Universidade do Minho
Escola de Engenharia

Bruna Antunes Parente

Development of a new functional fruit-based product

julho de 2021

Development of a new functional fruit-based product

Bruna Parente

UMinho | 2021


ColOsH
cLABEL+
CLEAN LABEL FOOD
COMPETE 2020
PORTUGAL 2020
FCT
Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR
UNIÃO EUROPEIA
Fundo Europeu de Desenvolvimento Regional

Universidade do Minho
Escola de Engenharia

Bruna Antunes Parente

Development of a new functional fruit-based product

Dissertação de Mestrado
Mestrado em Biotecnologia

Trabalho efetuado sob a orientação do
Professor Doutor José António Couto Teixeira
e da
Doutora Clarisse Salomé Nobre Gonçalves

julho de 2021

AGRADECIMENTOS

Gostaria de agradecer a todos aqueles que direta ou indiretamente contribuíram e colaboraram para a concretização desta dissertação bem como para o meu crescimento pessoal e profissional em todo o percurso académico.

Primeiramente ao meu orientador, Professor José Teixeira, pela oportunidade que me deu de integrar o seu grupo de investigação e por toda a partilha de conhecimentos e orientação.

À minha co-orientadora, Doutora Clarisse Nobre, pela orientação, disponibilidade, paciência e pelos conhecimentos transmitidos, tendo acreditado sempre nas minhas capacidades.

A todo o pessoal integrante do LF agradeço a boa receção, a ajuda, disponibilidade e paciência durante todo o tempo. Um obrigado especial ao Paulo Berni, Lívia Simões, Ruth Cuvas e Dalila Roupar por todo o conhecimento, apoio e companheirismo que me deram ao longo de todo este projeto.

Um obrigado também aos investigadores do LIP pela disponibilidade e paciência mesmo eu não pertencendo ao seu laboratório, em especial ao Rui Rodrigues pela ajuda e conhecimentos partilhados.

Aos meus companheiros de licenciatura (Andreia Brandão, Bruna Dias, Liliana Cerqueira, Mafalda Lopes, Pedro Silva e Tânia Leal), às colegas de mestrado (Bruna Freitas, Catarina Ferreira, Lúcia Roque e Márcia Marques),às camaradas de quarentena (Adelaide Rouceiro, Carla Rouceiro e Cátia Batista) e aos parceiros de vida (António Freitas, Inês Rego e Maria Costa), um grande obrigada pelo apoio incondicional em todas as fases, pelas dúvidas tiradas e pela paciência e conselhos dados ao longo de todo o percurso.

Ao Ricardo Oliveira pelo suporte nestes últimos 5 anos, tendo acompanhado todos os bons e maus momentos com uma palavra de tranquilização e positivismo a dar.

A toda a minha família, especialmente aos meus pais e avós, pela motivação e apoio que deram em todas as etapas, pela paciência e por fazerem tudo o que estava ao seu alcance para que eu chegasse sempre aos meus objetivos e que acreditasse sempre no que sou, independentemente das dificuldades.

Por fim, agradeço o financiamento deste trabalho pelos fundos nacionais através da FCT – Fundação para a Ciência e a Tecnologia, I.P., no âmbito do projeto "ColOsH - Novel concept for a dynamic modular Colon system including peristalsis simulation by Oscillatory-flow and Colon-Host interaction" com referência PTDC/BTM-SAL/30071/2017 (POCI–01–0145–FEDER–030071) e do Projeto cLabel+ (POCI-01-0247-FEDER-046080) co-financiado pelo Compete 2020, Lisboa 2020, Portugal 2020 e a União Europeia, através do Fundo Europeu de Desenvolvimento Regional (FEDER) e ainda à empresa FRULACT pelo fornecimento da preparação de morango.

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration. I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho

ABSTRACT

People are increasingly concerned about maintaining a healthy lifestyle. Thus, the search for products with low sugar content and additionally functionality has greatly increased in the last few decades. Also, overconsumption of sugar has proven to be a problem for public health. The development of functional food can be used as a strategy to reduce sugar content of consumed products. Prebiotics are efficient modulators of the intestinal microbiota, providing health benefits. The fortification of fruit-based products has been the most used strategy to incorporate prebiotics in food, but the *in situ* synthesis in the food itself has not been much explored. Fructo-oligosaccharides (FOS) are well-known prebiotics, which may be industrially produced from the transfructosylation reaction of sucrose (GF) by microbial enzymes, namely β-D-fructofuranosidase (FFase) and/or Fructosyltransferase (FTase).

In this work, FFase produced by *Aspergillus ibericus* was applied in a strawberry preparation to convert *in situ* its native GF content into FOS. FFase with maximum activity of 239 ± 6 U/mL was obtained in the crude extract of *A. ibericus* (38 h fermentation). For extraction of intracellular FFase, cells were subjected to ultrasonication. FFase maximum activity (79 ± 11 U/mL) was obtained using 20 W for 6 minutes. Regarding enzyme application, similar total FOS concentration were obtained with extracellular and intracellular extract which showed no significant advantages of adding the enzyme extraction step to the process. Operational parameters were optimized for the FFase crude extract with a 300 g/L GF solution, namely, reaction time, temperature, pH and ratio enzyme:substrate. After 24 h reaction, 50 °C, pH 5, 150 rpm agitation using 3 mL of FFase in 15 mL solution were obtained in a concentration of 138 ± 10 g/L of FOS and 0.53 ± 0.04 g_{FOS}/g_{GF} yield. Finally, FFase crude extract was added to the fruit preparation without adjustments of the food pH. At optimum conditions (50 °C, 5 g of FFase in total of 15 g) 59 ± 3 g/L of FOS were obtained which corresponds to a reduction of approximately 10 g/L of non-prebiotic sugars. The pH value and colour of the fruit preparation did not significantly vary, while °Brix increased and viscosity decreased.

In conclusion, FFase from *A. ibericus* demonstrated good potential for *in situ* FOS enzymatic synthesis in fruit preparations, allowing to reduce its intrinsic sugar content and to develop novel functional fruit preparations.

