, vol. 39, n. 8, p. 1315-1318, 28 Mar. 2016. Available from: https://doi.org/10.1007/s00449-016-1595-8.

ZHOU, X. *et al*. Integrated production of gluconic acid and xylonic acid using dilute acid pre-treated corn stover by two-stage fermentation. **Biochemical Engineering Journal**, vol. 137, p. 18-22, Sept. 2018. Available from: https://doi.org/10.1016/j.bej.2018.05.005.

ZHOU, Xuelian, *et al*. Efficient coproduction of gluconic acid and xylonic acid from lignocellulosic hydrolysate by Zn (II)-selective inhibition on whole-cell catalysis by *Gluconobacter oxydans*. **Bioresource Technology**, vol. 243, p. 855-859, Nov. 2017. Available from: https://doi.org/10.1016/j.biortech.2017.07.023.

ZNAD, H.; MARKOŠ, J.; BALEŠ, V. Production of gluconic acid from glucose by *Aspergillus niger*: growth and non-growth conditions. **Process Biochemistry**, vol. 39, n. 11, p. 1341-1345, July 2004. Available from: https://doi.org/10.1016/s0032-9592(03)00270-x.

3 GA PRODUCTION BY *ASPERGILLUS NIGER* WHOLE-CELLS FROM DIFFERENT SUBSTRATES

RESUMO

A revisão da literatura realizada no Capítulo 2 mostra que *Aspergillus niger* tem sido um dos microrganismos mais aplicados como biocatalisador utilizando células inteiras para produzir ácido glicônico. As células inteiras de *A. niger* são consideradas biocatalisadores promissores para a produção de ácido glicônico porque contêm naturalmente as enzimas constitutivas glicose oxidase (GOD) e catalase (CAT). Assim, esse microrganismo foi selecionado devido à sua disponibilidade em nosso laboratório. Além disso, a revisão permitiu a identificação de alguns substratos que não haviam sido utilizados anteriormente para produzir ácido glicônico por meio de células inteiras de *A. niger*. De acordo com lacunas identificadas na literatura, os substratos sacarose, dextrina e lactose foram selecionados para serem testados na produção de ácido glicônico por células inteiras de *A. niger* associadas às enzimas comerciais glicose-isomerase (GI), amiloglucosidase (AMG) e β-galactosidase (β-Gal), respectivamente. O substrato glicose foi utilizado como substrato padrão para os ensaios. Os rendimentos em ácido glicônico alcançados foram de 96,34 ± 1,9, 89,92 ± 8,5, 68,16 ± 1,3 e 10,20 ± 0,1%, a partir de 20 g/L de cada um dos substratos: glicose, dextrina, sacarose e lactose, respectivamente. Além da glicose, substrato amplamente aplicado na produção de ácido glicônico, o melhor rendimento nesse ácido foi de 89,92% ± 8,5% utilizando dextrina-10 como substrato. A dextrina é um polissacarídeo obtido a partir da hidrólise parcial do amido, amplamente utilizada em muitas indústrias devido às suas propriedades funcionais. O alto rendimento em ácido glicônico (%) a partir da dextrina (molécula menor e menos complexa do que o amido) abre uma nova perspectiva para um sistema multienzimático inovador para produção de ácido glicônico a partir de amido usando células inteiras de *A. niger* associadas a enzimas amilolíticas. Além disso, os resíduos amiláceos da indústria agroalimentar também poderiam ser explorados como matéria-prima para a produção de ácido glicônico pelo sistema multienzimático proposto.

Palavras-chave: Células inteiras; biocatalisador; *Aspergillus niger*; sacarose; dextrina, lactose; sistema multienzimático; ácido glicônico.

ABSTRACT

The literature review carried out in Chapter 2 shows that *Aspergillus niger* is one of the most applied microorganisms as a whole-cell biocatalyst to produce gluconic acid (GA). *A. niger* whole-cells are considered promising biocatalysts to produce gluconic acid because they naturally contain the constitutive enzymes glucose oxidase (GOD) and catalase (CAT). Thus, this microorganism was selected due to its availability in our laboratory. Additionally, the review allowed the identification of some substrates that had not previously been used to produce gluconic acid through *A. niger* whole-cell. According to gaps identified in the literature, the substrates sucrose, dextrin, and lactose were selected to be tested on GA production by *Aspergillus niger* whole cells associated with commercial enzymes glucose-isomerase (GI), amyloglucosidase (AMG) and β-galactosidase (β-Gal), respectively. The GA yields achieved were 96.34 ± 1.9, 89.92 ± 8.5, 68.16 ± 1.3, and 10.20 ± 0.1% from 20 g/L of each of the substrates: glucose, dextrin, sucrose, and lactose, respectively. In addition to glucose, the substrate widely applied to produce GA, the best GA yield was 89.92 ± 8.5% using dextrin-10 as substrate. Dextrin is a polysaccharide obtained from the partial hydrolysis of starch, widely used in many industries due to its functional properties. The high GA yield (%) from dextrin (a smaller and less complex molecule than starch) opens a new perspective for an innovative multi-enzymatic system to produce GA from starch using *A. niger* whole-cells associated with amylolytic enzymes. Additionally, agro-food industry starchy wastes could be exploited as feedstock to produce GA by the multi-enzymatic system proposed.

Keywords: Whole cells; biocatalyst; *Aspergillus niger*; sucrose; dextrin, lactose; multienzyme system; gluconic acid.

3.1 Introduction

The literature review carried out in Chapter 2 shows that *A. niger* is one of the most applied microorganisms as a whole-cell biocatalyst to produce gluconic acid (GA). *A. niger* whole-cells are considered excellent enzyme bags and promising biocatalysts to produce GA because they naturally contain the constitutive enzymes glucose oxidase (GOD) and catalase (CAT) (Witteveen; Veenhuis; Visser, 1992; Ramachandran *et al.*, 2007, 2008). Briefly, GA production using *A. niger* whole-cell biocatalysis occurs due to the oxidation of glucose by the enzymes GOD and CAT *in situ*, according to the mechanism highlighted in green in Figure 3.1, distinguishing it from the fermentation process due to non-growing cell conditions (Woodley, 2006).

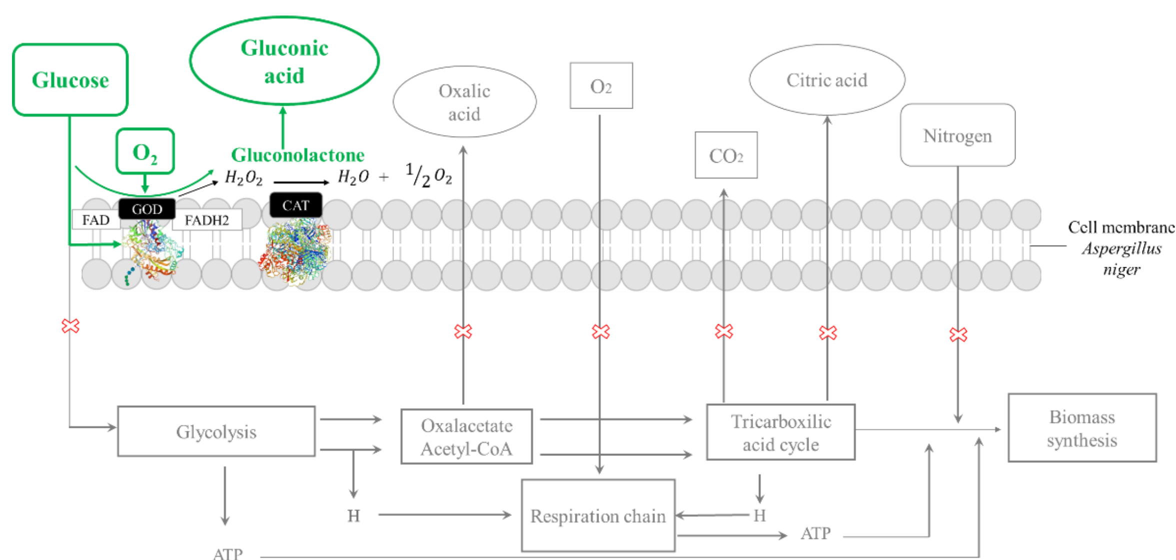


Figure 3.1. Gluconic acid production by *A. niger* whole -cells. Glucose oxidation by the constitutive enzymes GOD and CAT. GOD, FAD-dependent glucose oxidase (EC 1.1.3.4); CAT, catalase (EC 1.11.1.6). Adapted from Cameselle *et al*. 1998. Primary metabolic pathways essential for cell growth, development, and reproduction, such as glycolysis and the citric acid cycle, suppressed due to nitrogen starvation, are lowlighted in gray.

A wild-type strain of the filamentous fungus *A. niger 12* (National Register of Biological Collections number BRM028885) obtained from the Embrapa Food Technology collection (Rio de Janeiro, Brazil) was selected to be applied as a whole-cell biocatalyst in this study. *A. niger 12* was used in a previous study (Buffo *et al.*, 2021).

Buffo *et al.* (2021) reported that an initial pH between 4 and 6, combined with a spore concentration of up to 10^7 spores/mL of *A. niger*, resulted in cells with 100% pelletized morphology. Pellets are an attractive morphology for using whole-cells as biocatalysts since the agglomeration of hyphae can provide the enzymes with better protection. Furthermore, pellets tend to result in a broth with less viscous rheology when compared to dispersed hyphal

morphology, consequently improving oxygen transfer in the liquid phase (Rodríguez Porcel *et al.*, 2005). The downstream processing for biomass separation is also easier than with mycelial morphology (which tends to entangle) and can assist in the reuse of the cells (Ma *et al.*, 2022). Then, whole-cells pelletized morphology was selected to be applied as biocatalysts in this study.

The network analysis of the multi-enzymatic systems (Figure 2.16) for GA production allows mapping possible combinations between commercial enzymes that can hydrolyze/isomerize different substrates to glucose with intracellular enzymes from *A. niger* whole-cells (GOD and CAT) that oxidize glucose to gluconic acid. According to gaps in the literature identified by network analysis of the substrates (Figure 2.15), sucrose, dextrin, and lactose were tested for GA production by *A. niger* whole cells associated with commercial enzymes glucose-isomerase (GI), amyloglucosidase (AMG) and β-galactosidase (β-Gal), respectively. Although sucrose appears in the network analysis (Figure 2.15), it was not evaluated with resting cells of *A. niger* microorganism, thus presenting an innovative research opportunity. Figure 3.2 summarizes the literature gaps exploited in this chapter.

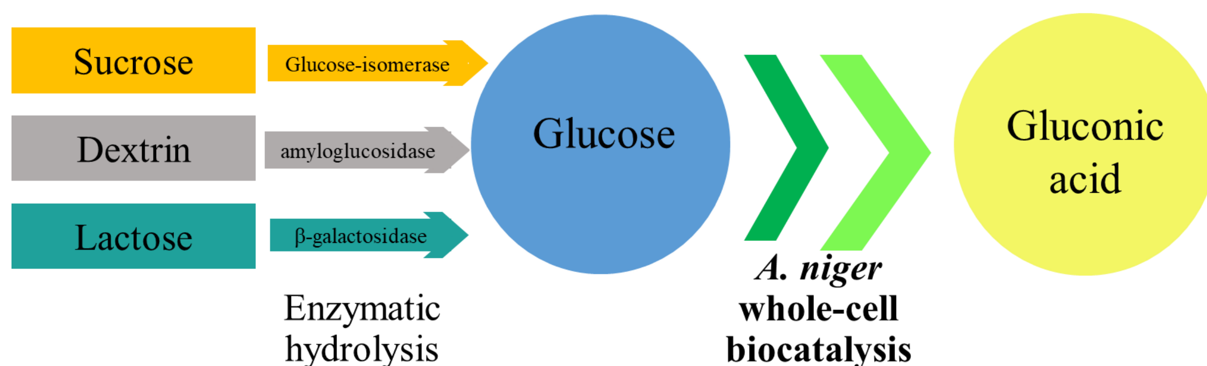


Figure 3.2. The general process to produce gluconic acid by *A. niger* whole cells from sucrose, dextrin, and lactose is associated with commercial enzymes glucose-isomerase, amyloglucosidase, and β-galactosidase, respectively.

3.2 Material and methods

3.2.1 Strain and medium for wc production

A wild-type strain of the filamentous fungus *A niger 12* (National Register of Biological Collections number BRM028885) was obtained from the Embrapa Food Technology collection (Rio de Janeiro, Brazil). The microorganism was maintained at -80 °C and was reactivated at least every 90 days by incubation in Petri dishes of potato dextrose agar medium for 5 days at 32 °C. Cryotubes with spore suspensions were prepared in 20% (v/v) glycerol, maintained at -80 °C, for use in the inoculum preparation (Buffo *et al.*, 2021). The concentration of the spore suspension was determined by counting in a Neubauer chamber (Brand, Germany). *A. niger*

whole-cells were prepared using the growth culture medium proposed by Rao & Panda (1994) for gluconic acid production. The culture medium consisted of (in g/L): glucose (50.0), $(NH_4)_2HPO_4$ (4.0), urea (1.8), KH_2PO_4 (3.3), and $MgSO_4.7H_2O$ (2.8). D-glucose and all other chemicals (analytical grade) were purchased from Synth (Diadema, SP, Brazil) and Vetec (Duque de Caxias, RJ, Brazil).

3.2.2 Commercial enzymes

Commercial enzyme extract Gensweet SGI (glucose-isomerase (EC5.3.1.5) from *Streptomyces rubiginosus*) donated by Genencor International (Palo Alto-CA-USA). Amyloglucosidase AMG 300L™ from *A. niger* (EC 3.2.1.3, glucoamylase, amyloglucosidase) (≥260 U/mL) was kindly donated by LNF Latino-Americana (Bento Gonçalves) and β-galactosidase (EC 3.2.1.23) from *Kluyveromyces fragilis* (Lactozym 3000 L) donated by Novo Nordisk, Brazil. Glucose, dextrin 10, sucrose, and lactose were purchased from Sigma-Aldrich. All other reagents used were of analytical grade.

3.2.3 Experimental procedure for whole-cell production

Erlenmeyer flasks (500 mL) containing 100 mL of culture medium were inoculated with an initial concentration adjusted to 10^6 spores/mL and incubated at 32 °C and 200 rpm, for 72 h, in an orbital shaker-incubator (model TE-421, Tecnal, Piracicaba, SP, Brazil). The pH of the medium was adjusted to pH 6.0 with 1.0 M HCl, before sterilization in an autoclave (model AV, Phoenix Luferco, Araraquara, SP, Brazil) at 121 °C for 20 min. The pH of the medium was adjusted to pH 6.0 and the initial spore concentration was set at 10^6 spores/mL, to obtain whole-cells with pelletized morphology. The resulting cells were separated from the culture medium by filtration, washed with distilled water, and resuspended in 0.01 M citrate-phosphate buffer at pH 6.5, to be used as biocatalysts in the GA production stage. The cells were analyzed using a stereomicroscope (model XT-3L-BI, Biofocus, Araucária, PR, Brazil) coupled to a digital camera. The images were processed with Image-J software (rsbweb.nih.gov/ij). The pellet diameters were obtained directly by image analysis using Image-J software (Buffo *et al.*, 2021).

3.2.4 Experimental procedure for GA production

During the GA production stage, 0.3% (w/v) whole cells associated with 1% (v/v) of each commercial enzyme (GI, AMG, and β-galactosidase) were applied as biocatalysts to produce GA from 20 g/L of the different substrates: sucrose, dextrin, and lactose, respectively. In the context of glucose isomerase, the salts $MgSO_4.7H_2O$ (50 mM) and $CoCl_2.6H_2O$ (2.5 mM)

were added since these metallic cofactors play a crucial role in catalyzing the conversion of glucose into fructose. Additionally, glucose was tested under the same conditions with no commercial enzyme added to check the capacity of whole-cells of the *A. niger 12* strain to produce GA.

Assays were performed in a 350 mL mini bubble bioreactor (8.50 × 3.33 cm, length × diameter) with a working volume of 200 mL, using a sintered filter as sparger to feed air at a specific flow rate of 3 vvm (0.6 L/min). The pH was kept constant at pH 6.0 by feeding a solution of 2.0 M NaOH from an automatic titrator (model 718 STAT Titrino, Metrohm AG, Herisau, Switzerland), to neutralize the GA produced (Figure 3.3). The temperature in the bioreactor was kept at 30 °C using a thermostatic bath (model 521/2D, Nova Ética, Vargem Grande Paulista, SP, Brazil). The reaction was run until PHSTAT stopped the titration, indicating the end of the reaction.

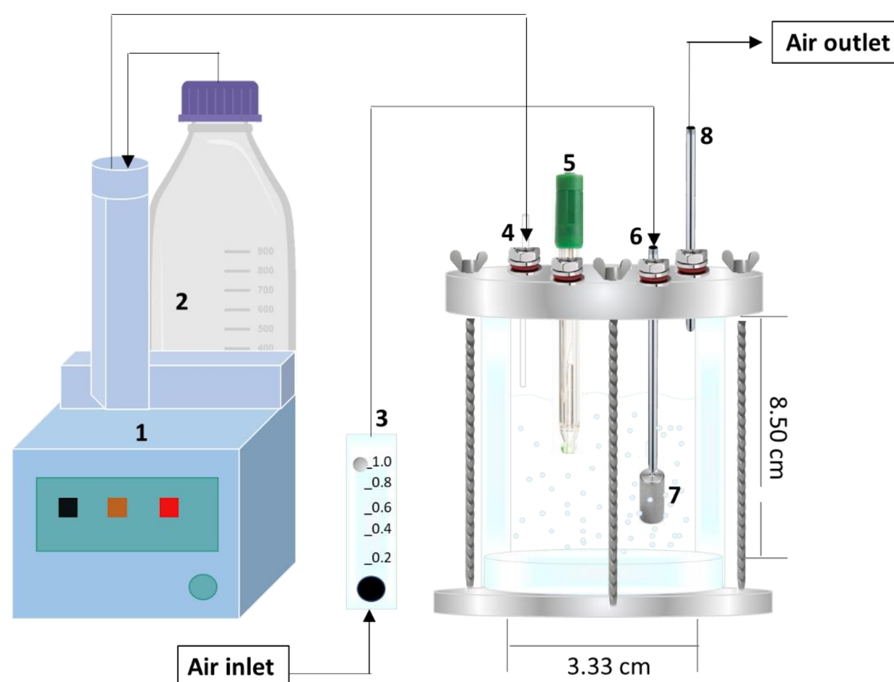


Figure 3.3. Experimental setup: 1) 718 STAT Titrino; 2) NaOH 2.0 M; 3) air flowmeter; 4) base inlet; 5) pH electrode; 6) air inlet; 7) air sparger 8) air outlet. Personal collection, 2023.

3.3 Results and discussion

The production of whole-cells from *A. niger* according to the experimental procedure described in Section 3.2.1 resulted in ca. 5 g/L of whole-cells with 100% pelletized morphology and a mean diameter of 1.9 mm (Figure 3.4).

Figure 3.4. a) Incubation in Petri dishes of potato dextrose agar medium for 5 days at 32 °C. b) Pelletized *A. niger* whole-cells obtained under the conditions detailed in Section 3.2.1. Personal collection, 2023.

The pelletized morphology obtained was consistent with the work of Buffo *et al.* (2021), who reported that an initial pH between 4 and 6, combined with a spore concentration of up to 10^7 spores/mL, resulted in cells with 100% pelletized morphology. The pH of the medium was adjusted to pH 6.0 and the initial spore concentration was set at 10^6 spores/mL, to obtain cells with pelletized morphology. Pellets are an attractive morphology to the whole-cells biocatalysis since the agglomeration of hyphae can provide the enzymes with greater protection, thus increasing mass transfer delays to the liquid phase. Furthermore, pellets generally result in a broth with less viscous rheology, compared to dispersed hyphal morphology, consequently improving oxygen transfer in the liquid phase (Rodríguez Porcel *et al*., 2005). The downstream processing for biomass separation is also easier than with mycelial morphology and can assist reuse of the cells (Ma *et al.* 2022). A possible problem in processes with pellet morphology is related to the internal mass transfer of nutrients and products (Krull *et al.*, 2013). Here, the pelletized morphology was selected for the whole-cells biocatalysis, with the concentration fixed at 3 g/L (0.3% w/v) for all the assays. The pelletized whole-cells produced were resuspended in 0.01 M citrate-phosphate buffer (pH 6.5), to be applied as biocatalysts in the GA production stage.

As described in section 3.2.4, different assays using 3 g/L whole cells associated with each commercial enzyme (GI, AMG, and β-Gal) were carried out to produce GA from 20 g/L of the different substrates: sucrose, dextrin, and lactose, respectively. Additionally, glucose was

tested under the same conditions with no commercial enzyme added to check the capacity of whole-cells of the *A. niger 12* strain to produce GA. Figure 3.5 summarizes the GA yield (%) resulting from each substrate.

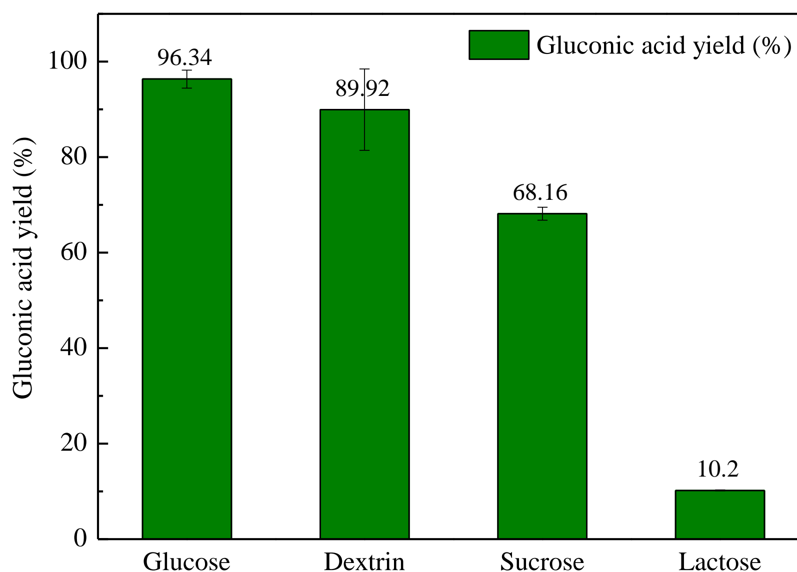


Figure 3.5. Gluconic acid yield (%) from substrates glucose, dextrin, sucrose, and lactose obtained in assays at 30°C, pH 6, and aeration of 3 vvm (0.6 L/min).

These conditions resulted in a GA yield of 96.34 ± 1.9% for glucose as substrate, proving the efficacy of the whole-cells of *A niger 12* strain to produce GA from glucose by GOD and CAT enzymes according to the mechanism simplified in Figure 3.6.

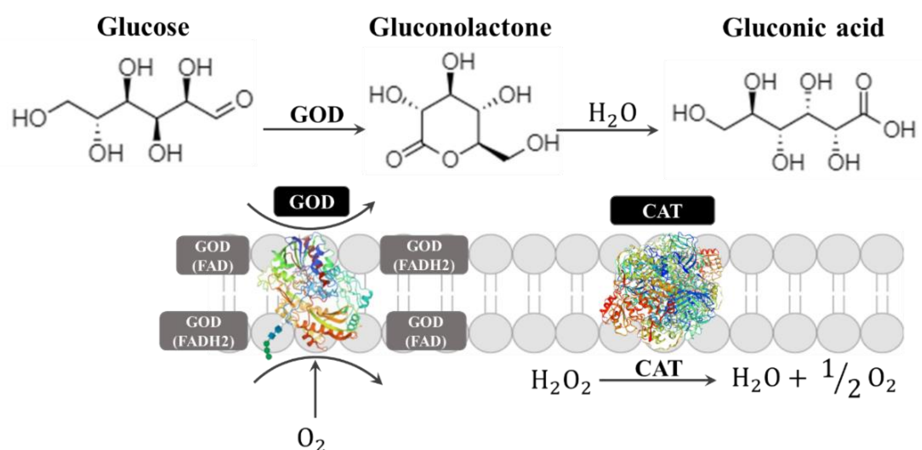


Figure 3.6. Gluconic acid production by *A. niger* whole-cells. Glucose oxidation by the constitutive enzymes GOD and CAT. GOD, FAD-dependent glucose oxidase (EC 1.1.3.4); CAT, catalase (EC 1.11.1.6). Personal collection, 2023.

Previous studies reported that the use of sucrose as a carbon source during *A. niger* cell growth induces the production of membrane-bound invertase (INV) (Rosenberg *et al.*, 1992). So, to be applied as biocatalysts in the GA production assays from the substrate sucrose, *A. niger* cells were grown in the medium described in section 3.2.1, replacing glucose with sucrose. Therefore, *A. niger* whole-cells can present the multi-enzymatic complex (INV, GOD, and CAT) to convert sucrose into GA and fructose (Silva; Tomotani; Vitolo, 2011; Taraboulsi; Tomotani; Vitolo, 2011; Mafra *et al.*, 2014).

Additionally, the commercial enzyme glucose isomerase/xylose isomerase (D-xylose ketol isomerase; EC 5.3.1.5), commonly referred to as glucose isomerase (GI), catalyzes the reversible isomerization of D-glucose to D-fructose (Bhosale; Rao; Deshpande, 1996). Considering that isomerization is a reversible reaction in which the equilibrium shifts to the substrate that is in lower concentration, the idea was that the GI enzyme acting synergistically with *A. niger* whole-cells (INV, GOD, and CAT) resulted in sucrose hydrolysis by INV, followed by the isomerization of fructose into glucose, simultaneously with the oxidation of glucose to GA, as illustrated by Figure 3.7.

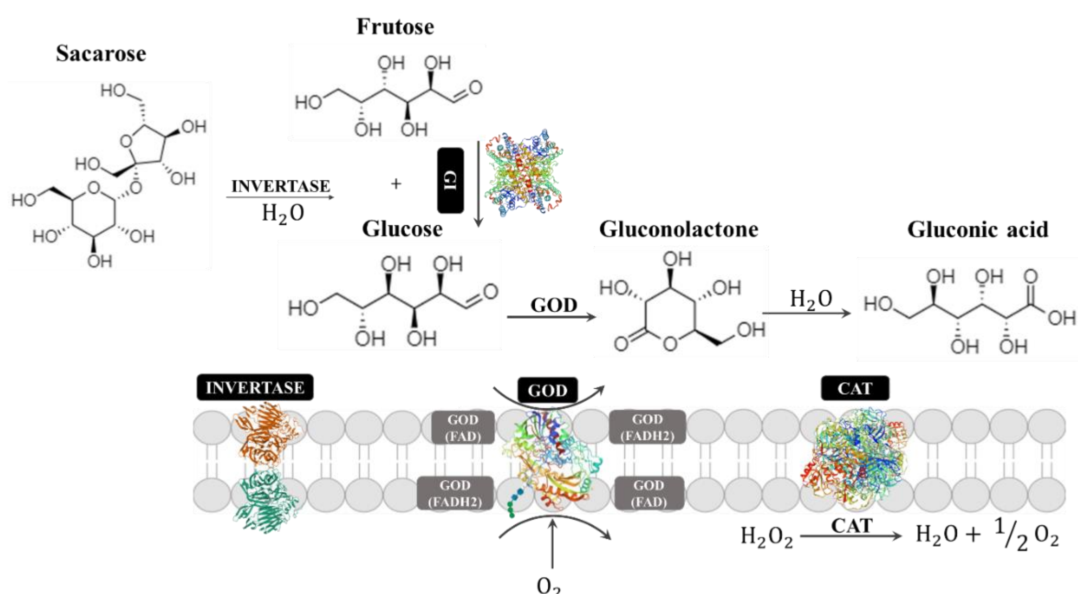


Figure 3.7. Multi-enzymatic reaction to produce gluconic acid from sucrose by *A. niger* whole-cells associated with the commercial enzyme GI (glucose-isomerase (EC 5.3.1.5) of *Streptomyces rubiginosus*. Invertase (E.C.3.2.1.26); GOD, FAD-dependent glucose oxidase (EC 1.1.3.4); CAT, catalase (EC 1.11.1.6). Personal collection, 2023.

The GA yield (%) from sucrose was 68.16 ± 1.3. Although all the sucrose has been consumed, a higher yield was not achieved most likely because of an inefficient catalytic action of GI in the presence of the GA produced, which is indeed known for its chelating potential,

capable of forming stable complexes with divalent cations such as Mg^{2+}, Mn^{2+}, and Co^{2+} (Anastassiadis; Morgunov, 2007). It is well documented that GI is a cation-divalent-dependent enzyme, especially Mg^{2+} (Bae; Hwang; Nam, 2017, 2018), which is an essential ion that activates and stabilizes the enzyme (Bhosale; Rao; Deshpande, 1996; Hobbs, 2009; Milessi-Esteves *et al.*, 2019), and is also able to enhance the enzyme-substrate affinity (Kasumi; Hayashi; Tsumura, 1982). This hypothesis is supported by the presence of fructose at the end of the reaction.

The multi-enzymatic reaction to produce GA from lactose as the substrate is illustrated in Figure 3.8.

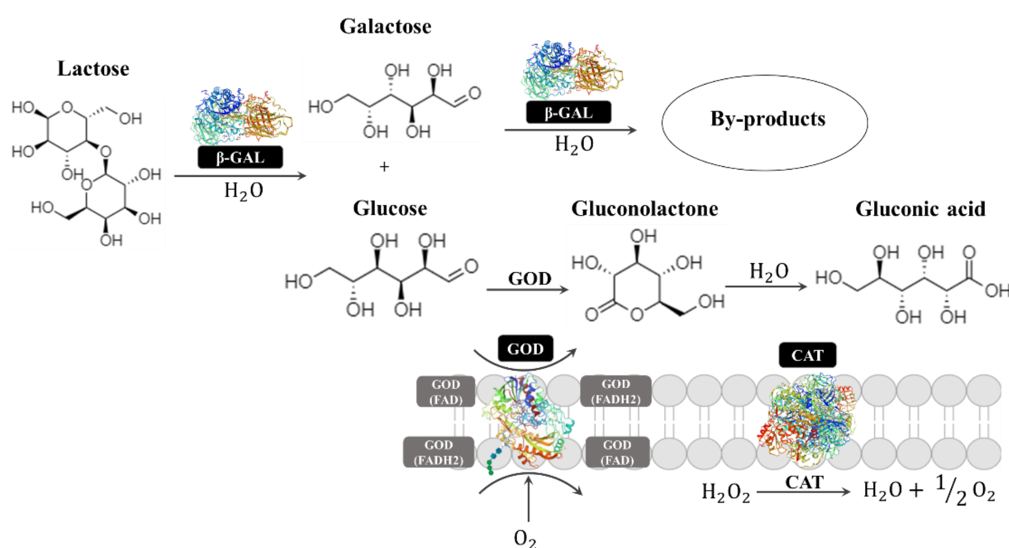


Figure 3.8. Multi-enzymatic reaction to produce gluconic acid from lactose by *A. niger* whole-cells associated with the commercial enzyme β-Gal, β-galactosidase (EC 3.2.1.23). GOD, FAD-dependent glucose oxidase (EC 1.1.3.4); CAT, catalase (EC 1.11.1.6). Personal collection, 2023.

The GA yield (%) from lactose was 10.20% ± 0.1. In addition to the approximately 40% of lactose that is not consumed, the lower GA yield from lactose was most likely due to the formation of by-products such as galactooligosaccharides and galactosyl, among others. Apart from the hydrolysis of the saccharide bond of lactose, β-galactosidases also catalyze transgalactosylation reactions, producing galactooligosaccharides (GOS) with prebiotic activity (Todea *et al.*, 2020). Additionally, the transglycosylation properties of β-galactosidase can also result in the galactosyl derivative of gluconic acid using lactose as a substrate (Wojciechowska *et al.*, 2017).

Finally, similar assays were performed using dextrin-10 as substrate, in the presence of amyloglucosidase, through the multi-enzymatic reaction to produce GA (Figure 3.9).

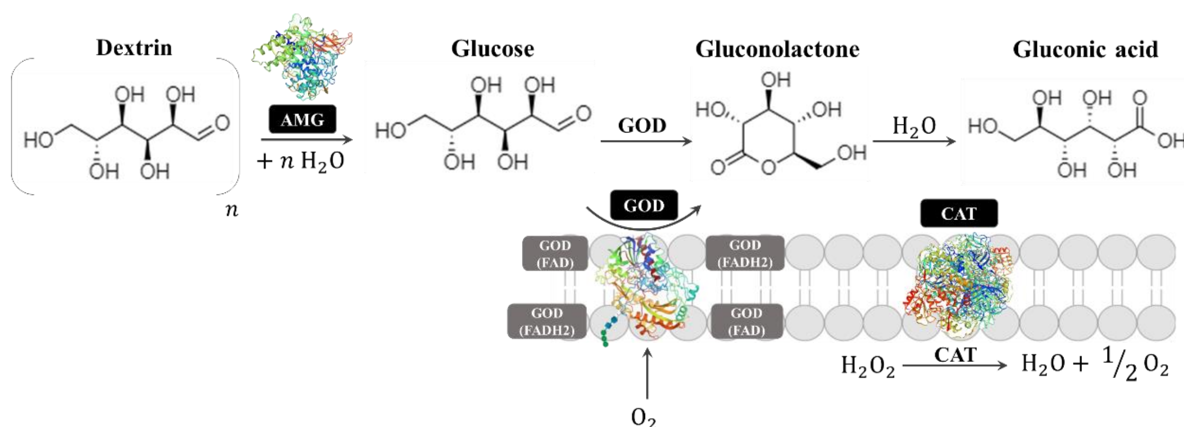


Figure 3.9. Multi-enzymatic reaction to produce GA from dextrin-10 by *A. niger* whole -cells associated with the commercial enzyme AMG (amyloglucosidase; glucoamylase; EC 3.2.1.3). GOD, FAD-dependent glucose oxidase (EC 1.1.3.4); CAT, catalase (EC 1.11.1.6). Personal collection, 2023.

The GA yield was 89.92 ± 8.5% from dextrin-10 as substrate. This good result approaches GA yield (%) from glucose. Dextrin is a soluble glucose polysaccharide obtained by partial hydrolyze of starch, widely used in many industries due to its functional properties (Lotfy, 2009). In the presence of AMG, the α-1,4 and α-1,6 glycosidic bonds at the non-reducing end of the dextrin chain are cleaved (Tomasik; Horton, 2012), releasing glucose capable to be converted into GA by *A. niger* whole-cells.

3.4 Conclusion

The high GA yield (%) from dextrin (partially hydrolyzed starch) than other glucose-containing substrates (e.g., sucrose and lactose) opens a new perspective for an innovative multi-enzymatic system to produce GA from starch using *A. niger* whole-cells associated with amylolytic enzymes. Additionally, agro-food industry starchy wastes also could be exploited as feedstock to produce GA by the multi-enzymatic system proposed.

REFERENCES

ANASTASSIADIS, S.; MORGUNOV, I. Gluconic Acid Production. **Recent Patents on Biotechnology**, vol. 1, n. 2, p. 167-180, 1 June 2007. Available from: https://doi.org/10.2174/187220807780809472.

BAE, J. Eun; HWANG, K. Y.; NAM, K. H. Structural analysis of substrate recognition by glucose isomerase in Mn^{2+} binding mode at M2 site in *S. rubiginosus*. **Biochemical and Biophysical Research Communications**, vol. 503, no. 2, p. 770-775, Sept. 2018. Available

from: https://doi.org/10.1016/j.bbrc.2018.06.074.

BAE, J. Eun; HWANG, K. Y.; NAM, K. H. Crystal structure of glucose isomerase in complex with xylitol inhibitor in one metal binding mode. **Biochemical and Biophysical Research Communications**, vol. 493, no. 1, p. 666-670, Nov. 2017. Available from: https://doi.org/10.1016/j.bbrc.2017.08.134.

BHOSALE, S. H.; RAO, M. B.; DESHPANDE, V. V. Molecular and industrial aspects of glucose isomerase. **Microbiological reviews**, vol. 60, n. 2, p. 280-300, 1996. Available from: https://doi.org/10.1128/mmbr.60.2.280-300.1996.

BUFFO, M. M. *et al*. Cellulolytic enzymes production guided by morphology engineering. **Enzyme and Microbial Technology**, vol. 149, p. 109833, Sept. 2021. Available from: https://doi.org/10.1016/j.enzmictec.2021.109833.

BUFFO, M. M. *et al*. Cellulolytic enzymes production guided by morphology engineering. **Enzyme and Microbial Technology**, vol. 149, p. 109833, Sept. 2021. Available from: https://doi.org/10.1016/j.enzmictec.2021.109833.

DICOSIMO, R. *et al*. Industrial use of immobilized enzymes. **Chemical Society Reviews**, vol. 42, n. 15, p. 6437, 2013. Available from: https://doi.org/10.1039/c3cs35506c.

KASUMI, T.; HAYASHI, K.; TSUMURA, N. Roles of magnesium and cobalt in the reaction of glucose isomerase from *Streptomyces griseofuscus* S-41. **Agricultural and Biological Chemistry**, vol. 46, no. 1, p. 21-30, 1982. Available from: https://doi.org/10.1271/bbb1961.46.21.

KRULL, R. *et al*. Characterization and control of fungal morphology for improved production performance in biotechnology. **Journal of Biotechnology**, vol. 163, n. 2, p. 112-123, Jan. 2013. Available from: https://doi.org/10.1016/j.jbiotec.2012.06.024.

LOTFY, S. Controlling degradation of low-molecular-weight natural polymer “dextrin” using gamma irradiation. **International Journal of Biological Macromolecules**, vol. 44, n. 1, p. 57-63, Jan. 2009. Available from: https://doi.org/10.1016/j.ijbiomac.2008.10.002.

MA, Y. *et al.* Production of Gluconic Acid and Its Derivatives by Microbial Fermentation: Process Improvement Based on Integrated Routes. **Frontiers in Bioengineering and Biotechnology**, vol. 10, 16 May 2022. Available from: https://doi.org/10.3389/fbioe.2022.864787.

MA, Y. *et al.* Production of Gluconic Acid and Its Derivatives by Microbial Fermentation: Process Improvement Based on Integrated Routes. **Frontiers in Bioengineering and Biotechnology**, vol. 10, 16 May 2022. Available from: https://doi.org/10.3389/fbioe.2022.864787.

MAFRA, A. C. O. *et al*. Gluconic acid production from sucrose in an airlift reactor using a multi-enzyme system. **Bioprocess and Biosystems Engineering**, vol. 38, n. 4, p. 671-680, 19 Oct. 2014. Available from: https://doi.org/10.1007/s00449-014-1306-2.

MILESSI-ESTEVES, T. *et al*. An Innovative Biocatalyst for Continuous 2G Ethanol Production from Xylo-Oligomers by Saccharomyces cerevisiae through Simultaneous

Hydrolysis, Isomerization, and Fermentation (SHIF). **Catalysts**, vol. 9, no. 3, p. 225, 1 Mar. 2019. Available from: https://doi.org/10.3390/catal9030225.

RAMACHANDRAN, S. *et al*. Gluconic Acid: Properties, Applications and Microbial Production. **Food Technology and Biotechnology**, v. 44, n. 2, p. 185-95, Apr. 2006.

RAMACHANDRAN, S. *et al.* Permeabilization and inhibition of the germination of spores of *Aspergillus niger* for gluconic acid production from glucose. **Bioresource Technology**, vol. 99, n. 11, p. 4559-4565, July 2008. Available from: https://doi.org/10.1016/j.biortech.2007.06.055.

RAMACHANDRAN, S. *et al.* Spores of *Aspergillus niger* as reservoir of glucose oxidase synthesized during solid-state fermentation and their use as catalyst in gluconic acid production. **Letters in Applied Microbiology**, vol. 44, n. 2, p. 155-160, Feb. 2007. Available from: https://doi.org/10.1111/j.1472-765x.2006.02051.x.

RAO, D. S.; PANDA, T. Comparative analysis of different whole cell immobilized aspergillus niger catalysts for gluconic acid fermentation using pretreated cane molasses. **Bioprocess Engineering**, vol. 11, n. 5, p. 209-212, Oct. 1994. Available from: https://doi.org/10.1007/bf00369632.

HOBBS, L. Sweeteners from Starch. In: HOBBS, Larry. Starch. [S. l.]: Elsevier, 2009. p. 797-832. Available from: https://doi.org/10.1016/b978-0-12-746275-2.00021-5.

RODRÍGUEZ PORCEL, E. M. *et al*. Effects of pellet morphology on broth rheology in fermentations of *Aspergillus terreus*. **Biochemical Engineering Journal**, vol. 26, no. 2-3, p. 139-144, Nov. 2005. Available from: https://doi.org/10.1016/j.bej.2005.04.011.

RODRÍGUEZ PORCEL, E. M. *et al*. Effects of pellet morphology on broth rheology in fermentations of *Aspergillus terreus*. **Biochemical Engineering Journal**, vol. 26, no. 2-3, p. 139-144, Nov. 2005. Available from: https://doi.org/10.1016/j.bej.2005.04.011.

ROSENBERG, M. *et al*. Importance of invertase activity for gluconic acid production from sucrose by *Aspergillus niger*. **Acta Biotechnologica**, vol. 12, n. 4, p. 311-317, 1992. Available from: https://doi.org/10.1002/abio.370120411.

SILVA, A. R.; TOMOTANI, E. J.; VITOLO, M. Invertase, glucose oxidase and catalase for converting sucrose to fructose and gluconic acid through batch and membrane-continuous reactors. **Brazilian Journal of Pharmaceutical Sciences**, vol. 47, n. 2, p. 399-407, June 2011. Available from: https://doi.org/10.1590/s1984-82502011000200022.

TARABOULSI, F. A.; TOMOTANI, E. J.; VITOLO, M. Multienzymatic Sucrose Conversion into Fructose and Gluconic Acid through Fed-Batch and Membrane-Continuous Processes. **Applied Biochemistry and Biotechnology**, vol. 165, n. 7-8, p. 1708-1724, 24 Sept. 2011. Available from: https://doi.org/10.1007/s12010-011-9389-6.

TODEA, A. *et al*. One-pot biocatalytic conversion of lactose to gluconic acid and galacto-oligosaccharides using immobilized β-galactosidase and glucose oxidase. **Catalysis Today**, Aug. 2020. Available from: https://doi.org/10.1016/j.cattod.2020.06.090.

TOMASIK, P.; HORTON, D. Enzymatic conversions of starch. **Advances in Carbohydrate Chemistry and Biochemistry**. [S. l.]: Elsevier, 2012. p. 59-436. Available from: https://doi.org/10.1016/b978-0-12-396523-3.00001-4.

WITTEVEEN, C. F. B.; VEENHUIS, M.; VISSER, J. Localization of Glucose Oxidase and Catalase Activities in *Aspergillus niger*. **Applied and Environmental Microbiology**, vol. 58, no. 4, p. 1190-1194, 1992. Available from: https://doi.org/10.1128/aem.58.4.1190-1194.1992

WOJCIECHOWSKA, A. *et al*. Synthesis of Galactosyl Derivative of Gluconic Acid with Transglycosylation Activity of β-galactosidase. **Food Technology and Biotechnology**, vol. 55, n. 2, 2017. Available from: https://doi.org/10.17113/ftb.55.02.17.4732.