KEYWORDS

β-fructofuranosidase, fructo-oligosaccharides, functional food, fruit preparation, sugar reduction

RESUMO

As pessoas estão cada vez mais preocupadas em manter um estilo de vida saudável. Assim, a procura por produtos funcionais e com baixo teor de açúcar tem aumentado muito nas últimas décadas. Além disso, o consumo excessivo de açúcar tem provado ser um problema para a saúde pública. O desenvolvimento de alimentos funcionais pode ser utilizado como uma estratégia para reduzir o teor de açúcar dos produtos. Os prebióticos são capazes de modular a microbiota intestinal, proporcionando efeitos benéficos na saúde. A fortificação de produtos à base de fruta tem sido a estratégia mais usada para incorporar prebióticos nos alimentos, mas a síntese *in situ* nos próprios alimentos não tem sido muito explorada. Fruto-oligossacarídeos (FOS) são prebióticos bem conhecidos, industrialmente produzidos a partir da reação de transfructosilação da sacarose (GF) por enzimas microbianas, nomeadamente a β-D-frutofuranosidase (FFase) e/ou Fructosiltransferase (FTase).

Neste trabalho, FFase produzida por *Aspergillus ibericus* foi usada numa preparação de morango para converter *in situ* o seu conteúdo de GF em FOS. A atividade máxima de FFase obtida no extrato bruto foi de 239 ± 6 U/mL (38 h fermentação). Para extrair a FFase intracelular, as células foram sujeitas a ultrasonicação. A atividade máxima de FFase (79 ± 11 U/mL) foi obtida aplicando 20 W durante 6 minutos. A quantidade de FOS produzidos com a enzima extracelular e intracelular foi similar, não evidenciando vantagens em extrair a enzima. Os parâmetros operacionais, nomeadamente tempo de reação, temperatura, pH e rácio enzima:substrato, foram otimizados para o extrato de FFase usando uma solução de GF 300 g/L. Após 24 h de reação a 50foi obtida uma concentração máxima de 153 ± 3 g/L de FOS, com um rendimento de conversão de 0,51 ± 0,03 g_{FOS}/g_{GF},. Após 24 h de reação a 50 °C, pH 5, 150 rpm de agitação e usando 3mL de FFase num volume total de 15 mL Foi obtida uma concentração de138 ± 10 g/L of FOS e rendimento 0.53 ± 0.04 g_{FOS}/g_{GF}. Finalmente, a FFase foi adicionada ao preparado de fruta, sem ajuste de pH. Nas condições ótimas, obteve-se um preparado com 59 ± 3 g/L de FOS, tendo havido uma redução de cerca de 10 g/L de açúcares não prébioticos. O valor de pH e a cor não variaram significativamente, enquanto o Brix aumentou e a viscosidade diminuiu.

Em conclusão, a FFase de *A. ibericus* demonstrou bom potencial para a síntese enzimática *in situ* de FOS em preparações de fruta, permitindo reduzir o seu teor de açúcar intrínseco e desenvolver novas preparações funcionais de fruta.

PALAVRAS-CHAVE

β-fructofuranosidase, fruto-oligossacarídeos, alimentos funcionais, preparados de fruta, redução de açúcares

TABLE OF CONTENTS

Direitos de Autor e Condições de utilização do trabalho por terceiros i
Agradecimentos ii
Statement of integrity iii
Abstract iv
Resumo v
List of Figures ix
List of Tables xi
List of Abbreviations xii
1. State of art 1
1.1 Fruit-based products 1
1.3 Probiotics and prebiotics 5
1.3.1 Prebiotics as food ingredients 7
1.3.2 Incorporation of prebiotic in fruit-based products 8
1.3.2.1. Fortification 9
1.3.2.2. *In situ* synthesis 10
1.4 Fructans 14
1.4.1 FFase production 16
1.4.2 Production of FOS by enzymatic synthesis 17
1.5 FOS safety, dose and health benefits 21
2. Goals 23
3. Materials and Methods 24
3.1 FFase production using a *A. ibericus* culture 24
3.1.1 Microrganism and culture conditions 24
3.1.2 FFase production in shake flasks 24
3.1.3 Extraction of intracellular FFase enzyme by ultrasonication 25
3.1.4 Sugar analysis 25
3.1.5 FFase activity 26
3.2 Optimization of enzymatic synthesis of FOS by A. ibericus FFase extract 26

3.2.1 Application of intracellular and extracellular FFase extract in GF solution 26
3.2.2 Effect of initial GF concentration 26
3.2.3 Effect of Temperature 27
3.2.4 Effect of pH 27
3.2.5 Optimization of the enzyme:GF solution ratio 27
3.3 Enzymatic synthesis of FOS in a fruit-based product 28
3.3.1 Fruit-based product 28
3.3.2 Effect of Temperature on the FOS synthesis 28
3.3.3 Effect of enzyme:product ratio on the FOS synthesis 29
3.4 Characterization of the functional fruit preparation 29
3.4.1 pH 29
3.4.2 Water activity (a_w) 29
3.4.3 Total Soluble Solids (TSS) 29
3.4.4 Sugars analysis 29
3.4.5 Colour analysis 30
3.4.6 Rheology analysis 30
3.5 Statistical analysis 30
4. Results and discussion 31
4.1 Characterization of fruit preparation 31
4.2 FFase production by *A. ibericus* 32
4.2.1 Extracellular FFase 32
4.2.2 Intracellular FFase 35
4.3 The potential of FFase extracts for the synthesis of FOS 36
4.4 Optimization of FOS enzymatic synthesis 39
4.4.1 Effect of initial GF concentration 39
4.4.2 Effect of Temperature 40
4.4.3 Effect of pH 42
4.4.4 Effect of enzyme:GF ratio 43
4.5 Production of the functional fruit-based product 45
4.5.1 Effect of temperature 45

4.5.2 Effect of ratio enzyme:fruit-based product 48
4.6 Characterization of functional fruit-based product 49
5. Conclusions 53
7. References 55
Annexes 67
Annex I– Sugars calibration for HPLC analysis 67
AI.1 Fructose (Fru) 67
AI.2 Glucose (Glc) 67
AI.3 Sucrose (GF) 68
AI.4 1-kestose (GF_2) 68
AI.5 Nystose (GF_3) 69
AI.6 1F-fructofuranosylnystose (GF_4) 69
Annex II– Specification sheet of strawberry preparation 70