WOODLEY, J. M. Microbial Biocatalytic Processes and Their Development. **Advances in Applied Microbiology**. Elsevier, 2006. p. 1-15. ISBN 9780120026623. Available from: https://doi.org/10.1016/s0065-2164(06)60001-4.

4 AN INNOVATIVE ONE-STEP MULTI-ENZYMATIC SYSTEM FOR GLUCONIC ACID PRODUCTION FROM STARCH USING *ASPERGILLUS NIGER* WHOLE CELLS

RESUMO

A utilização de sistemas multienzimáticos para a produção industrial de compostos químicos é considerada uma importante ferramenta verde na química orgânica sintética. O ácido glicônico é um ácido orgânico multifuncional amplamente utilizado nas indústrias química, farmacêutica, alimentícia, têxtil e de construção. Sua produção industrial a partir de glicose por fermentação utilizando *Aspergillus niger* ainda apresenta alguns gargalos tais como altos custos relacionados ao crescimento celular e manutenção da viabilidade celular. Este estudo apresenta um sistema multienzimático inovador de uma etapa para produção de ácido glicônico a partir de amido usando células inteiras de *Aspergillus niger* em associação com enzimas amilolíticas. Utilizando amido solúvel como substrato, foram alcançados os seguintes resultados para 96 h de reação: concentração de ácido glicônico de 134,5 ± 4,3 g/L, rendimento de ácido glicônico de 98,2 ± 1,3% e rendimento de biocatalisador de 44,8 ± 1,4 $g_{GA}/g_{células\ inteiras}$. Embora o processo tenha sido desenvolvido utilizando amido como matéria-prima, a abordagem é viável para qualquer substrato ou resíduo que possa ser hidrolisado em glicose.

Palavras-chave: Células inteiras; *Aspergillus niger*; amido; sistema-multienzimático; ácido glicônico; enzimas amilolíticas.

ABSTRACT

The use of multi-enzymatic systems for the industrial production of chemical compounds is currently considered an important green tool in synthetic organic chemistry. Gluconic acid is a multi-functional organic acid widely used in the chemical, pharmaceutical, food, textile, and construction industries. Its industrial production from glucose by fermentation using *Aspergillus niger* has drawbacks including high costs related to cell growth and maintenance of cell viability. This study presents an innovative one-step multi-enzymatic system for gluconic acid production from starch using *Aspergillus niger* whole-cells in association with amylolytic enzymes. Using soluble starch as substrate, the following results were achieved for 96 h of reaction: 134.5.±.4.3 g/L gluconic acid concentration, 98.2.±.1.3% gluconic acid yield, and 44.8.±.1.4 $g_{GA}/g_{whole\text{-}cells}$ biocatalyst yield. Although the process has been developed using starch as raw material, the approach is feasible for any substrate or residue that can be hydrolyzed to glucose.

Keywords: Whole-cells; *Aspergillus niger*; starch; one-step multi-enzymatic system; gluconic acid; amylolytic enzymes.

4.1 Introduction

Gluconic acid (GA) is a mild, non-corrosive, nontoxic, and easily biodegradable organic acid produced from the microbial oxidation of glucose. Its chemical and physiological characteristics make GA, and its alkali metal salts, especially sodium gluconate, highly useful in the chemical, textile, beverage, pharmaceutical (e.g., for iron and calcium deficiency), construction, and food industries (Ramachandran *et al.*, 2006; Anastassiadis; Morgunov, 2007). The most widely sold derivative is sodium gluconate, corresponding to more than 80% of gluconic acid production worldwide (Kornecki *et al.*, 2020). According to market research reports, the global gluconic acid market was valued at approximately USD 1 billion in 2020 and is estimated to reach USD 1.9 billion by 2028, growing at a compound annual growth rate (CAGR) of 5% during the period 2021-2028 (Ahuja; Singh, 2018).

Chemical and fermentative processes are the main techniques used to produce GA. Although the one-step conversion for the synthesis of GA by chemical and electrolytic oxidation is efficient, the high electrolysis cost, environmental toxicity, and biological hazards limit the industrial application of chemical approaches (Ma *et al* 2022; BioAcids Tech, 2023). Hence, current industrial processes are based on a fermentation process employing *Aspergillus niger* (*A. niger*) with glucose as substrate (Kornecki *et al.*, 2020). However, fermentative processes still present drawbacks: 1) during industrial fermentation, mycelia are entangled with protein flocculants, activated carbon, and other fermentation broth components, which hinders the reuse of strains; 2) inoculation of the spores of the fungus used for fermentation involves long and complex culture conditions, requiring rigorous aseptic procedures to avoid contamination; 3) there are additional supplementation costs for cell growth and maintenance of cell viability (Ma *et al* 2022). Although enzymatic processes have been studied to overcome these bottlenecks (Kornecki *et al.*, 2020), the high cost of commercial enzymes still hinders the application of these biocatalysts on a large scale (Sakhuja *et al.*, 2021). Therefore, there are several ongoing attempts to develop alternative processes for GA production, but the eco-friendly production of high-purity GA by a feasible process has remained a challenge (Kornecki *et al.*, 2020).

GA can also be produced through whole-cell biocatalysis (Kornecki *et al.*, 2020), as the GA production pathway is not directly linked to the primary metabolic pathways of cell growth, development, or reproduction, such as glycolysis or the citric acid cycle Cameselle *et al.* 1998. As such, it can be generated during the stationary phase or under conditions of non-growth, arising from nutrient starvation or stress (Znad; Markoš; Baleš, 2004).

In this context, conventional whole-cell biocatalysis primarily focuses on converting substrates into desired products at the expense of cell growth. This phenomenon can be harnessed by strategically modulating the availability of essential nutrients, thereby arresting cellular growth (de Carvalho, 2017; Lin; Tao, 2017). This biosynthetic strategy offers advantages such as high specificity and biocatalyst stability under gentle operational and environmentally friendly conditions. It also enables biocatalyst reuse, has low toxicity, and presents the potential to utilize eco-friendly waste as a substrate (Garzón-Posse *et al.*, 2018). These advantages position whole-cell biocatalysis as a highly significant tool for developing novel green techniques that exhibit better cost-effectiveness compared to traditional processes (de Carvalho, 2017; Cheng *et al*., 2020; Song *et al.*, 2020; Intasian *et al.*, 2021).

Whole cells can be applied as biocatalysts in several biochemical transformations, depending on the different enzyme sets of each microorganism. Examples include oxidation-reduction reaction by a whole-cell dehydrogenase (Klatte; Lorenz; Wendisch, 2013), hydrolysis and esterification using whole-cell lipases (dos Santos; da Silva Cruz; Tardioli, 2017), and isomerization reaction through whole-cell glucose-isomerase (Jia *et al.*, 2018). Consequently, whole-cell biocatalysis is a promising and emerging field of science based on advancements in biosystems engineering to synthesize relevant products such as chemicals, fuels, and pharmaceuticals through multi-enzymatic reactions with cofactor regeneration, with high regio- and stereo-selectivity, under mild operational and environment-friendly conditions (de Carvalho, 2017; Wachtmeister; Rother, 2016; Madavi *et al.* 2021).

The present study proposes an innovative multi-enzymatic system for gluconic acid production, with starch as a model substrate, employing *A. niger* whole-cells and amylolytic enzymes. Starch, one of the most abundant natural polysaccharides, is a sustainable material, due to its biodegradability and renewability, and is widely used industrially (Zeng *et al.* 2022). Enzymatic processing to transform raw starch into glucose is already widely applied on an industrial scale, where the main enzymes involved (α-amylase and amyloglucosidase) are extracellular enzymes commercialized at a cost that is not high when compared to intracellular enzymes or new enzymes (van der Maarel *et al.*, 2002; Cripwell *et al.*, 2020; Witteveen; Veenhuis; Visser, 1992).

Additionally, the use of wc containing enzymes for biocatalytic synthesis is attracting considerable industrial interest as a way to avoid costs associated with the purification of intracellular enzymes (Lin; Tao, 2017). *A. niger* whole-cells are considered excellent enzyme bags and promising biocatalysts for GA production because they contain the constitutive enzymes

glucose oxidase (GOD) and catalase (CAT) (Witteveen; Veenhuis; Visser, 1992; Vassilev *et al.*, 1993; Ramachandran *et al.*, 2007, 2008).

Briefly, the multi-enzymatic process proposed in this study for GA production from starch involves two key steps. First, starch hydrolysis takes place using commercial enzymes (α-amylase and amyloglucosidase). Following this, glucose oxidation is facilitated by the enzymes GOD and CAT within the *A. niger* whole-cells environment, as depicted in Figure 4.1. Notably, primary metabolic pathways essential for cell growth, development, and reproduction, such as glycolysis and the citric acid cycle Cameselle *et al.* 1998, are simplified and marked by the gray dotted area due to nitrogen starvation suppression.

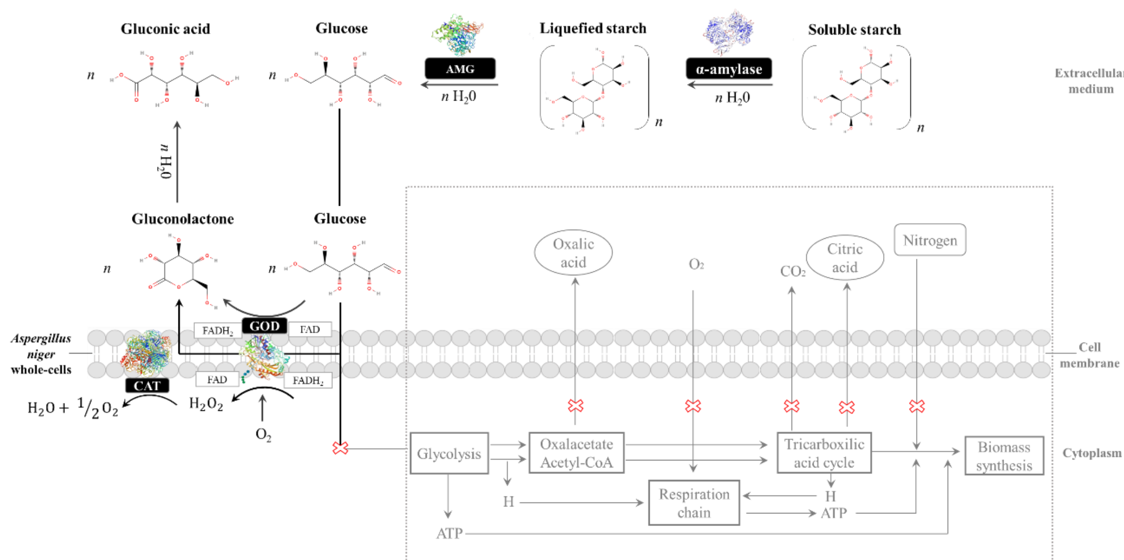


Figure 4.1. The innovative multi-enzymatic process for gluconic acid production from starch with commercial enzymes (α-amylase and amyloglucosidase) followed by the oxidation of glucose by the enzymes GOD and CAT in situ, in *A. niger* whole-cells. Primary metabolic pathways essential for cell growth, development, and reproduction, such as glycolysis and the citric acid cycle, are simplified and marked by the gray dotted area due to nitrogen starvation suppression. Alpha-amylase (4-α-d-glucan glucanohydrolase; EC 3.2.1.1); AMG (amyloglucosidase; glucoamylase; EC 3.2.1.3); GOD (FAD-dependent glucose oxidase; EC 1.1.3.4); CAT (catalase; EC 1.11.1.6). Adapted from Cameselle *et al.*1998.

This approach (Figure 4.1) can have great advantages for gluconic acid production, compared to the fermentation process: (1) economy with expensive nutrients for microorganism growth; (2) the possibility of maintaining operating conditions such as pH, temperature, and oxygen transfer at the optimum for enzymatic conversion, without precautions related to growth of the microorganism; (3) potential for greater conversion of substrate to the target product, since carbon from the substrate can be directed to acid gluconic production, avoiding other routes (growth, or production of another organic acid); (4) a higher purity product, reducing the

costs of downstream steps; (5) low risk of contamination, due to the absence of a nitrogen source.

Therefore, this study provides a new strategy for GA production, at a high yield and in a single step, using a low-cost multi-enzymatic biocatalyst and an inexpensive substrate. Firstly, *A. niger* whole-cells were used for GA production from glucose, to evaluate their potential for use as biocatalysts containing GOD and CAT associated with the fungus cell structure. In the next step, the operational conditions for GA production by simultaneous saccharification and oxidation using *A. niger* whole-cells combined with amyloglucosidase (AMG) were established by an experimental design using dextrin-10 as substrate and evaluating the following independent variables: initial substrate concentration, pH, and AMG concentration. The best conditions predicted by the model were then validated for soluble starch as substrate only with the addition of α-amylase. This polysaccharide was chosen because is easily converted to glucose and is highly abundant worldwide, including in agro-industrial wastes. Optimization was then performed of the process of simultaneous saccharification and oxidation, in terms of GA production (g/L). Finally, the production of GA at high purity (~96%) from soluble starch was achieved in a one-step process combining α-amylase and AMG with *A. niger* whole-cells.

4.2 Material and methods

4.2.1 Strain and medium for wc production

A wild-type strain of the filamentous fungus *Aspergillus niger 12* (National Register of Biological Collections number BRM028885) was obtained from the Embrapa Food Technology collection (Rio de Janeiro, Brazil). The microorganism was maintained at -80 °C and was reactivated at least every 90 days by incubation in Petri dishes of potato dextrose agar medium for 5 days at 32 °C. Cryotubes with spore suspensions were prepared in 20% (v/v) glycerol, maintained at -80 °C, for use in the inoculum preparation (Buffo *et al.* 2021). The concentration of the spore suspension was determined by counting in a Neubauer chamber (Brand, Germany). *A. niger* whole-cells were prepared using the growth culture medium proposed by Rao & Panda (1994) for gluconic acid production. The culture medium consisted of (in g/L): glucose (50.0), $(NH_4)_2HPO_4$ (4.0), urea (1.8), KH_2PO_4 (3.3), and $MgSO_4.7H_2O$ (2.8). D-glucose and all other chemicals (analytical grade) were purchased from Synth (Diadema, SP, Brazil) and Vetec (Duque de Caxias, RJ, Brazil).

4.2.2 Experimental procedure for wc production

Erlenmeyer flasks (500 mL) containing 100 mL of culture medium were inoculated with

an initial concentration adjusted to 10^6 spores/mL and incubated at 32 °C and 200 rpm, for 72 h, in an orbital shaker-incubator (model TE-421, Tecnal, Piracicaba, SP, Brazil). The pH of the medium was adjusted to pH 6.0 with 1.0 M HCl, before sterilization in an autoclave (model AV, Phoenix Luferco, Araraquara, SP, Brazil) at 121 °C for 20 min. The resulting wc were separated from the culture medium by filtration, washed with distilled water, and resuspended in 0.01 M citrate-phosphate buffer at pH 6.5, to be used as biocatalysts in the GA production stage. The cells were analyzed using a stereomicroscope (model XT-3L-BI, Biofocus, Araucária, PR, Brazil) coupled to a digital camera. The images were processed with Image-J software (rsbweb.nih.gov/ij). The pellet diameters were obtained directly by image analysis using Image-J software (Buffo *et al.* 2021).

4.2.3 Validation of A. niger whole-cells as biocatalyst for gluconic acid production from glucose

Assays were performed in a 350 mL mini bubble bioreactor (8.50 × 3.33 cm, length × diameter) with a working volume of 200 mL, containing a sintered filter used as a sparger for air supplied at a specific flow rate of 5 vvm (1 L/min), ensuring sufficient available oxygen. The pH was kept constant at pH 6.0 by feeding a solution of 2.0 M NaOH from an automatic titrator (model 718 STAT Titrino, Metrohm AG, Herisau, Switzerland), in order to neutralize the GA produced (Figure 4.2).

Figure 4.2. Experimental setup: 1) 718 STAT Titrino; 2) NaOH 2.0 M; 3) air flowmeter; 4) base inlet; 5) pH electrode; 6) air inlet; 7) air sparger 8) air outlet. Personal collection, 2023.

The capacity of the *A. niger 12* whole-cells as biocatalysts for GA production was evaluated using glucose at 50 g/L as substrate and wc at 3 g/L (0.3% w/v) in the mini bubble bioreactor, with pH control during GA production. The temperature in the bioreactor was kept at 40 °C, the optimal temperature for glucose oxidase from *A. niger* (Bhatti *et al.* 2006), using a thermostatic bath (model 521/2D, Nova Ética, Vargem Grande Paulista, SP, Brazil).

4.2.4 Optimization of simultaneous saccharification and oxidation of dextrin for gluconic acid production

4.2.4.1 Enzyme and production medium

Amyloglucosidase AMG 300L™ from *Aspergillus niger* (EC 3.2.1.3, glucoamylase, amyloglucosidase, Novozymes, Bagsvaerd, Denmark) (≥ 260 U/mL) was kindly donated by LNF Latino Americana (Bento Gonçalves, RS, Brazil). The GA production medium contained only dextrin-10 (Sigma-Aldrich, St. Louis, MO, USA) dissolved in distilled water at different concentrations. No nutritional supplementation was added to the GA production medium.

4.2.4.2 Experimental design

To optimize the process of simultaneous saccharification and oxidation of dextrin-10 to GA using AMG and *A. niger* whole--cells, an experimental design was carried out with three independent variables: dextrin-10 concentration (X_1), pH (X_2), and AMG concentration (X_3). The response variable was GA production (g/L) at 48 h. For all the experimental design assays, the biocatalyst (wc) concentration and temperature were fixed at 3 g/L (0.3% w/v) and 40 °C, respectively. Table 4.1 shows the real and coded values of the independent variables of the central composite rotatable design (CCRD), for 17 runs in random order (Rodrigues; Iemma, 2014).

Table 4.1. Real values and the corresponding coded values for each variable of the central composite rotational

Code	Parameter	Unit	-1.68	-1	0	1	+1.68
X_1	Dextrin-10 concentration	g/L	50.00	90.48	150.00	209.52	250.00
X_2	pH		4.50	5.11	6.00	6.89	7.50
X_3	AMG concentration	% (v/v)	0.10	0.28	0.55	0.82	1.00

A full quadratic polynomial regression model was used to correlate the experimental data, according to Equation 4.1.

$$y^* = \beta_0 + \sum_1^i \beta_i X_i + \sum_1^i \beta_{ii} X_i^2 + \sum_1^i \sum_2^j \beta_{ij} X_{ij} \quad (4.1)$$

where y* is the response variable (GA production (g/L) at 48 h), X_i are process factors including the initial dextrin-10 concentration (X_1), pH (X_2), and the AMG concentration (X_3), β_0 is the offset coefficient, β_i are linear coefficients, β_{ii} are quadratic coefficients, and β_{ij} are interaction coefficients. Simplifying for three variables, Equation 1 can be rewritten as Equation 4.2:

$$y^* = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (4.2)$$

Regression analysis of the experimental data was performed using Statistica software (version 7.0, StatSoft) to determine the model coefficients and their significance. The polynomial equations were used to plot three-dimensional (3D) surfaces and two-dimensional (2D) contours, enabling visualization of the individual and interactive effects of the process factors on the response variables, within their predefined ranges. The significance of the model was evaluated by the determination of the R^2 and adjusted R^2 coefficients. Subsequent validation experiments were conducted to confirm the response predicted by the fitted model.

4.2.4.3 Experimental procedure

Assays for GA production from dextrin-10 were carried out in a 350 mL mini bubble bioreactor (8.5 × 3.33 cm, length × diameter) with a working volume of 200 mL, containing a sintered filter used as a sparger for aeration at 5 vvm (1 L/min). The pH was kept constant (at different values for each assay) by supplying a solution of 2.0 M NaOH from an automatic titrator (model 718 STAT Titrino, Metrohm AG, Herisau, Switzerland) to neutralize the GA produced. For all assays, 3 g/L of wc were applied as biocatalysts and the temperature was maintained at 40 °C using a thermostatic bath (model 521/2D, Nova Ética, Vargem Grande Paulista, SP, Brazil). Validation assays were carried out in duplicate, and samples were with-drawn at 12 h intervals for quantification of GA and glucose by HPLC (Mafra *et al.* 2014).

4.2.5 Gluconic acid production from soluble starch

4.2.5.1 Enzymes and production medium

Amyloglucosidase AMG 300L™ from *Aspergillus niger* (EC 3.2.1.3, glucoamylase, amyloglucosidase) (≥ 260 U/mL) and α-amylase (BAN® 480L) (480 KNU/kg of activity), both from Novozymes (Bagsvaerd, Denmark) were kindly donated by LNF Latino Americana (Bento Gonçalves, RS, Brazil). The GA production medium contained only soluble starch (Synth, Diadema, SP, Brazil) dissolved in distilled water.

4.2.5.2 Experimental procedure

Using soluble starch as substrate, a validation assay was carried out, in duplicate, under the conditions previously optimized for dextrin-10. For this, a soluble starch solution (121.10 g/L) was fed into the mini bubble column bioreactor (200 mL working volume), with aeration at 5 vvm (1 L/min). Next, 0.10% (v/v) of α-amylase, 0.70% (v/v) of AMG, and 3 g/L (0.3% w/v) of wc were added simultaneously to the mini bioreactor. The pH was kept constant at 5.50 by dosing a solution of 2.0 M NaOH from an automatic titrator (model 718 STAT Titrino, Metrohm AG, Herisau, Switzerland). The temperature was kept at 40 °C using a thermostatic bath (model 521/2D, Nova Ética, Vargem Grande Paulista, SP, Brazil). Samples were with-drawn at 12 h intervals for quantification of GA and glucose by HPLC.

4.2.6 Gluconic acid yields

The GA yield was calculated according to Equation 4.3:

$$\text{GA yield (\%)} = \frac{\left(C_{GA} - C_{GA_0}\right) \times 0.918}{C_{Glc_0} - C_{Glc}} \times 100\% \qquad (4.3)$$

where, C_{GA} and C_{Glc} are the final concentrations (g/L) and C_{GA_0} and C_{Glc_0} are the initial

concentrations (g/L) of gluconic acid and glucose, respectively.

The biocatalyst yield, expressed as g_{GA}/g_{wc} was obtained using Equation 4.4:

$$\text{Biocatalyst yield } (g_{GA}/g_{wc}) = \frac{C_{GA}}{C_{wc}} \quad (4.4)$$

where, C_{wc} is the wc concentration (g/L).

4.2.7 Analytical methods

4.2.7.1 High-performance liquid chromatography (HPLC)

GA was quantified using an HPLC system (model 410, Waters, Milford, MA, USA) equipped with a UV detector (set at 210 nm) and an Aminex HPX87-H column maintained at 55 °C. The eluent was 5 mM sulfuric acid in Milli-Q water, at a flow rate of 0.6 mL/min. Before injection, all the samples were filtered through a 0.22 µm filter. The concentration of glucose was determined by HPLC (model 410, Waters, Milford, MA, USA) with a differential refractometer detector. The compounds were separated on a Sugar-Pak column kept at 80 °C, with Milli-Q water as the eluent at a flow rate of 0.5 mL/min (Mafra *et al.* 2023). For glucose quantification by HPLC, samples containing soluble starch were hydrolyzed with α-amylase (BAN 480L, 2 mL/kg starch) at 60 °C for 20 min. The temperature was set to 55 °C and soluble AMG (3 mL/kg starch) was added for saccharification of the liquefied starch to glucose (Fonseca, 2019).

4.2.7.2 Mass spectrometric method

Qualitative mass spectrometry was employed to confirm the identity of the gluconic acid produced. The standard (1.02 mg/mL) and the sample (1 mg/mL) were analyzed using a Xevo-TQD system (Waters, Milford, MA, USA) equipped with an electrospray ionization (ESI) source operated in negative mode, at atmospheric pressure. The mass spectrum was recorded using MassLynx software.

For optimization of the ionization conditions, the standard solution was infused into the system at a flow rate of 100 µL/min. The operating conditions were a capillary voltage of 3.8 kV, a desolvation temperature of 400 °C, and a desolvation gas flow rate of 1,000 L/h. The collision energy (CE) and cone voltage were optimized for each transition, using the IntelliStart autotuning tool of the MassLynx software. The mass spectrometer was operated in selected reaction monitoring (SRM) mode and the default transitions were 194.9 to 128.96 (cone: 30 V; CE: 12 eV) and 194.9 to 75.06 (cone: 30 V; CE: 12 eV).

The sample was analyzed under the same conditions as the gluconic acid standard, using the Intellistart autotuning tool to compare the mass spectra of the standard and the sample.

4.2.7.3 Gluconic acid purity

The product purity was calculated by dividing the pure sodium gluconate (SG) mass by the dry mass of the total sample (obtained by the oven-drying method) and multiplying by 100 (Equation 4.5).

$$\text{GA purity (\%)} = \frac{\text{mass of pure SG}}{\text{total sample mass}} \times 100\% \tag{4.5}$$

To calculate the mass of pure SG, the mass of gluconic acid (GA) was obtained by multiplying the GA concentration (determined by HPLC, according to the method described in Section 4.2.7.1 by the sample volume and multiplying by a factor of 1.112, which corresponds to the ratio between the molecular weights of SG (MW_{SG}) and GA (MW_{GA}) (Equation 4.6).

$$\text{mass of pure SG (g)} = \text{mass of pure GA (g)} \times \frac{MW_{SG}\ (\frac{g}{mol})}{MW_{GA}\ (\frac{g}{mol})} \tag{4.6}$$

4.3 Results and discussion

4.3.1 A. niger whole-cells production and characterization

The production of wc from *A. niger* according to the experimental procedure described in Section 4.2.1 resulted in ca. 5 g/L of wc with 100% pelletized morphology and a median diameter of 1.9 mm (Figure 4.3).

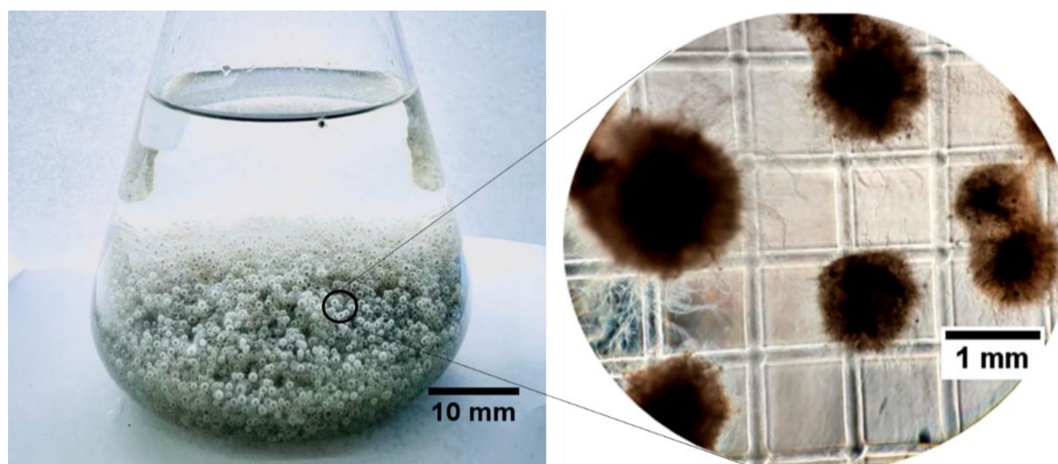


Figure 4.3. a) Pelletized *A. niger* whole-cells obtained under the conditions detailed in Section 4.2.1. b) Stereomicroscopy image of pelletized *A. niger* whole-cells. Personal collection, 2023.

The pelletized morphology obtained was consistent with the work of Buffo *et al.* (2021), who reported that an initial pH between 4 and 6, combined with a spore concentration of up to 10^7 spores/mL, resulted in cells with 100% pelletized morphology. The pH of the medium was adjusted to pH 6.0 and the initial spore concentration was set at 10^6 spores/mL, to obtain cells

with pelletized morphology. Pellets are an attractive morphology for the use of wc since the agglomeration of hyphae can provide the enzymes with greater protection. Furthermore, pellets generally result in a broth with less viscous rheology, compared to dispersed hyphal morphology, consequently improving oxygen transfer in the liquid phase (Rodríguez Porcel *et al.*, 2005). The downstream processing for biomass separation is also easier than with mycelial morphology (which tends to entangle) and can assist reuse of the cells (Ma *et al.* 2022). A possible problem in processes with pellet morphology is related to the internal mass transfer of nutrients and products (Krull *et al.*, 2013). Here, the pelletized morphology was selected for the wc, with the concentration fixed at 3 g/L (0.3% w/v) for all the assays. The pelletized wc produced were resuspended in 0.01 M citrate-phosphate buffer (pH 6.5), to be applied as biocatalysts in the GA production stage.

It is important to point out that the *A. niger* whole-cells were obtained based on a culture medium for gluconic acid production and that such cells may be derived from the reuse of *A. niger* cells employed industrially to produce gluconic acid or other bio-products. This is possible because the *A. niger* enzymes (GOD and CAT) capable of producing gluconic acid are present in the cell membrane of the grown microorganism (Witteveen; Veenhuis; Visser, 1992). Furthermore, *A. niger* cells can maintain GOD activity even after storage for 3 months at 20 °C, without any loss of enzyme activity (Ramachandran *et al.*, 2007).

4.3.2 Gluconic acid production from glucose: validation of A. niger 12 whole-cells as biocatalysts

GA production depends on the oxygen availability and pH of the production medium. Since GOD is inactivated at low pH, the medium must be kept at a pH between 4.5 and 6.5 to improve GA production (Ramachandran *et al.* 2006). For this reason, the experimental procedure described in Section 4.2.3 employed a high aeration condition (5 vvm) and controlled pH 6.0 in the preliminary assays. These conditions resulted in a final GA concentration of 48.9.±.0.3 g/L from 45.4.±.0.5 g/L of glucose, with a GA yield of 98.9.±.0.4% and a biocatalyst yield of 16.3.±.0.1 g_{GA}/g_{wc}, for 36 h of reaction. There was almost no formation of other organic acids. These results were much better than those reported by Rao & Panda (1994), who also used *A. niger* whole-cells as biocatalysts, resulting in a final GA concentration of 12.9 g/L and GA yield of 25.9% after 3 days of reaction. Hence, the wc pellets from *A. niger 12* showed excellent potential as biocatalysts for GA production. These results were achieved at a temperature of 40 °C and applying 3 g/L of wc, which were the conditions adopted in all the subsequent

assays.

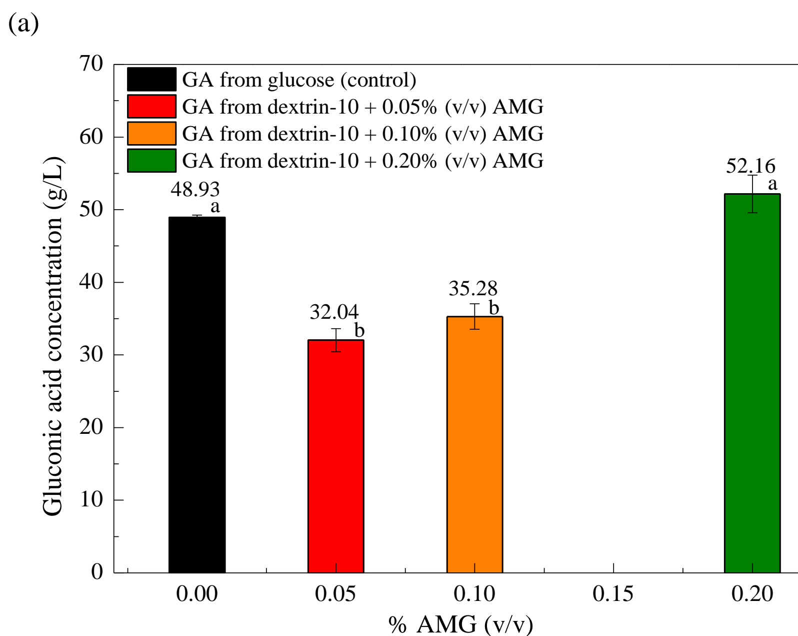
4.3.3 Optimization of simultaneous saccharification and oxidation for gluconic acid production from dextrin

Dextrin is one of several carbohydrates that have the same general formula as starch, but it is a smaller and less complex molecule, widely used in many industries due to its functional properties (Lotfy, 2009). A previous study reported that the saccharification of dextrin-10 by amyloglucosidase (AMG) generated glucose with nearly 100% yield (Amaral-Fonseca *et al.*, 2021). Hence, dextrin-10 was selected as the substrate to optimize the process of simultaneous saccharification and oxidation to GA using AMG and *A. niger* whole-cells.

4.3.3.1 Preliminary assays for gluconic acid production from dextrin-10

According to *Matsui et al.* (2013), 0.4% (v/v) of AMG was found to be suitable for saccharification of 300 g/L corn starch, avoiding the glucose feeding limitation of GA production during the fermentation. Preliminary assays were performed to evaluate the AMG percentage employed during GA production by wc, so that saccharification of dextrin-10 would not be the limiting step. A range of 0.05-0.20% (v/v) AMG was applied for 50 g/L dextrin-10 (Figure 4.4).

(a)



(b)

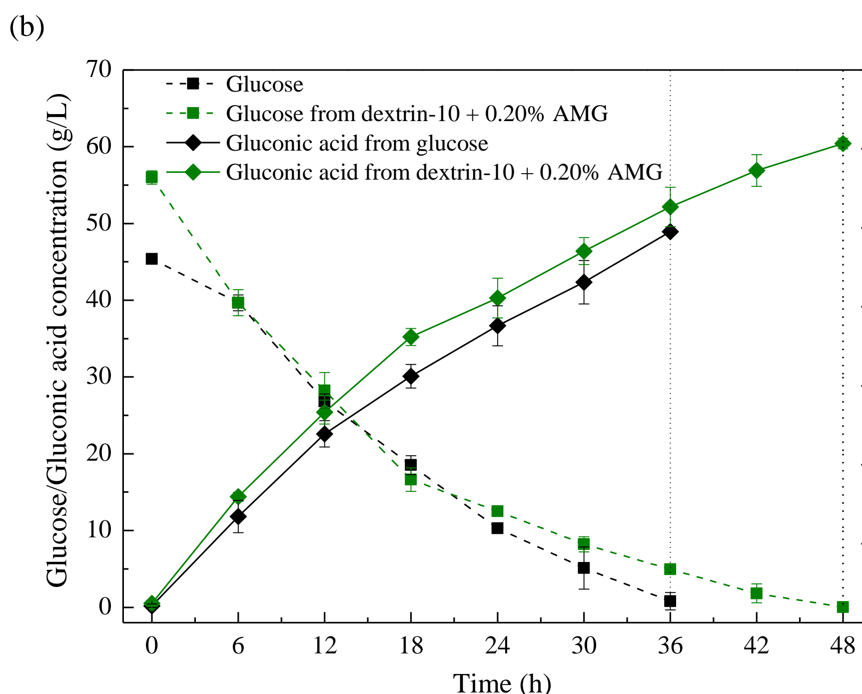


Figure 4.4. a) GA production from 50 g/L of dextrin-10 with different amounts of AMG (% v/v), for 36 h of reaction. Different letters above the bars indicate statistically significant differences. b) Temporal profiles for GA production from glucose and dextrin-10 + 0.20% (v/v) AMG. T = 40 °C; pH = 6.0; specific air flow rate = 5 vvm (1 L/min); 3 g/L of *A. niger* whole-cells.

As shown in Figure 4a, when 0.20% (v/v) of AMG was added, GA production using 50 g/L of dextrin-10 was not significantly different from GA production using glucose (Tukey's test). Figure 4b shows the temporal profiles of substrate consumption (for both substrates evaluated) and GA production. The use of dextrin-10 resulted in a final GA concentration of 60.4 ± 0.8 g/L and a yield of 98.0 ± 0.4%, for 48 h of reaction. Therefore, the reaction time was set at 48 h for all the experimental design (CCRD) assays, corresponding to the reaction time at which the assay with the lowest concentration of dextrin-10 (50 g/L) ended, enabling comparison between runs.

4.3.3.2 Development of a model for simultaneous saccharification and oxidation of dextrin-10 to GA by experimental design

For optimization of the simultaneous saccharification and oxidation of dextrin-10 to GA using AMG and *A. niger* whole-cells, an experimental design (CCRD) was carried out to evaluate the effects of three independent variables on GA production (g/L) after 48 h of reaction: dextrin-10 concentration (g/L), pH, and AMG concentration (% v/v).

A dextrin-10 concentration range of 50-250 g/L (Table 4.1) was selected in the CCRD, based on previously reported evidence that glucose concentrations in the range of 110-250 g/L

are optimal for GA production by the fermentative process (Ramachandran *et al.*,2006). The AMG concentration range used in the CCRD (0.1-1.0% v/v, Table 4.1) was chosen based on the results for dextrin-10 shown in Figure 4a. The pH range 4.5-7.5 was selected to cover the pH values corresponding to the best activities of the enzymes: AMG (pH 4.5), GOD (pH 6.0), and CAT (pH 7.0) (Kumar; Satyanarayana, 2009; Slivinski *et al.*, 2011; Miłek, 2020).

The parameters temperature, wc concentration, and specific air flow rate were kept constant in all the assays, at 40 °C, 3 g/L, and 5 vvm (1 L/min), respectively. The temperature was fixed at 40 °C, because this is the optimal temperature for the glucose oxidase activity of *A. niger* (Bhatti *et al.* 2006). Furthermore, at higher temperatures, the catalase activity decreases significantly (Milek, 2020), while lower temperatures than 40 °C are too distant from the optimum temperature for the AMG enzyme (60 °C) (Slivinski *et al.*, 2011).

Hence, to achieve the effective synergistic action of AMG and *A. niger* whole-cells in the production of GA from dextrin-10, a statistical experimental design with 17 runs (2^3+2(3)+3CP) was used to evaluate the effects of dextrin-10 concentration (g/L), pH, and AMG concentration (% v/v) on GA production (g/L) after 48 h of reaction.

Table 4.2 shows the CCRD experimental results, and the predicted values calculated from the second-order polynomial obtained by multiple regression analysis of the experimental data (using Statistica 7.0 software), for GA production under each evaluated condition of initial dextrin-10 concentration, pH, and AMG concentration. Although the model had been developed for GA production (g/L), enabling predicted values to be obtained using Equation 4.7, other important parameters including GA yield (%) and biocatalyst yield (g_{GA}/g_{wc}) and GA volumetric productivity (g_{GA}/ (L.h)) are also shown in Table 4.2.

Table 4.2. Experimental design for simultaneous saccharification and oxidation of dextrin-10. Experimental and predicted values for GA production (g/L) for 48 h of reaction.

Run	**Independent variable (X)**						**Response variable (*y*)**			**GA yields and productivity at 48 h of reaction**		
	X_1 (dextrin-10 concentration) (g/L)		X_2 (pH)		X_3 (AMG concentration) (% v/v)		GA production (g/L) at 48 h			GA yield (%)	Biocatalyst yield ($g_{GA}/g_{whole\text{-}cell}$)	GA productivity (g_{GA}/L.h)
							Experimental	Predicted				
1	-1	(90.48)	-1	(5.11)	-1	(0.28)	78.95	69.35	26.32	72.06	26.32	1.64
2	1	(209.52)	-1	(5.11)	-1	(0.28)	44.09	48.43	14.70	17.38	14.70	0.92
3	-1	(90.48)	1	(6.89)	-1	(0.28)	41.57	43.13	13.86	37.94	13.86	0.87
4	1	(209.52)	1	(6.89)	-1	(0.28)	31.45	22.21	10.48	12.39	10.48	0.66
5	-1	(90.48)	-1	(5.11)	1	(0.82)	81.51	80.23	27.17	74.40	27.17	1.70
6	1	(209.52)	-1	(5.11)	1	(0.82)	55.16	59.31	18.39	21.74	18.39	1.15
7	-1	(90.48)	1	(6.89)	1	(0.82)	75.83	74.89	25.28	69.21	25.28	1.58
8	1	(209.52)	1	(6.89)	1	(0.82)	52.64	53.97	17.55	20.75	17.55	1.10
9	-1.68	(50.00)	0	(6.00)	0	(0.55)	60.43	65.36	20.14	99.78	20.14	1.26
10	1.68	(250.00)	0	(6.00)	0	(0.55)	31.68	30.21	10.56	10.47	10.56	0.66
11	0	(150.00)	-1.68	(5.00)	0	(0.55)	79.07	79.36	26.36	43.53	26.36	1.65
12	0	(150.00)	1.68	(7.50)	0	(0.55)	49.56	52.84	16.52	27.29	16.52	1.03
13	0	(150.00)	0	(6.00)	-1.68	(0.10)	35.21	41.39	11.74	19.38	11.74	0.73
14	0	(150.00)	0	(6.00)	1.68	(1.00)	80.50	77.21	26.83	44.32	26.83	1.68
15	0	(150.00)	0	(6.00)	0	(0.55)	80.16	78.18	26.72	44.13	26.72	1.67
16	0	(150.00)	0	(6.00)	0	(0.55)	75.36	78.18	25.12	41.49	25.12	1.57
17	0	(150.00)	0	(6.00)	0	(0.55)	79.68	78.18	26.56	43.87	26.56	1.66

The final second-order polynomial predictive model for GA production (Y*, g/L) in 48 h, after the elimination of non-significant statistical terms, is given by Equation 4.7.

$$Y^* = 78.18 - 10.46\,X_1 - 10.77\,X_1{}^2 - 7.89\,X_2 - 4.28\,X_2{}^2 + 10.66\,X_3 - 6.69\,X_3{}^2 + 5.22\,X_2X_3 (R^2 = 0.9448) \quad (4.7)$$

Equation 4.7 shows that the model was influenced by all the linear and quadratic terms, as well as by the interaction of pH with AMG concentration. The pH-AMG interaction was probably significant because for pH far from the optimal value, alterations of the three-dimensional enzyme structure occur, due to changes in the ionization of amino acid residues. The modification of the enzyme conformation affects its interaction with the substrate, reducing its activity, so a higher enzyme concentration is required to catalyze the same amount of substrate (Robinson, 2015). The goodness of fit of the model was measured by the coefficient of determination (R^2), with a value of 0.9448 indicating that 94.48% of the variability of the observed responses could be explained by this model. Table 4.3 presents the analysis of variance (ANOVA, 95% confidence level) for the quadratic model with interactions, applied to GA production (Equation 4.7).

Table 4.3. Analysis of variance for the data is presented in Table 4.2.

Source of variation	**GL**	**SQ**	**QM**	**Fcal**	**Ftab**	**p-valor**
Regression	7	553.3778	79.0539	21.8711	3.2927	<0.0001
Residual	9	32.5309	3.6145			
Lack of fit	7	30.9778	4.4254	5.6989	19.3532	0.1574
Pure error	2	1.5530	0.7765			
Total	16	585.9087				

SS: sum of squares; df: degrees of freedom; MS: mean square; Fcal: calculated F value; Ftab: tabulated F value.

The calculated F value (21.87) for the model was higher than the tabulated F value (3.29), at a 5% significance level. The test based on the Fisher distribution (F-test) indicated that the fitted equation 4.7 was statistically significant ($F=21.87>3.29$), with a very low probability value ($p_{model}<0.0001$). The F-test and the high R^2 value showed that the model was able to satisfactorily represent the experimental GA production data. In addition, a lack-of-fit sum of squares ($F=5.70<19.35$) corroborated the good agreement between the predicted and experimental values, in the range evaluated for each variable.