LIST OF FIGURES

Figure 1. General scheme of fruit processing (Adapted from J. Barta, 2012) [8]. 3

Figure 2. Schematic representation of the direct and indirect mechanism of action by the prebiotics on host health (Adapted by Mohanty et. al.2018) [29]. 6

Figure 3. Chemical structure of (a) 1- kestose (GF_2), (b) nystose(GF_3), and (c) 1F-fructofuranosylnystose (GF_4) from ChemDraw 12.0.2. software. 15

Figure 4. Scheme of transfructosylation reaction carried out by β-fructofuranosidase (FFase) enzymes to produce fructo-oligosaccharides (FOS) from ChemDraw 12.0.2. software. 15

Figure 5. Culture plate of *Aspergillus ibericus* MUM 03.49. 24

Figure 6. Shake flask fermentation of sucrose by an *A. ibericus* monoculture performed at 37 °C, pH 6.2 and 150 rpm: Fru (red), Glc (light blue); GF (orange); GF_2 (purple), GF_3 (dark blue) and GF_4 (yellow), total of FOS (green). Results correspond to the average of two independent experiments ± standard deviation. 33

Figure 7. Activity profile of β-fructofuranosidase (FFase) from *A. ibericus* produced in shake flasks using a monoculture strategy. Results correspond to the average ± standard deviation of two independent experiments. 34

Figure 8. Sugars profile obtained for FOS enzymatic synthesis reaction performed at 50 °C, pH 5.0 and 150 rpm for 27 h by (a) extracellular and (b) intracellular FFase extract. Fru (white bars), Glc (dotted bars), GF (light grey bars), GF_2 (striped bars), GF_3 (dark grey bars), total FOS (○). Results correspond to the average of three independent assays ± standard deviation. 37

Figure 9. Effect of initial GF concentration in FOS enzymatic synthesis. Experiments carried out at 50 °C, pH 5.0 and 150 rpm for 27 h. (a) FOS production and (b) conversion yield obtained for 100 g/L (orange), 200 g/L (blue), 300 g/L (green) and 400 g/L (red) of initial GF concentration. Results correspond to the average of two independent assays ± standard deviation. 40

Figure 10. Effect of temperature in FOS enzymatic treatment by addition of 5 g of FFase extract. Experiments carried out at pH 5 and 150 rpm for 24 h. Effect of (a) 40, (b) 50 and (c) 60 °C in the sugar profile. Fru (red), Glc (orange); GF (yellow); GF_2 (dark blue bar), GF_3 (light blue bar) and total FOS (green). Results correspond to the average of two independent experiments ± standard deviation. 41

Figure 11. Effect of pH on FOS enzymatic synthesis. Experiments carried out at 50 °C and 150 rpm for 24 h. Sugar's profile under different pH values: (a) 3.6, (b) 4.3, (c) 5.0, (d) 5.7 and (e) 6.4. Fru (red),

Glc (orange); GF (yellow); GF_2 (dark blue bar), GF_3 (light blue bar), total FOS (green). Results correspond to the average of two independent experiments ± standard deviation. 43

Figure 12. Effect of the enzyme:GF ratio on FOS enzymatic production. Sugars profile obtained in assays with total volume of 15 mL and (a) 2, (b) 3, (c) 4, (d) 5 and (e) 6 mL of FFase from *A.ibericus*. Experiments carried out at 50 °C, pH 5 and 150 rpm for 24 h. Fru (red), Glc (orange); GF (yellow); GF_2 (dark blue bar), GF_3 (light blue bar), total FOS (green). Results correspond to the average of two independent experiments ± standard deviation. 44

Figure 13. Effect of temperature in the enzymatic treatment of a fruit preparation, by addition of 5 mL of FFase extract. Experiments carried out for 30 h with 150 rpm of agitation at (a) 40, (b) 50 and (c) 60 °C. Fru (red), Glc (orange); GF (yellow); GF_2 (dark blue bar), GF_3 (light blue bar), total FOS (green). Results correspond to the average of two independent experiments ± standard deviation. 46

Figure 14. Total FOS produced by enzymatic treatment for 24 h, 150 rpm at 40, 50 and 60 °C by addition of 5 mL of FFase extract. GF_2 (dark blue bar) and GF_3 (light blue bar). Results correspond to the average of duplicate experiments ± standard deviation. Statistical analysis was performed by one-way ANOVA. *$p<0.05$ 47

Figure 15. Effect of ratio enzyme:fruit preparation on FOS enzymatic synthesis performed at 50 °C and 150 rpm for 24 h. The volume (mL) of FFase extract addition was evaluated for a total mass of 15 g: (a) 4, (b) 5 and (c) 6. Fru (red), Glc (orange); GF (yellow); GF_2 (dark blue bar), GF_3 (light blue bar), total FOS (green). Results correspond to the average of two independent experiments ± standard deviation. 48

Figure 16. Influence of shear rate on the flow curve of strawberry preparation (a) non-treated and (b) treated and the influence of shear rate on the viscosity of strawberry preparation (c) non-treated and (d) treated. A three steps program (up-down-up) are performed, which corresponds to blue, red and green curves, respectively. Results correspond to the average of two independent experiments ± standard deviation. 51

LIST OF TABLES

Table 1. Sugars contents of some fruits, in grams per 100 grams of fruit, namely glucose (Glc), fructose (Fru) and sucrose (GF) (Adapted from Matthews *et al.*, 1987) [3]. 2

Table 2. Functional food categories according to the chemical composition (Adapted from Birch *et al.*, 2019) [25]. 5

Table 3. Different enzymatic treatments of fruit-based products used to convert the free sugar content of fruits into prebiotics. 11

Table 4. FOS synthesis by enzymatic treatment: conditions used and yields obtained (Adapted from Martins *et al.*, 2019) [88]. 19

Table 5. Proportions of volumes of GF solution and FFase crude extract. 27

Table 6 Nutritional information per 100 g of strawberry preparation provided by FRULACT. 28

Table 7. Proportions of mass of strawberry preparation and enzyme extract volume used in experiments. 29

Table 8. Summary of organoleptic characterization of strawberry preparation. Results correspond to the average of two independent assays ± standard deviation. 31