Additionally, accuracy metrics were used to evaluate the goodness of model predictions. The mean absolute percentage error (MAPE) is one of the most widely used metrics to measure the accuracy of a forecasting model for continuous variables due to its advantages of scale-independency and interpretability (Kim, S. Kim, H., 2016). It is recommended in most text-books (Koehler; O'Connell; Bowerman, 2004, Hanke; Reitsch, 1995) and was used as the primary measure in the M-competition (Makridakis *et al.* 1982). MAPE is the percentage equivalent of mean absolute error (MAE) (Myttenaere, A. *et al.* 2016). Here, errors are the differences between the predicted values (values predicted by our regression model) and the actual values of a variable. Then, MAE and MAPE are metrics to evaluate the performance of a predictive model, defined by Eqs. 8 and 9, respectively.

$$\text{MAE} = \frac{1}{n} \times \sum_{i=0}^{n} |y_i - x_i| \tag{8}$$

$$\text{MAPE} = \frac{1}{n} \times \sum_{i=0}^{n} \left| \frac{(y_i - x_i)}{y_i} \right| \times 100 \tag{9}$$

where:

y_i: the observed value for the ith observation.

x_i: the predicted value for the ith observation.

n: the total number of observations.

MAE and MAPE were performed by using the scikit-learn package available in Anaconda (Python 3.7 version, 2019.03). The values calculated by Anaconda software were approximately 3.42 g/L and 6.91%, corresponding to MAE and MAPE values, respectively.

The MAE is expressed in the same units as the target variable. In this case, MAE is expressed in g/L. A lower MAE indicates that the model is making more accurate predictions. Although this makes it easy to understand the magnitude of the error in the original data's scale, it cannot be compared across different models and datasets. However, by converting MAE to MAPE, it becomes possible to compare model performance as this error is returned as a percentage. By taking into consideration the MAPE-value, Lewis (1982) proposed an interpretation of the MAPE results to judge the accuracy of the forecasting model, as given in Table 4.4.

Table 4.4. Interpretation of MAPE results for forecasting accuracy (Lewis, 1982).

MAPE-value	Accuracy of forecast
Less than 10%	Highly accurate forecast
11% to 20%	Good forecast
21% to 50%	Reasonable forecast
More than 51%	Inaccurate forecast

According to Lewis (1982), the MAPE-value result of 6.91% indicates a highly accurate forecast, confirming that the data are reliable enough to be used in forecasting model development.

4.3.3.3 Effect of the process variables on GA production

Equation 4.7 was used to plot 3D response surfaces and the corresponding 2D contours, performed with Statistica 7.0 software. The response surfaces showed how two independent variables interacted with each other when the other variable was fixed at its central level (Figure 4.5).

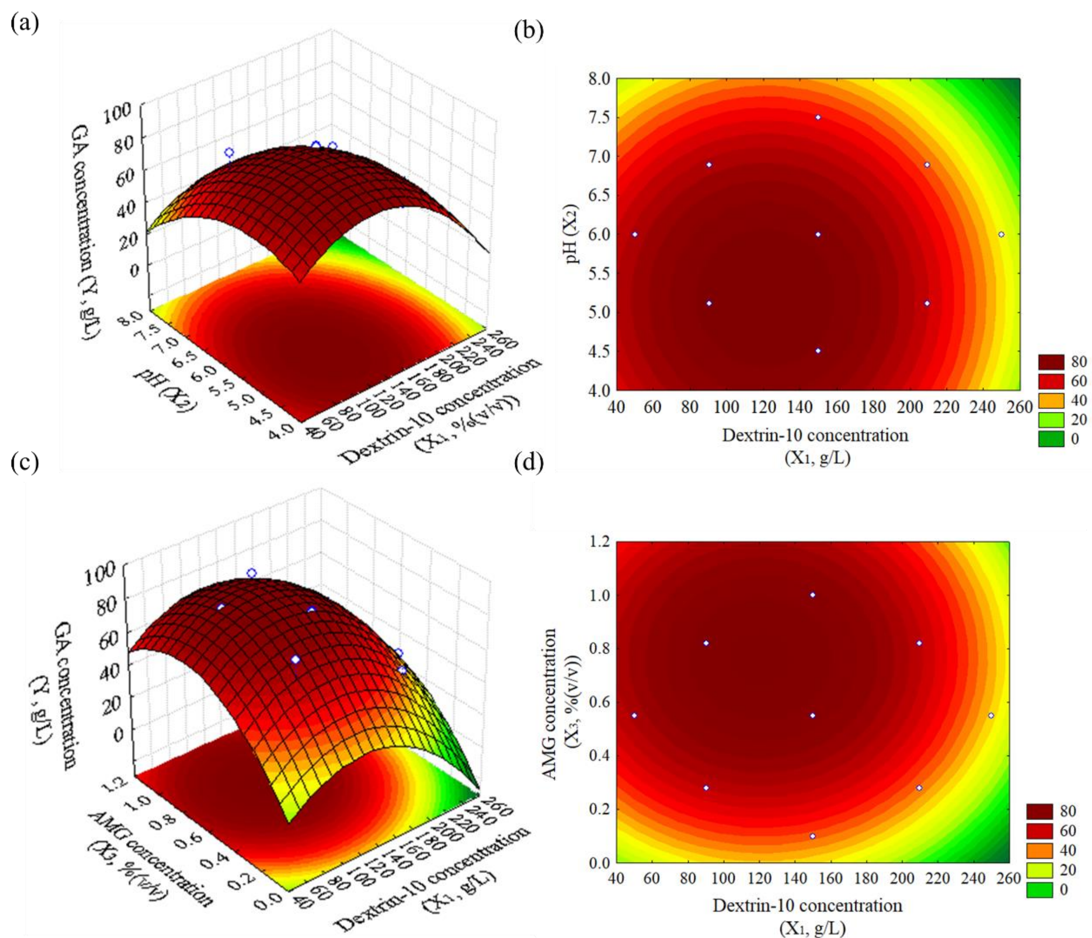


Figure 4.5. a) 3D response surface and b) the corresponding contour plot, showing the effects of dextrin-10 and pH on the GA yield for 48 h of reaction, with the AMG concentration fixed at 0.55% (v/v). c) 3D response surface and d) the corresponding contour plot, showing the effects of the dextrin-10 and AMG concentrations on the GA yield for 48 h of reaction, with fixed pH 6.

The response surface (Figure 4.5a) and the contour curve (Figure 4.5b) obtained from the model described by Equation 4.7, with the AMG concentration fixed at 0.55% (v/v), indicated that there was an optimal region of dextrin-10 concentration, between 80 and 160 g/L, where GA production (g/L) reached the highest values for 48 h of reaction. Above 160 g/L of dextrin-10, GA production started to decrease, probably due to inhibition by the substrate. This result was consistent with the current industrial fermentation process, where glucose is supplied in a fed-batch mode to overcome substrate inhibition (Kornecki *et al*., 2020).

It should be highlighted that although Figures 4.5a and 4.5b show that the optimum pH for simultaneous saccharification and oxidation of dextrin-10 was pH 5.50, an operational range between 4.50 to 6.50 only slightly affected the response variable, showing the robustness of the simultaneous saccharification and oxidation, considering pH variations. It should also be noted that Bhatti *et al.* (2006) reported pH 5.50 as being the optimal pH for GOD from *Aspergillus*, in full agreement with the optimum pH obtained here from the CCRD.

Figures 4.5c and 4.5d show that in order to obtain higher response variable values, the AMG concentration should be set between 0.55 and 0.95% (v/v), for a dextrin-10 concentration from 80.0 to 160.0 (g/L), when the pH was fixed at 6.0. This corresponds to the area confined in the smallest ellipse indicated by the dark red color in the contour diagram, with GA production up to the predicted maximum of 86.0 g/L for 48 h of reaction. Equation 4.7 also shows that the interaction between pH and AMG concentration was significant, as expected, due to the known pH dependence of enzyme-catalyzed reactions (Marangoni, 2003).

In addition to the important information highlighted previously, such as substrate concentrations that cause enzyme inhibition and the optimal pH range for operation, the use of experimental design tools can enable further optimization of the operating conditions. The second-order polynomial regression equation (Equation 4.7) obtained from the experimental data could be used to predict the GA production at any AMG concentration (% v/v), dextrin-10 concentration (g/L), and pH within the range of the experimental design. The maximum GA production predicted by the model was 86.0 g/L under the optimum conditions of pH 5.5, 0.70% (v/v) AMG concentration, and 121.10 g/L dextrin-10 concentration. In addition, it is important to highlight that this innovative process could potentially be even more efficient, if variables kept constant in this study, such as the concentration and size of the wc, were also optimized.

4.3.4 Validation of the mathematical model for GA production from dextrin-10 and starch

For validation of the GA predictive model (Equation 4.7), two experiments were carried

out (in duplicate) under the optimum conditions predicted by the model, using dextrin-10 or soluble starch as substrate, at pH 5.5, 0.70% (v/v) AMG concentration, and 121.10 g/L substrate (dextrin-10 or soluble starch) concentration.

The use of dextrin-10 as substrate resulted in GA production of 89.3 ± 5.9 g/L for 48 h of reaction. This was in good agreement with the value predicted by the model (86.0 g/L) for the same reaction time. A final GA concentration of 138.0±0.5 g/L, GA yield of 98.3 ± 2.2%, and biocatalyst yield of 46.0 ± 0.2 g_{GA}/g_{wc} were achieved for 96 h of reaction.

Similar results were obtained using soluble starch as substrate, under the predicted optimum conditions and with the addition of α-amylase (0.10% v/v). GA production of 85.6.±.1.4 g/L for 48 h of reaction was in good agreement with the value predicted by the model (86.0 g/L) for the same reaction time. A final GA concentration of 134.5±4.3 g/L, GA yield of 98.2.±.1.3%, and biocatalyst yield of 48.8.±.1.4 g_{GA}/g_{wc} were achieved for 96 h of reaction. Furthermore, a comparison between the mass spectra of the standard and a sample of GA produced from starch showed great similarity, confirming the GA identity (Figure S4.1, Supplementary Material).

These results demonstrated the robustness of the developed model, allowing the identification of optimal operating conditions for producing GA from both dextrin-10 and soluble starch as substrate. Figure 4.6 shows very similar behaviors of the profiles of GA production and glucose consumption, according to reaction time, for the two substrates, under the optimum conditions predicted by the model.

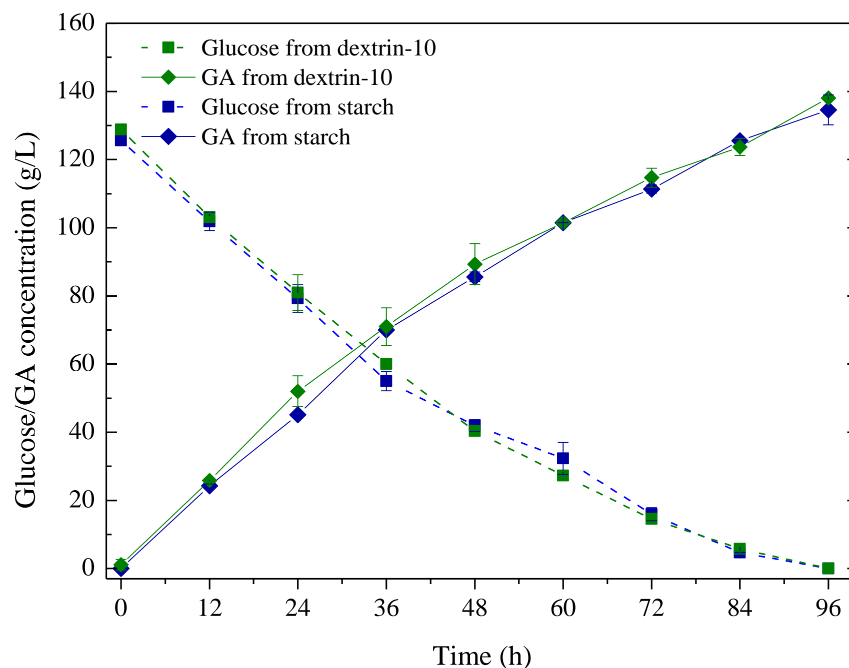


Figure 4.6. Temporal profiles of GA production and glucose consumption at 40 °C, using dextrin-10 and soluble starch as substrates, under the optimum conditions predicted by the model: 121.10 g/L of substrate, pH 5.50 and 0.70% (v/v) of AMG.

According to Woodley (2006), product concentration (g/L), biocatalyst yield ($g_{product}/g_{bio-catalyst}$), and product yield (%) are the main metrics that need to be considered in achieving an economically viable process. Accordingly, the proposed process proved to be efficient, when compared to other processes for GA production reported in the literature (Table 4.5

Table 4.5. Comparison of processes for gluconic acid production using different substrates as glucose sources.

Substrate	**Microorganism/ enzyme and support for immobilization**	**GA concentration (g/L)**	**Biocatalyst yield ($g_{GA}/g_{whole-cell}$)**	**$[Yield]_{GA}$ (%)**	**GA productivity (g_{GA} / (L.h))**	**Process time**	**Process**	**Reference**
Hydrolyzed starch	*A. niger* ORS-4.410	30.12	3.77	35.92	0.10	12 days	Batch culture/surface fermentation	(Singh; Kapur; Singh, 2005)
Hydrol	*A. niger* cells immobilized in polyurethane foam	143.00	34.56	95.00	2.04	70 h	Repeated batch	(Vassilev; Vassileva; Spassova, 1993)
Corn stover	*Aspergillus niger*	76.67	11.88	94.83	0.87	88 h	SHF	(Zhang; Zhang; Bao, 2016)
Liquefied starch	*A. niger* cells immobilized on nonwoven fabric	272.00	-	90.00	6.04	45 h	Repeated batch culture/SSF	(Matsui *et. al*, 2013)
Cassava starch	*Zymomonas mobilis* immobilized whole cells	193.00	19.30	97.30	4.02	48 h	Batch/SSO	(An; Hu; Bao, 2013)
Starch	GOD/CAT	0.92	2.30	92.12	0.69	80 min	Enzymatic process (nanoflowers)	(Han *et al.*, 2020)
Soluble starch	*A. niger* whole cells	134.56	48.88	98.25	1.40	96 h	Batch/SLSO	Present work

SSF: Simultaneous saccharification and fermentation. SLSO: Simultaneous liquefaction, saccharification, and oxidation. SSO: Simultaneous saccharification and oxidation. SHF: Sequential hydrolysis and fermentation.

In comparison to the enzymatic process proposed by Han *et al.* (2020), the multi-enzymatic process combining soluble amylolytic enzymes and *A. niger* whole-cells could be considered an attractive approach, since it showed a higher biocatalyst yield and the potential to reduce costs, relative to the use of isolated enzymes to catalyze cascade reactions. When compared to fermentation processes, such as those developed by Singh *et al.* (2005) and Matsui *et al.* (2013), the GA production yield was higher and there were no expenses associated with sources of supplementation. Additionally, there were no unwanted reactions during the innovative multi-enzymatic process proposed in this study.

Another significant process metric is GA volumetric productivity (g_{GA}/ (L.h)). The higher results for this parameter, as reported by studies cited in Table 4.5 (Vassilev *et al.*, 1993; Matsui *et al.* 2013; An; Hu; Bao, 2013), were likely attributed to distinct processes and unoptimized variables not considered in the current study. Vassilev *et al.* (1993) reported superior performance of immobilized cells over free cells and even optimized the mass of immobilized cell biocatalysts (Vassilev *et al.*, 1993). In contrast to their approach, the present study did not employ immobilization techniques and maintained a lower concentration of free cells. Matsui *et al.* employed immobilized whole cells in a pressurized reactor with pure oxygen Matsui *et al.* (2013). On the other hand, An *et al.* in addition to applying the form of immobilized biocatalyst, used a distinct microorganism (*Zymomonas mobilis*) (An; Hu; Bao, 2013). While the current study did optimize several crucial parameters, its primary accomplishment lies in successfully demonstrating the proof-of-concept for the proposed innovative multi-enzymatic system. Numerous aspects, including pellet size, biocatalyst concentration, bioreactor configurations, and operation modes, are still open for refinement.

Additionally, it is relevant to point out that the multi-enzymatic process to produce gluconic acid proposed in this study is analogous to the high-fructose corn syrup (HFCS) production industrial process as described in Figure 4.7, in which applied the most well-succeed immobilized biocatalyst whole-cell based (Parker; Salas; Nwosu; 2011).

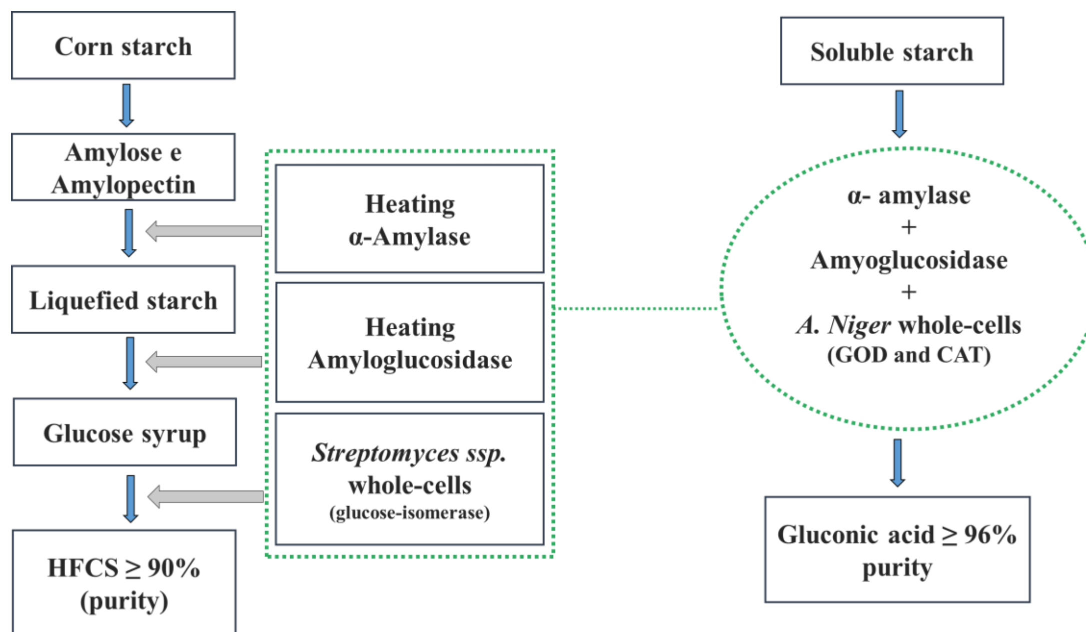


Figure 4.7. Flow chart showing the similarity between the GA production process proposed in this study and the industrial process for HFCS production from corn starch. The HFCS production schematic was adapted from (Parker; Salas; Nwosu; 2011).

The immobilized biocatalyst based on whole-cells of *Streptomyces* containing glucose isomerase (IGI) applied in the HFCS production industrial process is widely used in commercial applications and is considered the paradigm for an immobilized biocatalyst (Jensen; Rugh, 1987; Poulsen, 1984; Bhosale; Rao; Deshpande, 1996; DiCosimo *et al.*, 2013). Given this success, the similarity between both processes and the relevant process advantages that an immobilized biocatalyst offers, for example, enables continuous production, provides mechanical stability, and the absence of the biocatalyst in the product stream (DiCosimo *et al.*, 2013), the idea of the future works is the development of a similar immobilized biocatalyst whole-cell based, enabling continuous production in substitution to the current traditional fed-batch fermentation process (Kornecki *et al*., 2020).

4.4 Conclusions

This study presents an innovative and efficient single-step multi-enzymatic process for GA production from starch, using *A. niger* whole-cells together with amylolytic enzymes to efficiently convert starch to gluconic acid (98% yield). A final GA concentration of 134.5.±.4.3 g/L and a biocatalyst yield of 48.8 ± 1.4 g_{GA}/g_{wc} were achieved for 96 h of reaction under statistically predicted conditions (T = 40 °C, pH = 5.5, 0.70% (v/v) of AMG, 3 g/L (0.3% w/v)

of *A. niger* whole-cells, and specific air flow rate of 5 vvm (1 L/min)). Although the process was developed for starch, the approach has the potential to be applied to other materials that can be hydrolyzed to glucose. Therefore, the innovative proposed multi-enzymatic system opens new perspectives on GA production by whole-cell biocatalysis. Future research can explore a diverse combination of *A. niger* whole cells with other enzymatic cocktails. For example, by combining cellulolytic enzymes with *A. niger* whole-cells, there is potential to generate GA from lignocellulosic residues. This approach aligns the utilization of agri-food waste as cost-effective and renewable feedstock, tapping into abundant glucose sources for the proposed multi-enzymatic system.

Supplementary material

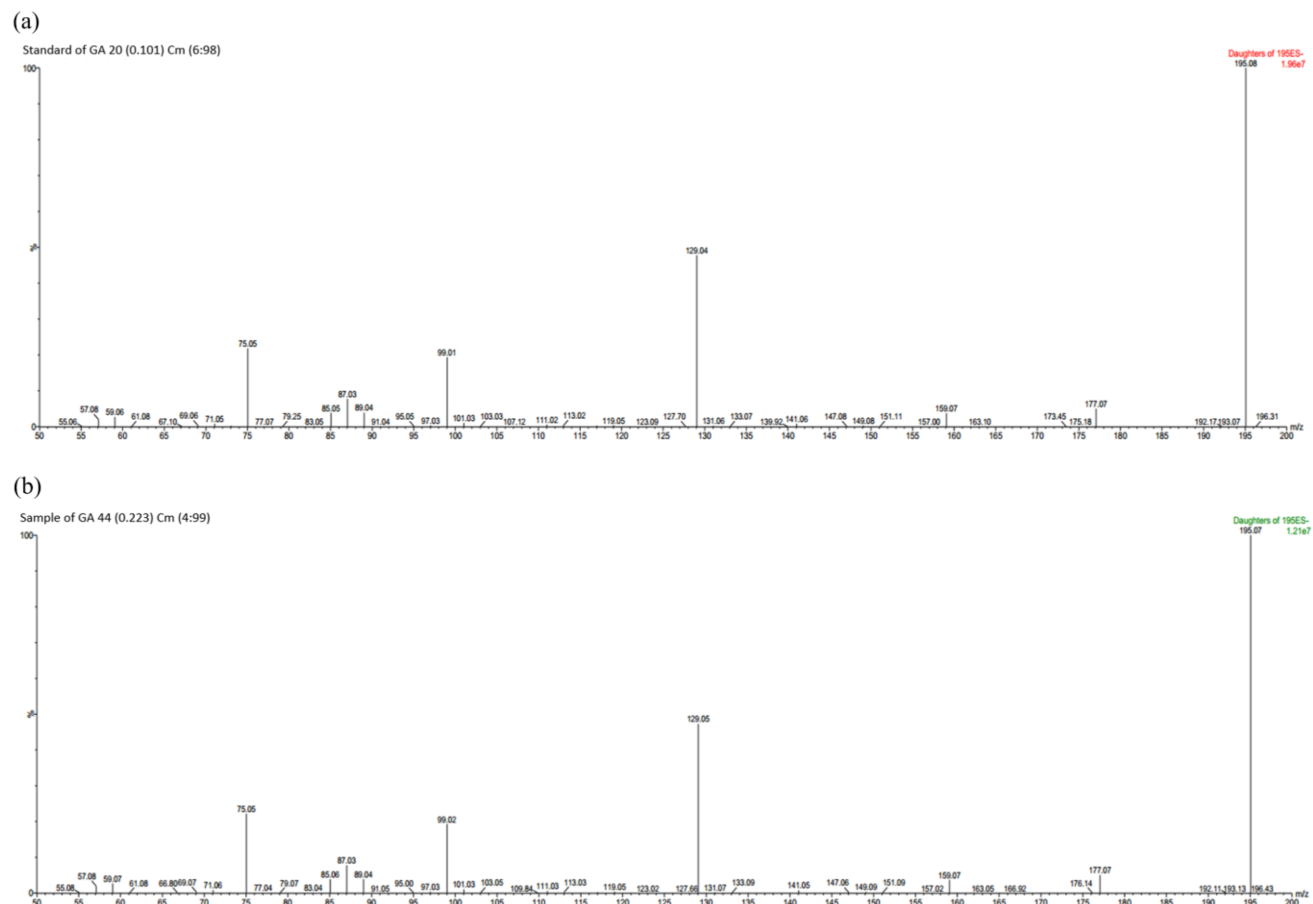


Figure S4.6. A comparison between the mass spectra of the standard (a) and a sample of GA produced from starch (b).

REFERENCES

AHUJA, K., SINGH, S. Gluconic Acid Market by Application, by Downstream Potential, Regional Outlook, Application Potential, Price Trend, Competitive Market Share & Forecast, 2018-2024. **Global Market Insights Inc.**, United States, p. 240, 2018.

AN, K.; HU, F.; BAO, J. Simultaneous Saccharification of Inulin and Starch Using Commercial Glucoamylase and the Subsequent Bioconversion to High Titer Sorbitol and Gluconic Acid. **Applied Biochemistry and Biotechnology**, vol. 171, no. 8, p. 2093-2104, 12 Sept. 2013. Available from: https://doi.org/10.1007/s12010-013-0278-z.

ANASTASSIADIS, S.; MORGUNOV, I. Gluconic Acid Production. **Recent Patents on Biotechnology**, vol. 1, n. 2, p. 167-180, 1 June 2007. Available from: https://doi.org/10.2174/187220807780809472.

BHATTI, H. N. *et al*. Purification and thermodynamic characterization of glucose oxidase from a newly isolated strain of *Aspergillus niger*. **Canadian Journal of Microbiology**, vol. 52, n. 6, p. 519-524, 1 June 2006. Available from: https://doi.org/10.1139/w05-158.

BHOSALE, S. H.; RAO, M. B.; DESHPANDE, V. V. Molecular and industrial aspects of glucose isomerase. **Microbiological reviews**, vol. 60, n. 2, p. 280-300, 1996. Available from: https://doi.org/10.1128/mmbr.60.2.280-300.1996.

BioAcids Tech Brazil. Technology and Production of Organic Acids – OUR PRODUCTS. Available from: <https://bioacidstech.com/en/products>. Accessed: 16 Apr. 2023.

BUFFO, M. M. *et al*. Cellulolytic enzymes production guided by morphology engineering. **Enzyme and Microbial Technology**, vol. 149, p. 109833, Sept. 2021. Available from: https://doi.org/10.1016/j.enzmictec.2021.109833.

C.D. Lewis. Industrial and Business Forecasting Methods, Butterworth-Heinemann, London, 1982.

CAMESELLE, C. *et al*. Oxalic acid production by *Aspergillus niger*. **Bioprocess Engineering**, vol. 19, n. 4, p. 247-252, 20 Oct. 1998. Available from: https://doi.org/10.1007/pl00009017.

CHENG, Jie, *et al*. Efficient whole-cell catalysis for 5-aminovalerate production from L-lysine by using engineered *Escherichia coli* with ethanol pretreatment. Scientific Reports, vol. 10, n. 1, 22 Jan. 2020. Available from: https://doi.org/10.1038/s41598-020-57752-x.

CRIPWELL, R. A. *et al*. Consolidated bioprocessing of raw starch to ethanol by *Saccharomyces cerevisiae*: Achievements and challenges. **Biotechnology Advances**, vol. 42, p. 107579, Sept. 2020. Available from: https://doi.org/10.1016/j.biotechadv.2020.107579.

DE CARVALHO, C. C. C. R. Whole cell biocatalysts: essential workers from Nature to the industry. **Microbial Biotechnology**, vol. 10, n. 2, p. 250-263, 3 May 2016. Available from: https://doi.org/10.1111/1751-7915.12363.

DE MYTTENAERE, A. *et al*. Mean Absolute Percentage Error for regression models. Neurocomputing, vol. 192, p. 38-48, June 2016. Available from: https://doi.org/10.1016/j.neucom.2015.12.114.

DICOSIMO, R. *et al*. Industrial use of immobilized enzymes. **Chemical Society Reviews**, vol. 42, n. 15, p. 6437, 2013. Available from: https://doi.org/10.1039/c3cs35506c.

DOS SANTOS, J. B. C.; DA SILVA CRUZ, R. G.; TARDIOLI, P. W. Production of Whole-Cell Lipase from *Streptomyces clavuligerus* in a Bench-Scale Bioreactor and Its First Evaluation as Biocatalyst for Synthesis in Organic Medium. **Applied Biochemistry and Biotechnology**, vol. 183, n. 1, p. 218-240, 24 Feb. 2017. Available from: https://doi.org/10.1007/s12010-017-2440-5.

FONSECA, M. A. *et al*. Optimization of simultaneous saccharification and isomerization of dextrin to high fructose syrup using a mixture of immobilized amyloglucosidase and glucose isomerase. **Catalysis Today**, vol. 362, p. 175-183, Feb. 2021. Available from: https://doi.org/10.1016/j.cattod.2020.03.021.

FONSECA, M. A. Simultaneous saccharification and isomerization of dextrin in the production of fructose syrup by the synergic action of magnetic CLEAs of amyloglucosidase and Sweetzyme®. **Ph.D. Thesis** - Federal University of São Carlos. Chemical Engineering Department. São Carlos. (2019), pp. 61.

GARZÓN-POSSE, Fabián *et al*. Whole Cells as Biocatalysts in Organic Transformations. **Molecules**, vol. 23, n. 6, p. 1265, 25 May 2018. Available from: https://doi.org/10.3390/molecules23061265.

HAN, J. *et al*. Construction of a Multienzymatic Cascade Reaction System of Coimmobilized Hybrid Nanoflowers for Efficient Conversion of Starch into Gluconic Acid. **ACS Applied Materials & Interfaces**, vol. 12, n. 13, p. 15023-15033, 11 Mar. 2020. Available from: https://doi.org/10.1021/acsami.9b21511.

HANKE J. E., REITSCH. A. G. Business forecasting (5th ed.). Englewood Cliffs, NJ: Prentice-Hall, California, 1995.

INTASIAN, Pattarawan *et al*. Enzymes, In Vivo Biocatalysis, and Metabolic Engineering for Enabling a Circular Economy and Sustainability. **Chemical Reviews**, vol. 121, n. 17, p. 10367-10451, 6 July 2021. Available from: https://doi.org/10.1021/acs.chemrev.1c00121.

JENSEN, V. J.; RUGH, S. Industrial-scale production and application of immobilized glucose isomerase. **Immobilized Enzymes and Cells**, Part C. [S. l.]: Elsevier, 1987. p. 356-370. ISBN 9780121820367. Available from: https://doi.org/10.1016/s0076-6879(87)36035-5.

JIA, Dong-Xu, *et al*. Whole cell immobilization of refractory glucose isomerase using tris(hydroxymethyl)phosphine as crosslinker for preparation of high fructose corn syrup at elevated temperature. **Journal of Bioscience and Bioengineering**, vol. 126, n. 2, p. 176-182, Aug. 2018. Available from: https://doi.org/10.1016/j.jbiosc.2018.03.001.

KIM, S.; KIM, H. A new metric of absolute percentage error for intermittent demand forecasts. **International Journal of Forecasting**, vol. 32, n. 3, p. 669-679, July 2016. Available from: https://doi.org/10.1016/j.ijforecast.2015.12.003.

KLATTE, S.; LORENZ, E.; WENDISCH, V. F. Whole cell biotransformation for reductive amination reactions. **Bioengineered**, vol. 5, n. 1, p. 56-62, 5 Dec. 2013. Available from: https://doi.org/10.4161/bioe.27151.

KOEHLER, A.; O'CONNELL, R.; BOWERMAN, B. L. Forecasting, Time Series, and Regression (Forecasting, Time Series, & Regression). 4th ed. [S. l.]: **Duxbury Press**, 2004. 696 p. ISBN 9780534409777.

KORNECKI, J. F. *et al*. Enzyme production of d-gluconic acid and glucose oxidase: successful tales of cascade reactions. **Catalysis Science & Technology**, vol. 10, n. 17, p. 5740-5771, 2020. Available from: https://doi.org/10.1039/d0cy00819b.

KRULL, R. *et al*. Characterization and control of fungal morphology for improved production performance in biotechnology. **Journal of Biotechnology**, vol. 163, n. 2, p. 112-123, Jan. 2013. Available from: https://doi.org/10.1016/j.jbiotec.2012.06.024.

KUMAR, P.; SATYANARAYANA, T. Microbial glucoamylases: characteristics and applications. **Critical Reviews in Biotechnology**, vol. 29, n. 3, p. 225-255, 27 Aug. 2009. Available from: https://doi.org/10.1080/07388550903136076.

LIN, B.; TAO, Y. Whole-cell biocatalysts by design. **Microbial Cell Factories**, vol. 16, n. 1, 13 June 2017. Available from: https://doi.org/10.1186/s12934-017-0724-7.

LOTFY, S. Controlling degradation of low-molecular-weight natural polymer "dextrin" using gamma irradiation. **International Journal of Biological Macromolecules**, vol. 44, n. 1, p. 57-63, Jan. 2009. Available from: https://doi.org/10.1016/j.ijbiomac.2008.10.002.

MA, Y. *et al.* Production of Gluconic Acid and Its Derivatives by Microbial Fermentation: Process Improvement Based on Integrated Routes. **Frontiers in Bioengineering and Biotechnology**, vol. 10, 16 May 2022. Available from: https://doi.org/10.3389/fbioe.2022.864787.

MADAVI, T. B. *et al*. Whole-cell biocatalysis: Advancements toward the biosynthesis of fuels. Biofuels, **Bioproducts and Biorefining**, 11 Dec. 2021. Available from: https://doi.org/10.1002/bbb.2331.

MAFRA, A. C. O. *et al*. Gluconic acid production from sucrose in an airlift reactor using a multi-enzyme system. **Bioprocess and Biosystems Engineering**, vol. 38, n. 4, p. 671-680, 19 Oct. 2014. Available from: https://doi.org/10.1007/s00449-014-1306-2.

MAKRIDAKIS, S. *et al*. The accuracy of extrapolation (time series) methods: Results of a forecasting competition. **Journal of Forecasting**, vol. 1, n. 2, p. 111-153, Apr. 1982. Available from: https://doi.org/10.1002/for.3980010202.

MARANGONI, A. G. Enzyme Kinetics: A Modern Approach. [S. l.]: Wiley & Sons, Incorporated, John, 2003. 248 p. ISBN 9780471461418.

MATSUI *et al*. Simultaneous Saccharification of Corn Starch in Gluconic Acid Production by *Aspergillus niger* Immobilized on Nonwoven Fabric in a Pressurized Reactor. **Journal of Microbial & Biochemical Technology**, vol. 05, n. 04, 2013. Available from: https://doi.org/10.4172/1948-5948.1000106.

MIŁEK, J. Thermodynamics and kinetics of thermal deactivation of catalase *Aspergillus niger*. **Polish Journal of Chemical Technology**, vol. 22, no. 2, p. 67-72, 1 June 2020. Available from: https://doi.org/10.2478/pjct-2020-0018.

PARKER, K.; SALAS, M.; NWOSU, V. C. High fructose corn syrup: Production, uses and public health concerns. **Biotechnol**. Mol. Biol. Rev. 5(5) (2011) 71-78. https://doi.org/10.5897/BMBR2010.0009.

POULSEN, P. B. Current Applications of Immobilized Enzymes for Manufacturing Purposes. **Biotechnology and Genetic Engineering Reviews**, vol. 1, n. 1, p. 121-140, Feb. 1984. Available from: https://doi.org/10.1080/02648725.1984.10647783.

RAMACHANDRAN, S. *et al*. Gluconic Acid: Properties, Applications and Microbial Production. **Food Technology and Biotechnology**, v. 44, n. 2, p. 185-95, Apr. 2006.

RAMACHANDRAN, S. *et al.* Permeabilization and inhibition of the germination of spores of *Aspergillus niger* for gluconic acid production from glucose. **Bioresource Technology**, vol. 99, n. 11, p. 4559-4565, July 2008. Available from: https://doi.org/10.1016/j.biortech.2007.06.055.

RAMACHANDRAN, S. *et al.* Spores of *Aspergillus niger* as reservoir of glucose oxidase synthesized during solid-state fermentation and their use as catalyst in gluconic acid production. **Letters in Applied Microbiology**, vol. 44, n. 2, p. 155-160, Feb. 2007. Available from: https://doi.org/10.1111/j.1472-765x.2006.02051.x.

RAO, D. S.; PANDA, T. Comparative analysis of different whole cell immobilized aspergillus niger catalysts for gluconic acid fermentation using pretreated cane molasses. **Bioprocess Engineering**, vol. 11, n. 5, p. 209-212, Oct. 1994. Available from: https://doi.org/10.1007/bf00369632.

ROBINSON, P. K. Enzymes: principles and biotechnological applications. **Essays in Biochemistry**, vol. 59, p. 1-41, 26 Oct. 2015. Available from: https://doi.org/10.1042/bse0590001.

RODRIGUES, M. I.; IEMMA, A. F. Experimental Design and Process Optimization. [S. l.]: CRC Press, 2014. E-book. ISBN 9780429161865. Available from: https://doi.org/10.1201/b17848

RODRÍGUEZ PORCEL, E. M. *et al*. Effects of pellet morphology on broth rheology in fermentations of *Aspergillus terreus*. **Biochemical Engineering Journal**, vol. 26, no. 2-3, p. 139-144, Nov. 2005. Available from: https://doi.org/10.1016/j.bej.2005.04.011.

SAKHUJA, D. *et al*. Cost-effective production of biocatalysts using inexpensive plant biomass: a review. 3 **Biotech**, vol. 11, n. 6, 20 May 2021. Available from: https://doi.org/10.1007/s13205-021-02847-z.

SINGH, O. V.; KAPUR, N.; SINGH, R. P. Evaluation of agro-food byproducts for gluconic acid production by *Aspergillus niger* ORS-4.410. **World Journal of Microbiology and Biotechnology**, vol. 21, n. 4, p. 519-524, June 2005. Available from: https://doi.org/10.1007/s11274-004-2395-x.

SLIVINSKI, C. T. *et al.* Biochemical characterization of a glucoamylase from *Aspergillus niger* produced by solid-state fermentation. Brazilian Archives of Biology and Technology, vol. 54, no. 3, p. 559-568, June 2011. Available from: https://doi.org/10.1590/s1516-89132011000300018.

SONG, J. W. *et al*. Design and engineering of whole-cell biocatalytic cascades for the valorization of fatty acids. **Catalysis Science & Technology**, vol. 10, n. 1, p. 46-64, 2020. Available from: https://doi.org/10.1039/c9cy01802f.

VAN DER MAAREL, M. J. E. C. *et al.* Properties and applications of starch-converting enzymes of the α-amylase family. **Journal of Biotechnology**, vol. 94, no. 2, p. 137-155, Mar. 2002. Available from: https://doi.org/10.1016/s0168-1656(01)00407-2.

VASSILEV, N. B; VASSILEVA, M. C.; SPASSOVA, D. Production of gluconic acid by *Aspergillus niger* immobilized on polyurethane foam. **Applied Microbiology and Biotechnology**, vol. 39, n. 3, June 1993. Available from: https://doi.org/10.1007/bf00192079.

WACHTMEISTER, J.; ROTHER, D. Recent advances in whole cell biocatalysis techniques bridging from investigative to industrial scale. **Current Opinion in Biotechnology**, vol. 42, p. 169-177, Dec. 2016. Available from: https://doi.org/10.1016/j.copbio.2016.05.005.

WITTEVEEN, Cor F. B.; VEENHUIS, M.; VISSER, J. Localization of Glucose Oxidase and Catalase Activities in *Aspergillus niger*. **Applied and Environmental Microbiology**, vol. 58, no. 4, p. 1190-1194, 1992. Available from: https://doi.org/10.1128/aem.58.4.1190-1194.1992.

WOODLEY, J. M. Microbial Biocatalytic Processes and Their Development. **Advances in Applied Microbiology**. Elsevier, 2006. p. 1-15. ISBN 9780120026623. Available from: https://doi.org/10.1016/s0065-2164(06)60001-4.

ZENG, L. *et al.* Structure and functionality of cassava starch in different deep eutectic solvents/water mixtures: A comparative study. **Industrial Crops and Products**, vol. 177, p. 114453, Mar. 2022. Available from: https://doi.org/10.1016/j.indcrop.2021.114453.

ZHANG, H.; ZHANG, J.; BAO, Jie. High titer gluconic acid fermentation by *Aspergillus niger* from dry dilute acid pretreated corn stover without detoxification. **Bioresource Technology**, vol. 203, p. 211-219, Mar. 2016. Available from: https://doi.org/10.1016/j.biortech.2015.12.042.

ZHONG, P. *et al*. Piezoelectric sensing of glucose oxidase activity of *Aspergillus niger* spores pretreated by different methods. **Food Chemistry**, vol. 370, p. 130901, Feb. 2022. Available from: https://doi.org/10.1016/j.foodchem.2021.130901.

ZNAD, H.; MARKOŠ, J.; BALEŠ, V. Production of gluconic acid from glucose by *Aspergillus niger*: growth and non-growth conditions. **Process Biochemistry**, vol. 39, n. 11, p. 1341-1345, July 2004. Available from: https://doi.org/10.1016/s0032-9592(03)00270-x.

5 PRELIMINARY ASSAYS OF THE MULTI-ENZYMATIC SYSTEM PROPOSED TO PRODUCE GLUCONIC ACID FROM STARCH ON A HIGHER SCALE

RESUMO

Embora o oxigênio seja um substrato na reação de oxidação da glicose, a avaliação do oxigênio dissolvido não foi considerada anteriormente no presente estudo devido ao mini biorreator, com volume de trabalho de 200 mL, não ser adequado para utilização de sonda de monitoramento de oxigênio dissolvido. No entanto, como o sistema proposto teve sucesso na produção de ácido glicônico, neste capítulo os ensaios foram conduzidos em um biorreator de coluna de bolhas em escala de bancada com volume de trabalho de 2 L para monitorar a disponibilidade de oxigênio no meio. Os ensaios mostraram que a transferência de oxigênio para o meio líquido provavelmente é a etapa limitante do processo. Muito provavelmente, o processo é limitado por reações enzimáticas ou limitações difusionais dentro das células inteiras. Isto pode ser resolvido aumentando a concentração de células inteiras e/ou reduzindo o tamanho dos pellets de células inteiras. Embora os resultados sejam preliminares, este capítulo visa destacar potenciais desafios que podem ser encontrados em estudos futuros focados na ampliação deste processo e oferecer insights sobre parâmetros pertinentes que podem aumentar a eficácia do sistema multienzimático proposto.

Palavras-chave: oxigênio dissolvido, etapa limitante reação, biorreator, k_La.

ABSTRACT

Although oxygen is a substrate in the glucose oxidation reaction, the evaluation of this dissolved oxygen was not previously considered in the present study due to the mini bioreactor, with a working volume of 200 mL, not being suitable for a dissolved oxygen monitoring probe. Nevertheless, as the proposed system was successful in producing gluconic acid, in this chapter assays were conducted in a bench-scale bubble column bioreactor with a working volume of 2 L to monitor oxygen availability in the medium. The assays showed that the oxygen transfer was not the limiting step in the process. Most likely, the process is limited by enzymatic reactions or diffusional limitations within the whole-cells. This can be resolved by increasing the concentration of whole cells and/or reducing the size of the whole-cell pellet. While the results are preliminaries, this chapter aims to highlight potential challenges that could be encountered by future studies focused on scaling up this process and offer insights into pertinent parameters that may enhance the efficacy of the proposed multi-enzymatic system.