Table 9. Sugar's profile of extracellular FFase extract from *A. ibericus* performed at 37 °C, pH 6.2 at optimum fermentation time (38 h). Results correspond to the average of thirteen independent experiments replicas ± standard deviation. 34

Table 10. FFase activity determined for all intracellular extracts obtained by ultrasonication. The enzyme activity was measured in duplicate for each treatment and the present results are the average ± standard deviation. 36

Table 11. Summary of organoleptic and functional characterization of strawberry preparation before and after enzymatic treatment performed at 50 °C, 150 rpm for 24 h by addition of 5 g of FFase. Results correspond to the average of two independent assays ± standard deviation. 50

List of Abbreviations

a_w	Water Activity
DP	Degree of Polymerization
FFase	β-fructofuranosidase
Fru	Fructose
FTase	Fructosyltransferase
FOS	Fructo-oligosaccharides
GF	Sucrose
GF_2	1-Kestose
GF_3	Nystose
GF_4	1F-fructofuranosylnystose
GI	Gastrointestinal
Glc	Glucose
GOS	Galacto-oligosaccharides
IMO	Isomalto-oligosaccharides
LAB	Lactic Acid Bacteria
MOS	Malto-oligosaccharides
SCFA	Short-Chain Fatty Acids
TSS	Total Soluble Solids
XOS	Xylo-oligosaccharides

1. STATE OF ART

1.1 FRUIT-BASED PRODUCTS

Fruits are known to contain essential components for human diet such as nutrients, minerals, vitamins, fibres, among others. The main component of fruit is water, with contents varying between 70 – 95 % depending on the type of fruit and degree of ripeness [1].

Carbohydrates is the second most abundant nutrient, representing more than 90 % of the dry matter of fruits. Carbohydrates are the main source of energy in the human diet. Upon ingestion, carbohydrates are directly used to cover immediate energy needs or transformed into fat for energy storage. Regarding fruits carbohydrates metabolism, there is a translocation of sucrose (GF) from the leaf and peel which leads to an accumulation of sugars that are stored as starch. During maturation, the starch is converted mainly into glucose (Glc), fructose (Fru) and GF, which are mostly associated to the characteristic sweet taste of fruits. Hence, the abundance of these sugars varies according to the type of fruit and its degree of ripeness [1]–[4]. Examples of fruits sugars contents are presented in **Table 1**.

Regarding protein and fat, its content in fruits is low. Proteins play essentially regulatory and plastic roles in the human body because they are a good source of amino acids. Furthermore, proteins are structural components of all cells, being necessary to build and repair tissues, synthesize enzymes, hormones, among others. Fats besides being an energy source, assists as a carrier of fat-soluble vitamins and of some of the bioactive compounds present in fruits, such as phytoestrogens and carotenoids [1], [5].

In addition to the essential nutrients mentioned above, fruits provide a variety of bioactive compounds such as vitamins, dietary fibres, minerals, among others. These compounds are commonly known for their antioxidant activity and ability to eliminate free radicals, which leads to beneficial health effects. Thus, since human beings are not able to synthesize these bioactive compounds, their food supply is indispensable. Factors such as genotype, climate, cultural practices, maturity, harvesting methods and post-harvest management directly influence their concentration in the fruit [1], [2].

Vitamins are complex organic substances essential in small amounts for the normal functioning of the body. In recent years, great interest has been focused on antioxidant vitamins (A, C, and E), particularly because of their role in coronary heart disease and cancer risk reduction. Vitamin A is commonly present in fruits as pro-vitamin A carotenoids, which are precursors to the diet. For example, mango and papaya are good sources of this vitamin due to the high level of β-carotene accumulation. Vitamin C is found in

high quantities in citrus fruits, but other fruits, including kiwi, papaya, and strawberries, also contain this vitamin in a varied amount. Vitamin E naturally occurs in low quantities in fruit [5].

Table 1. Sugars contents of some fruits, in grams per 100 grams of fruit, namely glucose (Glc), fructose (Fru) and sucrose (GF) (Adapted from Matthews *et al.*, 1987) [3].

Fruit	Glc	Fru	GF	Total
Core Fruits				
Apple	2.3	7.6	3.3	13.3
Pears	1.9	6.4	1.8	10.5
Pip Fruits				
Apricots	1.6	0.7	5.2	9.3
Cherries, Sweet	8.1	6.2	0.2	14.6
Cherries, Sour	4.2	3.3	0.5	8.1
Mango	0.7	2.9	9.9	14.8
Nectarines	1.2	0	6.2	8.5
Peach	1.2	1.3	5.6	8.4
Plum	2.7	1.8	3.0	7.5
Citrus Fruit				
Grapefruit	1.3	1.2	3.4	6.2
Oranges	2.2	2.5	4.2	9.2
Lemon	1.0	0.8	0.6	2.5
Limes	0.2	0.2	0	0.4

Fruit	Glc	Fru	GF	Total
Berries				
Blackberries	3.1	4.1	0.4	8.1
Blueberries	3.5	3.6	0.2	7.3
Grapes	6.5	7.6	0.2	18.1
Raspberries	3.5	3.2	2.8	9.5
Strawberries	2.2	2.5	1.0	5.8
Melons				
Cantaloupe	1.2	1.8	5.4	8.7
Watermelon	1.6	3.3	3.6	9.0
Tropical Fruits				
Banana	4.2	2.7	6.5	15.6
Figs	3.7	2.8	0.4	6.9
Guava	1.2	1.9	1.0	6.0
Kiwi	5.0	4.3	1.1	10.5
Pineapple	2.9	2.1	3.1	11.9
Pomegranate	5.0	4.7	0.4	10.1

Fibres play a regulatory and protective role in the human body, being effective against diseases of the gastrointestinal (GI) tract, circulation related diseases, metabolic diseases, among others. The most common fibres in fruits are cellulose, hemicelluloses, and pectin polysacharides. For example, pectin can be modified, which leads to changes in the nutritional value, texture, rheology and palatability of the final product. Hence, these compounds have a relevant role in technological processes [6].

Minerals have several functions with high importance. Their performance as cofactors in enzymatic and metabolic processes, mainly by magnesium, and their important role in bone density, mainly by calcium, stands out within their roles. The most abundant mineral in fruits is potassium, followed by base-forming elements calcium and magnesium and the acid-forming element phosphorous. Also, manganese, zinc, iron, copper, and sodium are present in small amounts [2], [5].