Keywords: Dissolved oxygen, reaction limiting step, bioreactor, kLa.

5.1 Introduction

The biotransformation of glucose to gluconic acid by whole-cell biocatalysis represents a simple dehydrogenation reaction without the involvement of complex metabolic cell pathways. In this reaction, oxygen and glucose are consumed only to produce gluconic acid, unlike the fermentation process in which oxygen and glucose are also consumed for biomass growth and byproducts such as other organic acids (Znad; Markoš; Baleš, 2004).

The formation of gluconic acid differs from most other organic acids since it is formed outside the cytoplasmic membrane by the enzyme glucose oxidase (Goldberg; Rokem, 2009). At least for fungi known to accumulate gluconic acid as *Aspergillus niger* (*A. niger*), this enzyme is localized in the cell wall (Witteveen; Veenhuis; Visser, 1992). The oxidation of glucose in the medium occurs in a two-step reaction to gluconic acid. First, glucose oxidase oxidizes D-glucose to D-glucono-1,5 lactone with the formation of hydrogen peroxide, acted upon by catalase present in the living cells, and is decomposed to water and oxygen. The lactone hydrolysis occurs spontaneously in gluconic acid in aqueous solutions (Goldberg; Rokem, 2009). The overall whole-cell biocatalysis mechanism by *A. niger* can be simplified by the enzymatic reaction as shown in Figure 5.1:

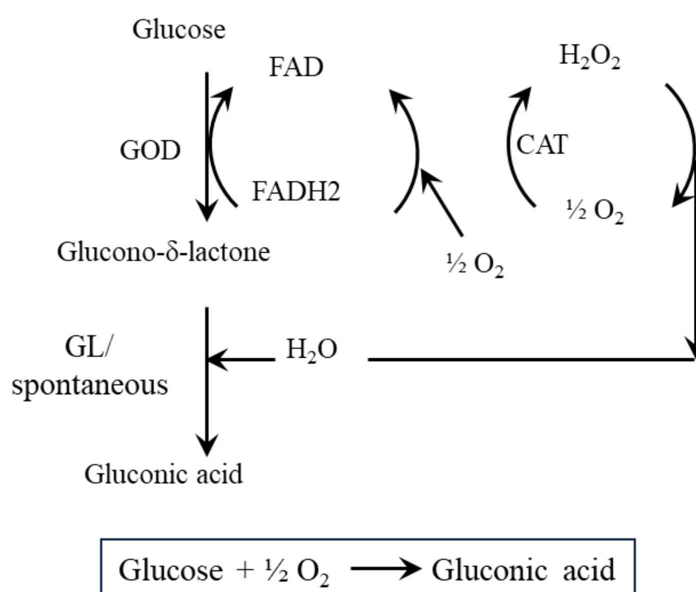


Figure 5.1. Fungal gluconic acid biosynthetic pathway. Adapted from Ramachandran *et al.* 2006. GOD, FAD-dependent glucose oxidase (EC 1.1.3.4); GL, gluconolactonase (EC 3.1.1.17); CAT, catalase (EC 1.11.1.6). GL, gluconolactonase (EC 3.1.1.17).

As summarized by Figure 5.1, oxygen is a substrate key in the oxidation of glucose, as glucose oxidase uses molecular oxygen in the bioconversion of glucose. Usually, oxygen is

provided in the form of air, but pure oxygen or hydrogen peroxide are options reported for efficient conversion (Sankpal; Kulkarni, 2002). Although oxygen is a key substrate to oxidize glucose, the present study did not consider the evaluation of this parameter previously due to the mini bioreactor of 200 mL working volume in which the process was developed not being suitable for the utilization of a dissolved oxygen monitoring probe. However, since the proposed system proved to be well-succeed in producing gluconic acid, the next step should be ensuring that oxygen, as a crucial substrate in the glucose oxidation reaction, is provided in quantity to support efficient gluconic acid production.

To monitor the availability of oxygen in the medium, preliminary assays were carried out in a bench-scale bubble column bioreactor of 2 L working volume. It is relevant to highlight that although the assays were carried out on a larger bioreactor, they do not effectively characterize a scale-up process for the bioreactor. Scaling up a bioreactor is a complex process that requires careful planning, experimentation, and attention to detail. It consists of increasing the size of the vessel while keeping all key parameters (e.g., impeller speed, aeration rate) proportionally to vessel size (scale) to maintain the same power per unit volume, which helps maintain similar mixing conditions.

Although the results are preliminary, the idea of this chapter is to signalize possible challenges that could be faced by future studies dedicated to scaling up this process and provide insights into relevant parameters that can improve the productivity of the proposed multi-enzymatic process.

5.2 Material and methods

5.2.1 Bubble column bioreactor of 2-L working volume

To measure dissolved oxygen tension, assays were carried out in a bench scale bubble column bioreactor of 2-L working volume (H_L=0.45 m, H_T=0.60 m, and $\emptyset$=0.125 m) provided with a stainless-steel cross-type sparger (84 orifices of 0.5 mm diameter) (Figure 5.2).

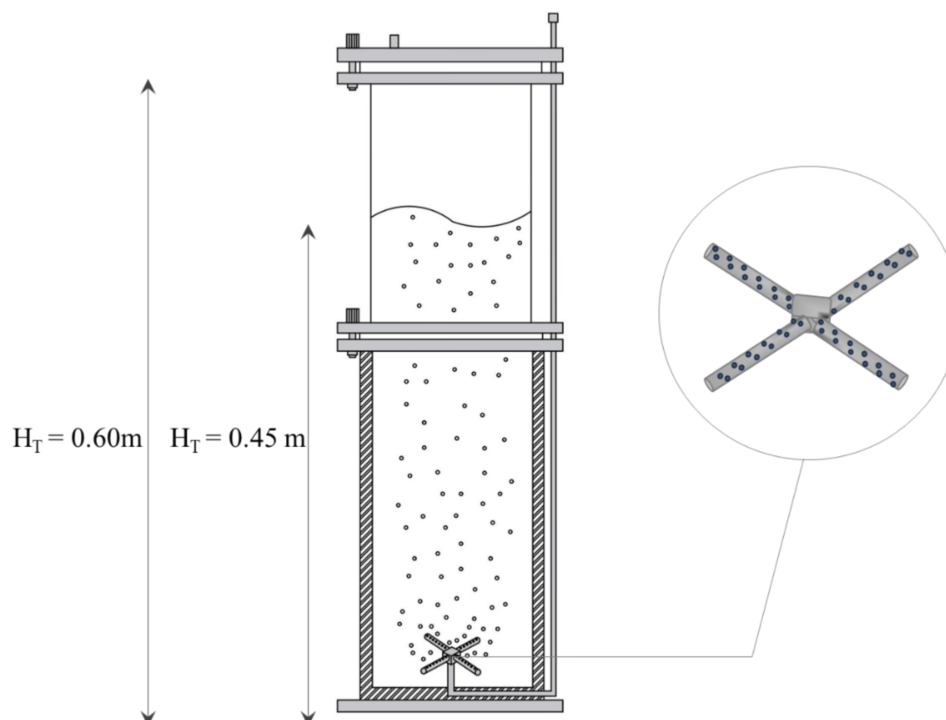


Figure 5.2. Scheme of the bubble column bioreactor: Personal collection, 2023.

5.2.2 Experimental procedure

The dissolved oxygen was evaluated by an assay carried out under the same conditions for both scales within the optimal operational region previously determined by the model (Eq. 4.7, Section 4.3.3). A soluble starch solution of 91.40 g/L was fed in the bubble column bioreactor under 2 vvm aeration (4 L/min). Following, 0.01% (v/v) of α-amylase, 0.64% (v/v) of AMG, and 0.3% (w/v) of whole cells were simultaneously added to the bioreactor. The system was kept at a temperature of 40 °C and pH 6.0 by feeding a solution of 2.0 M NaOH with Bioflo IIC, New Brunswick Scientific (Figure 5.2). An identical assay was performed in the 350 mL mini bubble bioreactor (8.50 × 3.33 cm, length × diameter) with a working volume of 200 mL, but with a specific flow rate of 5 vvm (1 L/min). A lower specific rate (2 vvm) was set up in the 2 L reactor to avoid problems faced on the smaller scale, such as excessive foam formation and bioreactor overflow. In addition, if dissolved oxygen in the liquid phase is not limiting at a lower specific aeration rate (2 vvm), it was most likely not limiting in the assays performed at a specific rate of 5 vvm.

5.3 Analytical methods

5.3.1 Dissolved oxygen tension measurement

Dissolved oxygen tension (DOT) is a critical parameter in bioprocess engineering commonly measured using a specialized probe or sensor designed to monitor the concentration or partial pressure of dissolved oxygen in a culture or reactional medium in bioprocesses (Hansen *et al.*, 2011). The DOT (%) relative to saturation with air oxygen was monitored by a polarographic O_2 sensor coupled to the O_2 Transmitter 4500 (Mettler Toledo, Switzerland).

5.3.2 Experimental determination of the volumetric oxygen transfer coefficient (k_La)

The volumetric oxygen transfer coefficient (k_La) was determined under the same conditions as the assay performed in the bubble column bioreactor (2 L). A soluble starch solution of 91.40 g/L, 0.01% (v/v) of α-amylase, and 0.64% (v/v) of AMG under 2 vvm aeration (4 L/min) and at 40 °C. The k_La was determined by the gassing-out method (Chisti, 1989), one of the most widely used experimental methods for k_La evaluation. When using this method, the medium inside the vessel first is stripped from oxygen. This can be done by introducing a second soluble gas (nitrogen is most commonly used). After the oxygen level in the medium stabilizes at zero, or somewhat close to zero value, the supply of nitrogen is switched off. Next, air is introduced into the medium at a constant flow rate, and then the increase in dissolved oxygen concentration is monitored with simultaneous and continuous measurement of the DO concentration by the electrode until saturation is reached. The electrode sensitivity (k_e) considered was $k_e = 0.14\ s^{-1}$ as determined by a previous study of our research group (Cerri *et al.*, 2016).

5.3.3 High-performance liquid chromatography (HPLC)

GA concentration was determined using a Waters Model 410 HPLC equipped with a UV detector (210 nm) and an Aminex HPX87-H column maintained at 55 °C. The eluent was 5 mM sulfuric acid in Milli-Q water at a flow rate of 0.6 mL/min. All samples were filtered using a 0.22 μm filter previously to be injected into the equipment. The concentration of glucose was determined using a Waters Model 410 HPLC equipped with a differential refractometer detector. The compounds were separated on a Sugar-Pak column at 80 °C, using Milli-Q water as the eluent at a flow rate of 0.5 mL/min. For HPLC glucose quantification, samples containing soluble starch were hydrolyzed with α-amylase (BAN 480L, 2 mL/kg starch) at 60 °C for 20 min. The temperature was fitted to 55 °C and soluble AMG (3 mL/kg starch) was added to the

saccharification of liquefied starch on glucose (Fonseca, 2019).

5.3.4 Antibiotic susceptibility test

To eliminate contamination troubles in the assays performed on a higher scale, the antibiotic susceptibility test of *Bacillus subtilis* cells (*B. megaterium* CNPMS B119) to different antibiotics was performed (Gajic *et al.*, 2022). Aliquots of the frozen stock of the microorganism were plated out on LB agar medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 15 g/L agar) containing each of the following antibiotics individually: ampicillin 80 µg/mL, kanamycin 50 µg/mL, chloramphenicol 25 µg/mL, streptomycin 50 µg/mL, tetracycline 25 µg/mL, and gentamicin 20 µg/mL. The Petri dishes were incubated for 72 h at 37°C.

5.4 RESULTS AND DISCUSSION

5.4.1 Solving contamination troubles

One of the main advantages of the proposed innovative multi-enzymatic process is the low risk of contamination due to the absence of nitrogen. It occurs because microorganisms, including bacteria, fungi, and algae, require nitrogen for various cellular processes and to build essential biomolecules such as amino acids, DNA, RNA, and ATP, among others (Grzyb; Wolna-Maruwka; Niewiadomska, 2021). Therefore, no sterilization procedures were needed in the previous assays performed in the mini bubble column reactor (200 mL working volume), all with no contamination incidents since the absence of nitrogen inhibits the proliferation of most microorganisms.

Depending on the organism, nitrogen, nitrates, ammonia, or organic nitrogen compounds can be used as a nitrogen source (Herrero; Muro-Pastor; Flores, 2001). Although there was no nitrogen source in the reaction medium, composed only of an aqueous starch solution, unknown products were formed in the 2 L bubble column bioreactor, indicating contamination by another microorganism. Afterward, the contamination was confirmed in the assays performed on a 2L scale by the presence of bacillus detected using a phase contrast microscope (Olympus, model BX51, Japan) (Figure 5.3).

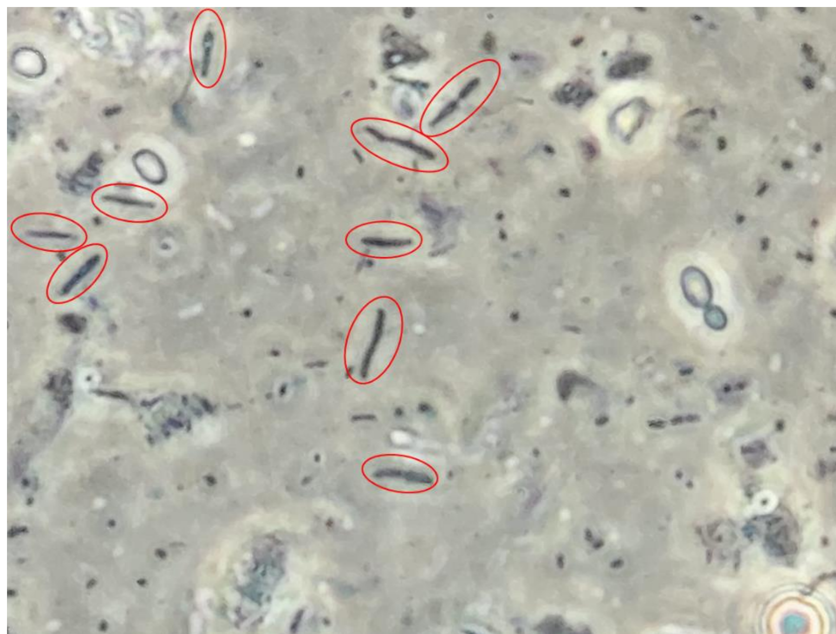


Figure 5.3. Bacillus was detected in the reactional medium using a phase contrast microscope (Olympus, model BX51, Japan). Personal collection, 2023.

The images obtained with a phase contrast microscope indicate that most likely, the microorganism was correspondent to *B. megaterium* which was utilized in co-worker research. Although *Bacillus* species are not the primary group of nitrogen-fixing microorganisms, some species of *Bacillus* bacteria are known for their ability to fix atmospheric nitrogen. The *B. megaterium* CNPMS B119 strain was proven to be able to fix nitrogen in a previous study (Velloso *et al.*, 2020). In addition, the *B. megaterium* CNPMS B119 growth in the reaction medium composed of starch is coherent with the fact that this strain was isolated from maize (Velloso *et al.*, 2023).

At that point, even with a sterilization procedure that was not part of the protocol in the mini-column bubble bioreactor, the assays performed on a higher scale (2 L) still presented contamination troubles. Then, an antibiotic susceptibility test procedure was performed to determine which antibiotic or antibiotics would be effective in solving the contamination by *Bacillus* as described in Section 2.3.4. The antibiotics tested were chloramphenicol, kanamycin, streptomycin, tetracycline, gentamicin, and ampicillin (Figure 5.4).

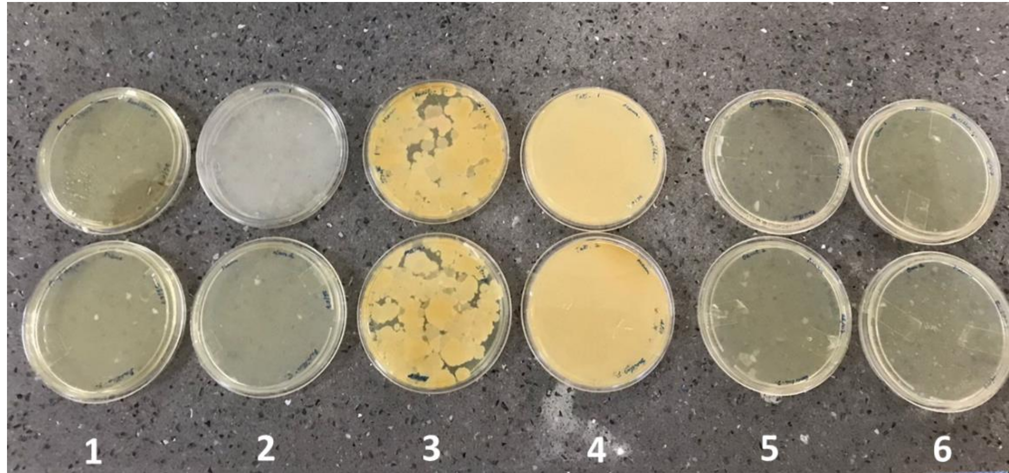


Figure 5.4. The antibiotic susceptibility tests. From left to right: 1) chloramphenicol 2) kanamycin 3) streptomycin 4) tetracycline 5) gentamicin 6) ampicillin. Personal collection, 2023.

As shown in Figure 5.4, the microorganism grew in the presence of streptomycin (3) and tetracycline (4). On the other hand, the antibiotic susceptibility test showed that chloramphenicol (1), kanamycin (2), gentamicin (5), and ampicillin (6) can inhibit the growth of the *B. megaterium*. Chloramphenicol was selected to be used in the higher-scale assay due to its ease of availability and low cost (Sood, 2016).

5.4.2 Volumetric oxygen transfer coefficient (k_La)

The k_La parameter is used to characterize the oxygen mass transfer. The volumetric oxygen mass transfer coefficient (k_La) is the parameter related to oxygen transfer from the gas into the liquid phase (Vanags; Suleiko, 2022). k_La shows numerically how efficiently oxygen, which is introduced through a sparger in the vessel, is transferred to the cultivation broth. The notion of k_La arises from the two-film theory developed by Lewis & Whitman 1(1924), which postulates that the mass transfer between two phases takes place through a boundary layer, between those two phases (see Figure 5.5).

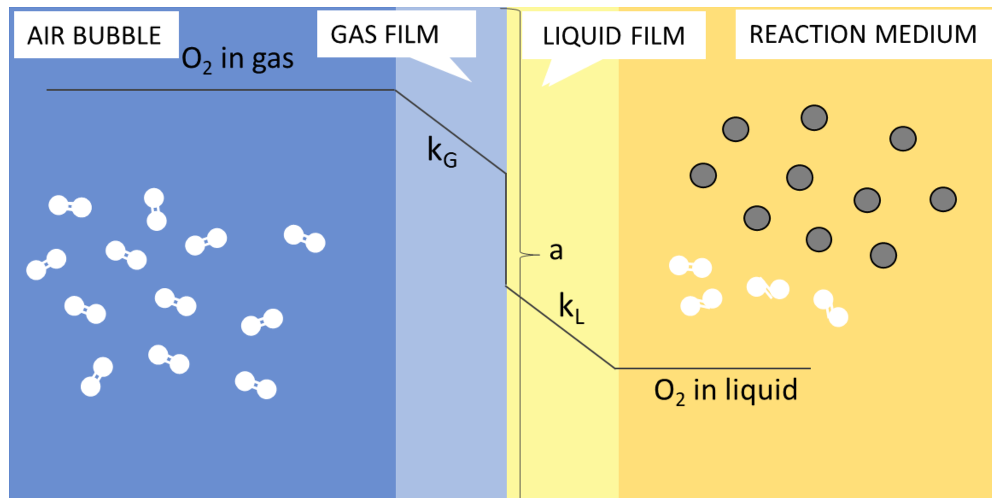


Figure 5.5. Gas-liquid oxygen mass transfer according to the two-film theory. Adapted from (Vanags; Suleiko, 2022).

The rate of diffusion of a component between phases is dependent on the mass transfer coefficient. For liquids, this coefficient is written as 'k_L'. The overall mass transfer rate between two phases apart from k_L is also dependent on the contact area between those two phases, termed as 'a'. When we combine the two, the volumetric oxygen mass transfer coefficient k_La is obtained (Lewis; Whitman, 1924). Additionally, k_La is related to a transient oxygen balance for the bioreactor volume by the following Equation 5.1 (Robinson *et al.*, 2002).

$$\frac{dC_L}{dt} = OTR - OUR = k_La \times (C^* - C_L) - OUR \tag{5.1}$$

where:

OTR – oxygen transfer rate from gas to the liquid medium

OUR – oxygen uptake rate

C^* – saturated dissolved oxygen concentration

C_L –current concentration of dissolved oxygen in the medium

Equation 5.1 shows that the change in the oxygen concentration in the liquid medium (dC_L/dt) is dependent on k_La and the difference between the current (C_L) and maximal possible (or sometimes termed equilibrium, C^*) oxygen concentrations. Simultaneously, the change of the oxygen concentration (dC_L/dt) is equal to the difference between the oxygen transfer rate to the medium (OTR) and the oxygen uptake rate (OUR) due to cellular respiration. During biomass growth, the microorganism oxygen demand increases. This demand is characterized

by the volumetric oxygen uptake rate OUR (Robinson *et al.*, 2002). Assuming the multi-enzymatic system has no microorganism growth, the oxygen uptake rate (OUR) can be considered zero (Eq. 5.2).

$$\mathrm{OTR} = k_L a \times (C^* - C_L) \qquad (5.2)$$

where:

C^* = 6.41 mg/L at 40 °C (equilibrium oxygen concentration according to dissolved oxygen and Henry's Law).

kLa = 150 h^{-1} (determined by the gassing-out method as described in Section 5.4.2).