Thus, the inclusion of fruits in the daily diet is fundamental to improve human health and well-being. Although, considering the high total sugar content of most fruits, their consumption should be moderate.

In food industry, fruits are used as food components in several sectors, such as dairy, pastry, confectionery, canned, frozen and distilleries. They are not only used as fresh, whole or chopped form, but also in preserves, jams, jellies, dehydrated or crystallized products, juices, concentrates, among others [7].

The general production of fruit-based products is presented in **Figure 1**. The process is initiated by a post-harvest treatment that aims to preserve the freshness of the raw material. Then, primary processing is carried out, resulting in semi-finished products such as paste, puree, pulp and dry products. Subsequently, secondary products can be manufactured depending on market demand such as juices, jam, ice cream, dairy and confectionery products, muesli, among others [8].

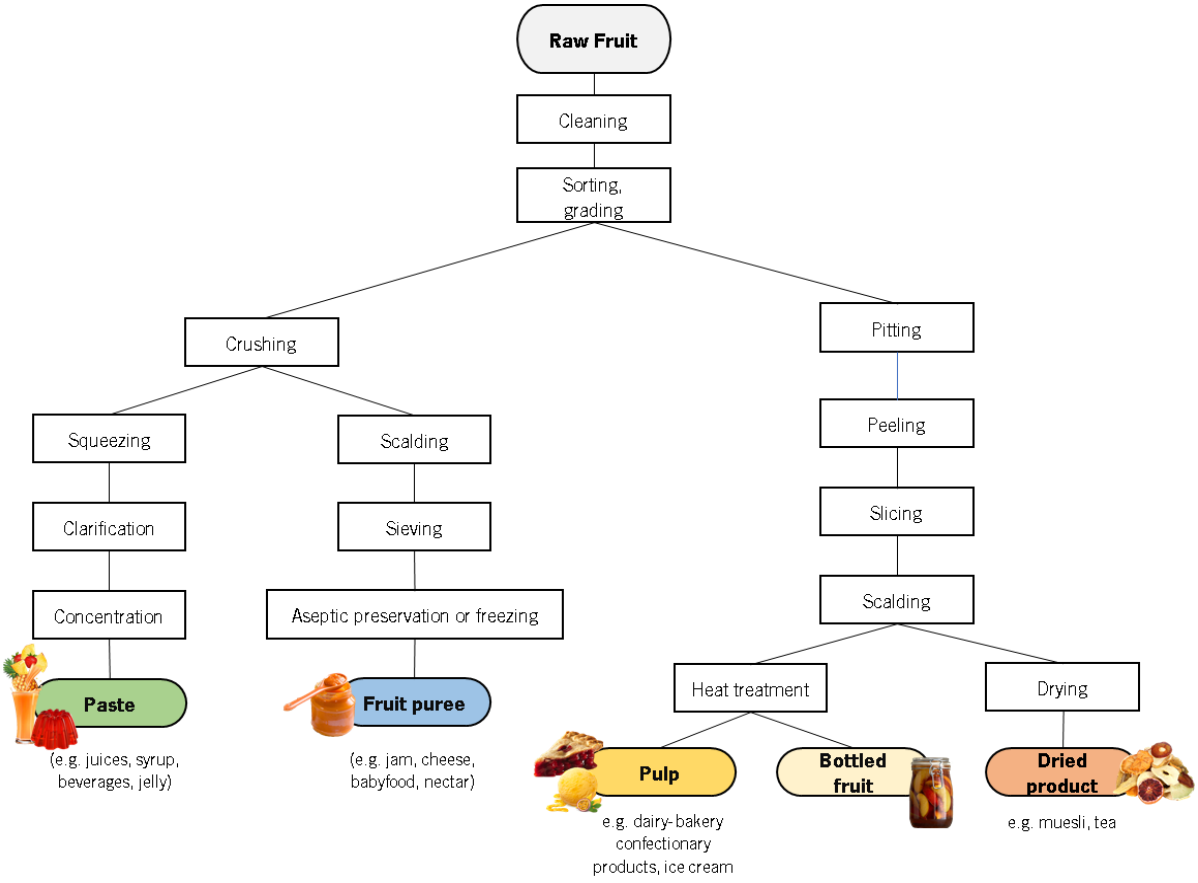


Figure 1. General scheme of fruit processing (Adapted from J. Barta, 2012) [8].

Traditionally, preservatives, acidulants, antioxidants, food colours, flavours, sweeteners, hydrocolloids, emulsifiers, among others food additives are added to the food during the fruit processing, to improve

shelf-life, cost efficiency, nutrition and sensory properties, such as colour, aroma, taste and texture. However, these additives are considered unnatural and unhealthy, which negatively influences the acceptance of the product by the consumers [9], [10].

Over the years, the demand for health-promoting foods, in addition to their nutritional quality, has been increasing. Health and well-being are clearly two of the priorities in everyone's daily life. Thus, one of the major concern's health associated with the consumption of fruit-based products is the high intake of sugars derived from the native sugars content of the fruit, along with the sugars added during processing.

Since 1975, the incidence of obesity has tripled worldwide, with sugar overconsumption being one of the main contributors [11], [12]. High sugar intake is also associated with an increased risk of cancer, oxidative stress, and inflammation, as well as cognitive deficiencies, variations in neuroplasticity, and emotional disorders [13]–[17]. To prevent all these health-related risks, the World Health Organization (WHO) recommends reducing the consumption of free sugar to less than 10 % of the total energy intake, which corresponds to 50 g for a healthy person who needs to consume 2,000 calories per day. Additional health benefits can be promoted if the reduction would achieve less than 5 % of the total energy [18].

The reduction of sugar consumption has been stimulated by several strategies, such as the implementation of food taxes, campaigns, labelling and nutrition education [19], [20]. However, more effective results have been verified through food reformulation, micronutrient fortification or functionalization of products [21].

In the last few decades, it has been developed new strategies to balance the free sugar content in fruit-based products, *i.e*, to reduce the sugars that naturally occur in fruits and also the sugars added by the manufacturer. One of the most interesting strategies is the development of functional foods, by reducing the sugar level of the products and providing additional health benefits simultaneously.[22].