C_L = 0 mg/L assuming that the reaction rate is fast enough that the oxygen that is transferred to the liquid medium is immediately consumed in the oxidation reaction of glucose.

$$\mathrm{OTR} = 150\ \mathrm{h^{-1}} \times (6.41 - 0)\frac{\mathrm{mg\ O_2}}{\mathrm{L}}$$

$$\mathrm{OTR} = 961.5\frac{\mathrm{mg\ O_2}}{\mathrm{L.h}}$$

By dividing by the oxygen molar mass, the OTR can be converted from mg.L^{-1}.h^{-1}) to mmol.L^{-1}.h^{-1}:

$$\mathrm{OTR} = \frac{961.5\frac{\mathrm{mg\ O_2}}{\mathrm{L.h}}}{32\frac{\mathrm{mg\ O_2}}{\mathrm{mmol\ O_2}}}$$

$$\mathrm{OTR} = 961.5\frac{\mathrm{mg\ O_2}}{\mathrm{L.h}} \times \frac{1}{32}\ \frac{\mathrm{mmol\ O_2}}{\mathrm{mg\ O_2}}$$

$$\mathrm{OTR} = 30.04\frac{\mathrm{mmol\ O_2}}{\mathrm{L.h}}$$

The OTR calculated based on k_La enables us to estimate the maximum theoretical productivity of GA (g_{GA}.L^{-1}.h^{-1}) for the system, based on the stoichiometry of the glucose oxidation reaction (Eq. 5.3).

$$C_6H_{12}O_6 + \frac{1}{2}O_2 \rightarrow C_6H_{12}O_7 \qquad (5.3)$$

Considering the stoichiometry of the glucose oxidation reaction (Eq. 5.3) and assuming that the rate of reaction is such that as oxygen enters the solution is immediately consumed in the oxidation reaction of glucose, the OTR of 30.04 $mmol_{O2}$.L^{-1}.h^{-1} has the potential to produce

60.09 $mmol_{GA}.L^{-1}.h^{-1}$. Multiplying by the GA molar mass (196.16 $g.mol^{-1}$), it is possible to calculate the maximum theoretical productivity of GA ($g_{GA}.L^{-1}.h^{-1}$) for the system:

$$\text{GA theoretical productivity} = 60.09 \times 10^{-3} \frac{\text{mol}_{\text{GA}}}{\text{L . h}} \times 196.16 \frac{\text{g}_{\text{GA}}}{\text{mol}_{\text{GA}}}$$

$$\text{GA theorical produtivity} = 11.79 \frac{\text{g}_{\text{GA}}}{\text{L . h}}$$

Theoretically, the specific aeration rate of 2 vvm used in a bench scale bubble column bioreactor (2 L working volume) has the potential to result in a GA productivity of 11.79 $g_{GA}.L^{-1}.h^{-1}$. However, it is relevant to point out that anti-foaming was not added in the k_La assay determination. The high degree of aeration required in the process to produce GA gives rise to the undesirable phenomenon of foam formation. Antifoam agents are designed to break down or inhibit foam formation by reducing the liquid surface tension. In addition to causing a collapse of bubbles in the foam, they also favor the coalescence of bubbles with the liquid phase, which results in larger bubbles with reduced surface area to volume ratios reducing the OTR (Vanags; Suleiko, 2022).

5.4.3 Assay performed on a higher scale

After solving the contamination issues, an assay was performed under the same conditions in the mini-reactor and in a higher scale (2 L reactor) as described in Section 5.4.2, to verify if the dissolved oxygen is limiting for the process.

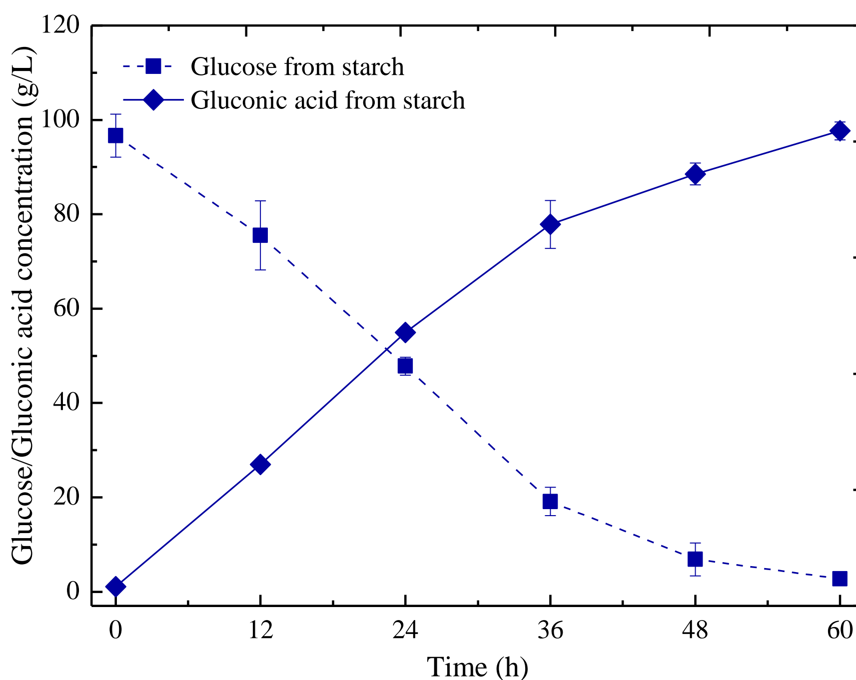


Figure 5.6. Gluconic acid production in the mini bubble column bioreactor at 40° C, 0.3% (w/v) of whole-cells, and 5 vvm (1 L.min). pH 5.50, 0.64% (v/v) AMG, and 91.40 g/L of soluble starch.

The experiment on a smaller scale resulted in a final GA concentration of 97.6 ± 1.9 g/L with a product yield of 98.3 ± 1.7% ($g_{GA}/g_{glucose}$), a biocatalyst yield of 32.5 ± 0.6 ($g_{GA}/g_{whole\text{-}cells}$) and a GA volumetric productivity of 1.6 ± 0.1 $g_{GA}.L^{-1}.h^{-1}$. The assay for GA production under the same experimental conditions as the previous one using soluble starch was performed in a bench-scale bubble column bioreactor of 2 L working volume, but with an aeration flow rate of 2 vvm (4 L/min). Figure 5.7 illustrates the GA production, glucose consumption, and dissolved oxygen tension (DOT) throughout the assay.

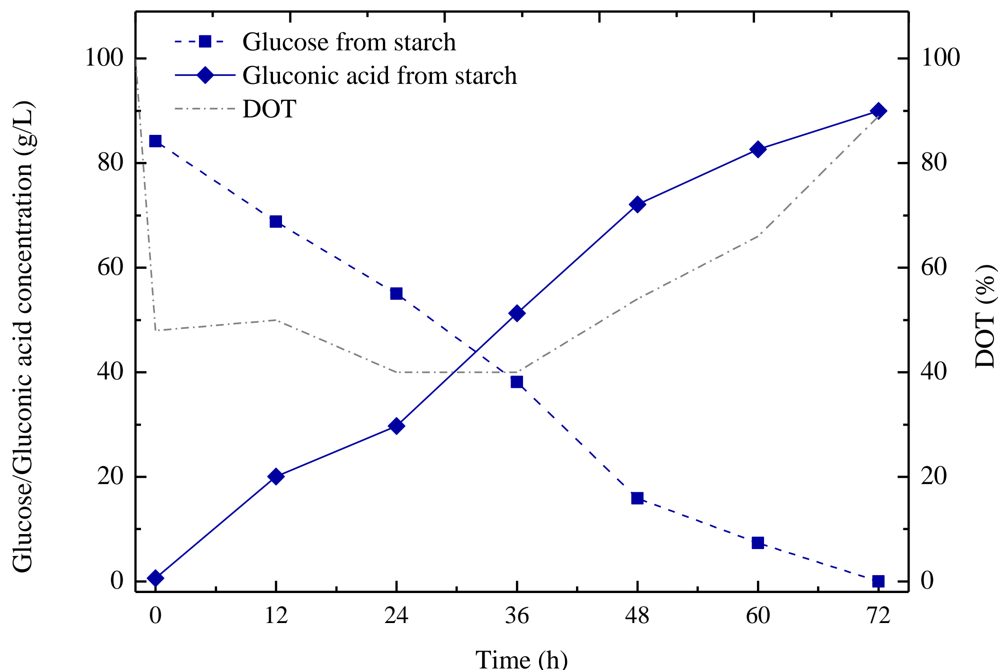


Figure 5.7. Gluconic acid production in a bench scale bubble column bioreactor at 40° C, 0.3% (w/v) of whole-cells, and 2 vvm (4 L/min) under the conditions: pH 5.50, 0.64% (v/v) AMG, and 91.40 g/L of soluble starch.

The experiment on a higher scale resulted in a final GA concentration of 89.96 g/L with a product yield of 98.1% ($g_{GA}/g_{glucose}$), a biocatalyst yield of 29.98 ($g_{GA}/g_{whole\text{-}cells}$), and a GA volumetric productivity of 1.25 $g_{GA}.L^{-1}.h^{-1}$.

The comparison between the assays performed on both scales under the same conditions showed similar results in terms of yield (%), biocatalyst yield ($g_{GA}/g_{whole\text{-}cells}$), and production (in g/L), but slightly different in terms of productivity. Most likely, a lower aeration rate (2 vvm) in the 2 L column bioreactor may have impacted the homogenization of the medium and, consequently, the reaction kinetics, resulting in a longer assay.

The DOT signal was always above 40% during the reaction for the assay performed in a bench-scale bubble column bioreactor (2 L), indicating a sufficient oxygen supply, even at a lower specific aeration flow rate (2 vvm) than applied in the mini bubble reactor (5 vvm). This parameter indicates that probably the process is not limited by external O_2 transfer (bulk). Additionally, it is relevant to point out that the decrease in DOT to below 50% between 12 and 36 h was due to the addition of antifoam. Like surface-active agents, antifoam agents can decrease the oxygen transfer rate dramatically and thus play an important role in the oxygen

transfer process (Li *et al.*, 1994). Even so, DOT did not reach values below 40%, indicating a sufficient oxygen supply.

Therefore, because the DOT has remained above 40% even with anti-foaming addition and the GA productivity obtained (1.25 $g_{GA}.L^{-1}.h^{-1}$) is much lower than the theoretical GA productivity calculated for this bench-scale bioreactor aeration system (11.79 $g_{GA}.L^{-1}.h^{-1}$), probably the reaction system is not limited by mass transfer of dissolved oxygen from the bulk phase to the phase boundary. However, the morphology of fungal pellets has a significant influence on mass transfer processes, since the rate of a chemical reaction can be limited both by mass transfer of the substrate from the bulk phase to the phase boundary and by mass transfer within the filamentous structure (Hille *et al*., 2009). Pellet morphology often presents problems with the internal transport of substrates and products, depending on the size and compactness of pellets (Dynesen; Nielsen 2003). Diffusion limitation of mass transfer within the filamentous structure often occurs when the reactants must pass through a porous or complex network of channels within the structure, leading to limitations in the transport of reactants to the reaction sites. In such cases, the rate of the overall reaction is determined not only by the intrinsic kinetics of the reaction but also by the rate at which the reactants can access the reactive sites within the intricate filamentous structure. To overcome this limitation, it is essential to optimize the design and structure of the filamentous material, improve the transport properties, or even employ techniques such as stirring or changing the operating conditions to enhance mass transfer. Understanding and overcoming mass transfer limitations is critical to maximizing the efficiency of reactions occurring within filamentous structures (Schmideder *et al.*, 2019).

Most likely, the process is limited by diffusion problems in the whole cells or enzymatic reactions since fungal cells have a thick cell wall composed of interconnected polysaccharides, proteins, and glycoproteins through which substrates and products pass to access membrane enzymes (Garcia-Rubio *et al.*, 2020). Difusional problems could be overcome by partially disrupting the fungal wall to facilitate access to these cell wall enzymes, reducing the size of the whole cell pellet, and/or increasing the concentration of whole cells, which was fixed at a lower level (3 g/L). As mentioned in the previous chapter, many aspects such as pellet size, biocatalyst concentration, bioreactor configurations, and operating modes are still open for refinement.

REFERENCES

CERRI, M. O. *et al*. A new approach for kLa determination by gassing-out method in pneumatic bioreactors. **Journal of Chemical Technology & Biotechnology**, vol. 91, n. 12, p. 3061-3069, 24 Mar. 2016. Available from: https://doi.org/10.1002/jctb.4937.

CHISTI, Y. Airlift Bioreactors (Elsevier Applied Biotechnology). [S. l.]: Springer, 1989. 346 p. ISBN 9781851663200.

DYNESEN, J.; NIELSEN, J. Surface Hydrophobicity of *Aspergillus nidulans* Conidiospores and Its Role in Pellet Formation. **Biotechnology Progress**, vol. 19, n. 3, p. 1049-1052, 6 June 2003. Available from: https://doi.org/10.1021/bp0340032.

GAJIC, I. *et al*. Antimicrobial Susceptibility Testing: A Comprehensive Review of Currently Used Methods. **Antibiotics**, vol. 11, n. 4, p. 427, 23 Mar. 2022. Available from: https://doi.org/10.3390/antibiotics11040427.

GARCIA-RUBIO, R. *et al*. The Fungal Cell Wall: Candida, Cryptococcus, and Aspergillus Species. **Frontiers in Microbiology**, vol. 10, 9 Jan. 2020. Available from: https://doi.org/10.3389/fmicb.2019.02993.

GOLDBERG, I.; ROKEM, J. S. Organic and Fatty Acid Production, Microbial. **Encyclopedia of Microbiology**. [S. l.]: Elsevier, 2009. p. 421-442. ISBN 9780123739445. Available from: https://doi.org/10.1016/b978-012373944-5.00156-5.

GRZYB, A.; WOLNA-MARUWKA, A.; NIEWIADOMSKA, A. The Significance of Microbial Transformation of Nitrogen Compounds in the Light of Integrated Crop Management. **Agronomy**, vol. 11, n. 7, p. 1415, 15 July 2021. Available from: https://doi.org/10.3390/agronomy11071415.

HANSEN, S. *et al*. Potential errors in conventional DOT measurement techniques in shake flasks and verification using a rotating flexitube optical sensor. **BMC Biotechnology**, vol. 11, n. 1, p. 49, 2011. Available from: https://doi.org/10.1186/1472-6750-11-49.

HERRERO, A.; MURO-PASTOR, A. M.; FLORES, E. Nitrogen Control in Cyanobacteria. Journal of Bacteriology, vol. 183, n. 2, p. 411-425, 15 Jan. 2001. Available from: https://doi.org/10.1128/jb.183.2.411-425.2001.

HILLE, A. *et al*. Effective diffusivities and mass fluxes in fungal biopellets. **Biotechnology and Bioengineering**, vol. 103, no. 6, p. 1202-1213, 15 Aug. 2009. Available from: https://doi.org/10.1002/bit.22351.

LEWIS, W. K.; WHITMAN, W. G. Principles of Gas Absorption. **Industrial & Engineering Chemistry**, vol. 16, n. 12, p. 1215-1220, Dec. 1924. Available from: https://doi.org/10.1021/ie50180a002.

LI, T. *et al*. A Study of Oxygen-vectors with a Model of Oxidation of Glucose in the Presence of Glucose Oxidase and Catalase. **Journal of Chemical Engineering of Japan**, vol. 27, n. 2, p. 205-210, 1994. Available from: https://doi.org/10.1252/jcej.27.205.

ROBINSON, K. K. *et al.* Mass transfer and cell growth kinetics in a bioreactor. Northwestern

University. vol. 36, n. 3 (2002).

SANKPAL, N. V.; KULKARNI, B. D. Optimization of fermentation conditions for gluconic acid production using *Aspergillus niger* immobilized on cellulose microfibrils. **Process Biochemistry**, vol. 37, n. 12, p. 1343-1350, July 2002. Available from: https://doi.org/10.1016/s0032-9592(01)00335-1.

SCHMIDEDER, S. *et al*. From three-dimensional morphology to effective diffusivity in filamentous fungal pellets. **Biotechnology and Bioengineering**, vol. 116, no. 12, p. 3360-3371, 8 Oct. 2019. Available from: https://doi.org/10.1002/bit.27166.

SOOD, S. Chloramphenicol – A Potent Armament Against Multi-Drug Resistant (MDR) Gram Negative *Bacilli*? **Journal of Clinical and Diagnostic Research**, 2016. Available from: https://doi.org/10.7860/jcdr/2016/14989.7167.

VANAGS, J.; SULEIKO, A. Oxygen Mass Transfer Coefficient Application in Characterization of Bioreactors and Fermentation Processes. **Latvian Journal of Physics and Technical Sciences**, vol. 59, n. 5, p. 21-32, 1 Oct. 2022. Available from: https://doi.org/10.2478/lpts-2022-0038.

VELLOSO, C. C. V. *et al*. High yield of heat-resistant spores of *Bacillus megaterium* in bioreactors. **Biochemical Engineering Journal**, p. 109030, July 2023. Available from: https://doi.org/10.1016/j.bej.2023.109030.

VELLOSO, C. C. V. *et al*. Genome-guided insights of tropical Bacillus strains efficient in maize growth promotion. **FEMS Microbiology Ecology**, vol. 96, n. 9, 12 Aug. 2020. Available from: https://doi.org/10.1093/femsec/fiaa157.

WITTEVEEN, Cor F. B.; VEENHUIS, Marten; VISSER, Jaap. Localization of Glucose Oxidase and Catalase Activities in *Aspergillus niger*. **Applied and Environmental Microbiology**, vol. 58, no. 4, p. 1190-1194, 1992. Available from: https://doi.org/10.1128/aem.58.4.1190-1194.1992.

ZNAD, H.; MARKOŠ, J.; BALEŠ, V. Production of gluconic acid from glucose by *Aspergillus niger*: growth and non-growth conditions. **Process Biochemistry**, vol. 39, n. 11, p. 1341-1345, July 2004. Available from: https://doi.org/10.1016/s0032-9592(03)00270-x.

6 GENERAL CONCLUSIONS

This study presented an intermediate method between classic fermentation and purely enzymatic processes to produce high-purity gluconic acid from starch. Regarding purely enzymatic processes, an advantage of this process is that the intracellular enzymes GOD and CAT, which would be more expensive, were used *in situ* without the associated purification costs. The use of enzymes *in situ* can also contribute to better stability of these biocatalysts due to the natural protection of the cell envelope. In addition, the cells will recycle co-factor for re-dox reactions with existing machinery. Compared to classic fermentation, this process eliminates the costs of supplementing salts for the reactional medium, which consists only of the carbon source, reducing the risk of contamination due to the absence of nitrogen.

The innovative multi-enzymatic system proposed occurs in one step for gluconic acid production from starch using whole cells of *Aspergillus niger* associated with amylolytic enzymes. The process resulted in high yield and product purity typical of a purely enzymatic process, for example, a gluconic acid concentration of 134.5 ± 4.3 g/L, a gluconic acid yield of 98.2 ± 1.3%, biocatalyst yield of 44.8 ± 1.4 $g_{GA}/g_{whole\text{-}cells}$, and product purity of c.a. 96% were achieved during 96 h of reaction. The process proved to be effective for glucose, dextrin, and lastly starch, one of the most abundant polysaccharides in nature. Although the process has been developed for starch, the approach is feasible for any material that can be hydrolyzed to glucose. This approach aligns the utilization of agri-food waste as cost-effective and renewable feedstock, tapping into abundant glucose sources for the proposed multi-enzymatic system. Therefore, the innovative proposed multi-enzymatic system opens new perspectives on GA production by whole-cell biocatalysis.

Given the results obtained, the following is suggested for future work:

a) Future research can explore a diverse combination of *A. niger* whole-cells with other enzymatic cocktails. For example, by combining cellulolytic enzymes with *A. niger* whole-cells, there is potential to generate GA from lignocellulosic residues. The association of commercial enzymes with whole-cells can provide different multi-enzymatic systems with better cost-effectiveness than an entirely enzymatic process. These multi-enzymatic systems can convert a wide variety of glucose-based feedstocks to GA through hydrolysis followed by whole-cell biocatalysis.
b) The multi-enzymatic process to produce gluconic acid proposed in this study is analogous to the industrial process of high-fructose corn syrup (HFCS) production, in which applied the most well-succeed whole-cell-based immobilized biocatalyst. The

immobilized biocatalyst based on whole-cells of *Streptomyces* containing glucose isomerase (IGI) applied in the HFCS production industrial process is widely used in commercial applications and considered the paradigm for an immobilized biocatalyst. Given this success, the similarity between both processes and the relevant process advantages that an immobilized biocatalyst offers, for example, enables continuous production, provides mechanical stability, and the absence of the biocatalyst in the product stream, future research can work on the development of a similar whole-cell-based immobilized biocatalyst currently used in the industrial production of HFCS.

c) Development of a biocatalyst through cell disruption and encapsulation of cellular debris in an attempt to reduce diffusional problems due to the complexity of the cell wall, which is composed of interconnected polysaccharides, proteins, and glycoproteins through which substrates and products pass to access membrane enzymes.

d) Although the current study has optimized some crucial parameters, its primary accomplishment lies in successfully demonstrating the proof-of-concept for the proposed novel multi-enzymatic system. Numerous aspects, including pellet size, biocatalyst concentration, bioreactor configurations, and operating modes, are still open for refinement.