1.2 FUNCTIONAL FOOD

Functional food is a food targeted to a specific function in the body, aiming at promoting human health. The functionality is assigned by the increase of a specific physiological response and/or by the decrease of the disease risk. At the same time, the food provides energy and nutrients to the host [23].

Functionalization of food was clearly designed as a strategy to counterbalance a set of health concerns, nutritional anxieties, and other distresses. In most developed countries the increasing spread of chronic non-infectious degenerative diseases, also known as XXI century's diseases, have been mostly related with the Western diet and worldwide lifestyle, like diabetes, overweight, cancer, irritable bowel disease,

Alzheimer and cardiovascular and immune functions. Although the concept of functional food has been introduced in the earliest 80s, just in the most recent decades its consume has been highlighted [24].

Functional foods can be classified according to the degree of modification of their chemical composition, as represented in **Table 2**.

Table 2. Functional food categories according to the chemical composition (Adapted from Birch *et al.*, 2019) [25].

Type of functional food	Definition
Natural products	Native high concentration of nutrients or components.
Altered products	Undesirable and/or harmful components have been removed, reduced or replaced with a well-promoting substance.
Fortified products	Incorporation of new nutrients such as minerals and vitamins.
Enriched products	Addition of unusual nutrients such as prebiotics and probiotics.
Enhanced products	Component enhanced through optimization of growing conditions, new feed formulations, genetic manipulation or other methods.

The functional food market has been growing over the past decades and was estimated at USD 161.49 billion in 2018. During the forecast period from 2019 to 2025, a compound annual growth rate of 7.9 % is expected. Among functional ingredients, probiotics and/or prebiotics became ubiquitous in the marketplace over the past decades, due to the health benefits evidence provided on gut health through dietary modulation of the human gut microbiota [25].

1.3 PROBIOTICS AND PREBIOTICS

The definition of probiotic is clear since 2001 and consists in "Live microorganisms which when administered in adequate amounts confer a health benefit on the host". The bacteria that have been mostly used in functional foods and dietary supplements, due to its probiotic properties, are the Bifidobacteria and some strains of Lactic acid bacteria (LAB) [26].

The concept of prebiotic has been modified over the years. In 2017, it was defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit". The definition englobes all health-promoting bacteria with a selective metabolism. Furthermore, it also redirected the prebiotic applications to the entire GI tract, including microbial communities present in the urogenital tract, skin, and even in the upper digestive tract. The expansion of the concept also covers the use of prebiotics by animals, which implies that *in vivo* experiments may be conducted according to the target host. Fructo-

oligosaccharides (FOS), galacto-oligosaccharides (GOS), inulin and lactulose are known as confirmed prebiotics since their functionalities have been extensively shown by *in vivo* evidence. Notwithstanding, pectin, cellulose, resistant starch, xylo-oligosaccharides (XOS), isomalto-oligosaccharides (IMO), malto-oligosaccharides (MOS), raffinose, polyphenols, gluco-oligosaccharides, and β-glucans appear as promising prebiotic candidates [27].

The mechanism of action by prebiotics on host health is presented in **Figure 2**. Prebiotics promote a direct inhibition of several pathogenic bacteria and cancer cells, reduction of cholesterol and the risk of cardiovascular diseases, prevent obesity and constipation, among others. While indirectly, they also provide nutrients and nourishment to the gut microbiota contributing to the maintenance of the gut health [28], [29].

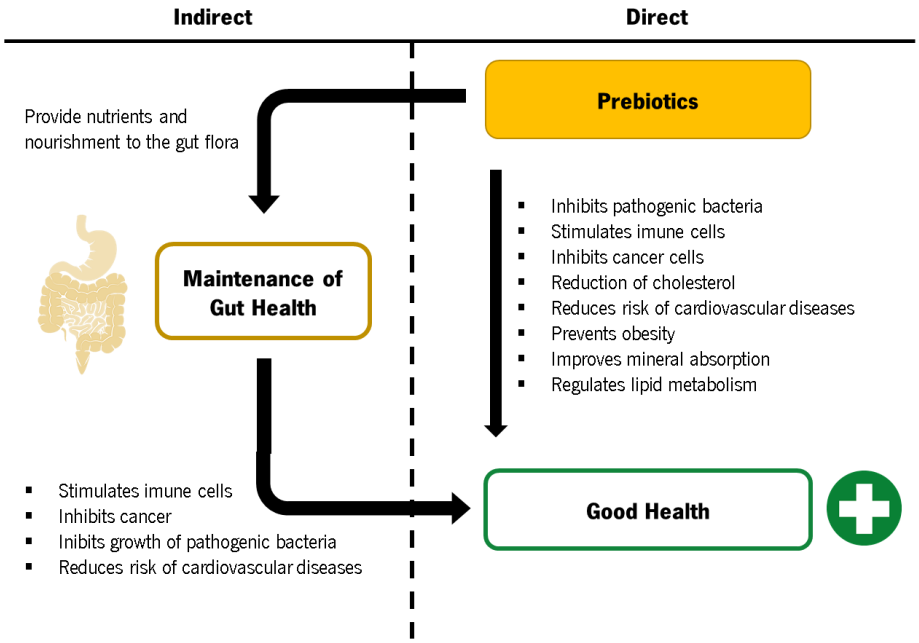


Figure 2. Schematic representation of the direct and indirect mechanism of action by the prebiotics on host health (Adapted by Mohanty et. al.2018) [29].

It is important to consider all the advantages and disadvantages when making a decision on the application of probiotics and prebiotics. The application of probiotics has several disadvantages essentially related to viability issues, limiting the range of suitable food matrices. Viability is compromised by environmental stresses, food processing steps including heating, the probiotic metabolites resulted from storage such as acids, as well as GI conditions essentially due to gastric acid and bile salts. In addition, the input of bacteria in the human intestine, which are not necessarily native, may promote some competition by colonization sites among other residents. Due to this fact, the restoration of probiotic bacteria levels might be compromised. In turn, prebiotics are not digestible throughout the GI tract

reaching the gut as a portion of food for the existing microbiota. Consequently, it has applicability in a wide range of food products such as dairy and bakery products, beverages, breakfast cereals and bars, some confectionery items, among others. Thus, the intestinal microbiota can be more efficiently modulated with the incorporation of prebiotics in the food than probiotics [30]–[32].

1.3.1 Prebiotics as food ingredients

Considering all the evidence related to the beneficial health effects promoted by prebiotics, their incorporation in food has been explored over the years.

FOS are considered food ingredients by law, instead of additives. Due to their low energy are commonly used as a fat replacer. FOS are known to improve properties such as product shelf-life, organoleptic characteristics, in addition, to enable browning reduction [33], [34]. They are water-soluble, highly hygroscopic and have a light sweetness (40 to 60 % of GF), which is reduced in FOS with longer chain. Notwithstanding, FOS are less stable than other prebiotics to low pH and high temperatures, which can lead to the partial hydrolyzation of the β-(2→1) glycosidic bonds. Its decomposition showed a first-order kinetic rate for FOS and GF hydrolysis, but with the much lower activation energy for FOS, indicating that less energy is required for prebiotic hydrolysis [35], [36]. In food industries, FOS are mainly used in beverages (tea, coffee, fruit drinks and alcoholic beverages), dairy products (fermented milk, ice cream and instant powders), also in light jam and confectionery products [37]. They provide crispness to low-fat cookies, decreases the freezing point in frozen desserts, contribute to give moisture to soft baked products and body to dairy products, and acts as a binder in nutritional or granola bars providing a similar effect of sugar, but with the added benefit of fewer calories, fibre enrichment, among other nutritional properties [38].

GOS are applicable as food ingredients due to their low glycaemic index, low calories, the ability to form colourless solutions, non-carcinogenicity, solubility, and good stability under acidic pH values and high temperatures [31], [39], [40]. For example, the resistance of 99 % of GOS in acidic fruit juices (pH 2.7 – 4.1) when subjected to a pasteurization process, which implies the use of high temperatures ranging between 88 – 95 °C, has been reported [41]. GOS have a light sweetness (40 % as sweet as GF), thus, they have been used as sugar substitutes. They can be easily incorporated in a wide range of food product such as infant nutrition, dairy products, beverages and bakery products [42].

IMO have high moisture retention, low water activity, light sweetness (60 % as sweet as GF), low viscosity, no residual flavour, and structural stability when subjected to low pH conditions and moderately high temperature and are non-digestible by yeasts. Considering the mentioned characteristics, IMO's are

suitable to replace sugar or fat, to provide organoleptic functionality, or even to provide a slow release of energy [43], [44].

XOS have only recently received considerable attention and consequently are less explored as a food ingredient. Nevertheless, they have shown stability to both acidity and heat in food processing, allowing their application in low pH food products. Moreover, it was reported that XOS are 0.25 times sweeter than 5 % GF solution [45].

1.3.2 Incorporation of prebiotic in fruit-based products

Regardless of the methodology used, to include the prebiotic in the fruit-based matrix, the prebiotic stability has to be maintained during its processing. In general, fruit processing is carried out under high temperatures, low pH, or a combination of both, which stimulates Maillard reactions and consequently have a negative impact in the final prebiotic content [46].

The functional profile of the final product is fundamental to recognize it as a prebiotic or not. A minimal oral dose of 3 g per day is required to have prebiotic functionality. A formulation that contains a lower prebiotic content is not recognized as prebiotic, unless it proves the selective metabolism of the microbiota and promotes a health effect [27].

Furthermore, good organoleptic and functional characteristics of the food are essential to obtain a well-accepted product by consumers. Significant changes that impair nutritional, textural, sensory, and rheological parameters compared to the original product are undesirable to guarantee consumer acceptance [22], [46].

The compatibility between prebiotics and product ingredients in terms of physicochemical, sensory, nutritional, and functional properties is an essential analysis to obtain optimized product formulations.

The physicochemical profile can be characterized by total soluble solids (TSS), organic acids, and colour, for example. TSS and organic acids are linked to the sensory properties of the fruit, and the colour is the first quality parameter evaluated by consumers and it is critical to product acceptance [47], [48].

In terms of the nutritional properties, parameters as the ascorbic acid content, total phenolics compounds, antioxidant activity, vitamins, and others need to be evaluated by comparison of the values obtained with the original and final product.

Sensory analysis of the final product is usually performed by a group of panellists specialized in the matrix under investigation with the aims to detect changes in organoleptic characteristics. Notwithstanding, the

aroma of the product is related to the volatile fruit profile, which consists in a complex mixture of hundreds of volatile organic compounds [49].

The incorporation of prebiotics in fruit-based products may follow two different methodologies: 1) addition of the prebiotic carbohydrate to the food matrix – fortification or 2) *in situ* syntheses of the prebiotic in the food.

1.3.2.1. FORTIFICATION

The fortification of fruit-based products involves the addition of the purified prebiotics to the formulation. Inulin and FOS have been the most studied prebiotics in terms of the impact on physicochemical and organoleptic characteristics. They are also the best accepted by the consumers.

Several studies report successful fortification, without significantly altering the overall quality of the product. Pineapple, mango and orange juices were successfully fortified by the addition of approximately 4 g of FOS to 100 g of juice [50]. The shelf-life of the product was studied after 4 and 6 months' storage, at ambient and refrigerated temperature. At the end of 6 months storage, a significant amount of FOS was retained in the fruit juice beverages stored at refrigerated temperature ($\approx$ 2 $g_{FOS}/100\ g_{juice}$) in comparison with those stored at ambient temperatures ($\approx$ 0.5 $g_{FOS}/100\ g_{juice}$). It was also observed constant pH, TSS, titrable acidity and viscosity of the fruit juice beverages along with the storage, which indicated no spoilage of the product due to microbial or enzymatic reaction.

Similar results were obtained with other formulations, regarding sensory liking and adequacy of chemical parameters. The successful formulations consist of additions of 6 g of sugar and 6 g of inulin to 100 g of product, or with additions of 8 g of sugar, 2 g of inulin and 2 g of FOS to 100 g of product. However, the authors verified that, although preference mappings showed that functional nectars were as popular as nectars containing only sugar, increasing the proportion of sugars led to the increase of the taste, sweetness and overall acceptability of the nectars [51]. Similar results were reported to apple and orange juices fortified with FOS for 2 months and storage at a refrigerated temperature [52]. Besides the good acceptance of sensory, physicochemical and microbial characteristics, it was found that the taste and odour were improved by the increasing amount of sugar and FOS added, respectively.

Considering these results, in combination with the high cost of FOS, fortification through the combination of sugar and FOS has been suggested to obtain a better flavour of the food and also low costs on the process at industrial scale. Nevertheless, it is important to keep in mind that the proportions of sugar and

FOS must be optimized for each product, and that the final formulation must have the minimum amount of prebiotics required for the product be considered as a functional food [51], [52].

As an alternative to sugar, a functional formulation of mango nectar with stevia as a low-calorie natural sweetener, and inulin as a textured prebiotic, has been investigated [53]. The complete replacement of GF by stevia and inulin was able to compensate the GF omission defects on the rheological characteristics of the product. The optimum formulation determined, where no changes on physicochemical and organoleptic characteristics were identified, was 6 % (w/w) inulin and 3 % (w/w) stevia.

On the other hand, a functional peach yoghurt drink was successfully formulated with 740 g/kg of skimmed or whole milk yoghurt, 246 g/kg of mashed peach syrup and 14 g/kg of FOS. The incorporation of prebiotics revealed a general intensification of aroma, flavour, and mouthfeel in the final formulation. The whole milk drink containing FOS, was the consumer's preference compared to skimmed ones, due to the richer mouthfeel and sweet and fruity flavours [54].

Recently, the incorporation of XOS in a fruit-based product has been studied. An optimized formulation with 5 % (w/w) XOS concentration resulted in minimal changes in rheology, colour, water activity (a_w), pH and TSS as compared to the original product. This supplementation with XOS may also be an equally viable solution to increase the dietary fibre intake of consumers [55].

1.3.2.2. *IN SITU* SYNTHESIS

The mechanism of prebiotic *in situ* synthesis includes an enzymatic action. The reaction converts the sugars of the product into prebiotic carbohydrates. Consequently, there is a reduction in the sugar levels of the final product and its caloric value, besides to the acquired functional properties. All of this, coupled with the fact that prebiotic purification steps are eliminated, makes this strategy more nutritionally and economically attractive compared to fortification [46]. Finished fruit juices, pulps and preparations have been used as raw materials of the developed functional juices [56]. **Table 3** provides a summary of the outcomes of recent studies on enzymatic conversion of fruit-based products sugar into prebiotics.

Table 3. Different enzymatic treatments of fruit-based products used to convert the free sugar content of fruits into prebiotics.

Enzyme source	Prebiotic	Fruit-Based product	T (°C)	pH	Reaction Time (h)	Amount of product	Enzyme amount	Prebiotics concentration	Conversion yield (%, w/w)	DP [d]	Reference
Microorganism											
Dextransucrase of *Leuconostoc. mesenteroides* B512F	IMO	Acerola juice	30	5.2	24	25 mL (1:2)	1 IU/mL	130.77 g/L	87.18 [a]	3-12	[57]
		Lemon juice	30	5.2	24	10 mL	0.5 IU/mL	94.81 g/L	63.21 [a]	up to 11	[58]
		Orange juice	30	5.2	24	Diluted (1:7)	1 IU/mL	130.17 g/L	86.78 [a]	3 - 7	
		Pineapple juice	30	5.2	24	Diluted (1:2)	1 IU/mL	141.10 g/L	94.06 [a]	3 - 6	[59]
		Melon pulp	30	5.2	24	Diluted (1:2)	1 IU/mL	138.52 g/L	92.35 [a]	3 -10	
		Orange juice	30	5.2	24	800 mL	0.05 IU/mL	11.55 g/L	7.70 [a]	3-8	[60]
		Cashew apple juice	30	5.2	24	10 mL	0.5 IU/mL	104.73 g/L	69.82 [a]	2-5	[61]
	MOS and IMO	Mandarin juice	16	3.6	7	n.i	1 U/mL	45.00 g/L	50.70 [a]	2-7	[62]
		Mandarin concentrate	16	3.3	c	n.i	3 U/mL	315.70 g/L	46.60 [a]	2-7	
		Orange juice	30	3.7	2	n.i	3 U/mL	28.10 g/L	35.00 [a]	2-5	[63]
		Orange concentrate	30	3.4	2	n.i	3 U/mL	157.40 g/L	31.70 [a]		
Dextransucrase of *Weissella cibaria*	IMO	Mango concentrate	30	5.4	24	2 mL	200 µl	14.96 g/L	12.00 [a]	2-5	[64]
		Pineapple concentrate	30	5.4	24	2 mL	200 µl	11.12 g/L	9.88 [a]	2-5	
Commercial											
Glucansucrase GTF180	IMO	Apple concentrate	50	4.5	1,5	20 mL	14.47 U/g GF	103.30 g/L	61.86 [b]	2-3	[65]
		Orange concentrate	50	4.5	1,5	20 mL	14.47 U/g GF	90.90 g/L	30.61 [b]	2-3	
Viscozyme L	FOS	Longan pulp	55	6.0	2	50 g	300 mg	49.49 mg/g	28.40 [a]	2-3	[56]
			55	6.0	5	50 g	300 mg	61.59 mg/g	35.06 [a]	2-3	

(a) Yield: Prebiotics produced / Total sugars ; (b) Yield: Prebiotics produced / initial GF concentration; (c) Until GF consumption reaches 95 %; (d) DP: Degree of polymerization

Most of the reports used a dextransucrase (EC 2.4.1.5) to produce IMO, MOS. Enzymes of LAB, from the genera *Lactobacillus*, *Leuconostoc*, *Streptococcus*, *Pediococcus* and *Weissella*, have been used.

Dextransucrase uses GF as a substrate, releases Fru, and transfers Glc to certain acceptors, linking α-(1→6) -glucosyl residues to form dextrans. Galactose, isomaltose and maltose are the acceptors to synthesise GOS, IMO and MOS, respectively. Maltose has shown the best results as an acceptor, providing an effective synthesis [66]–[68].

Several works report the use *Leuconostoc mesenteroides* B512F to produce dextransucrase. Fermentation is run in fed-batch, at 30 °C, pH 6.5, with a mechanical agitation of 150 rpm and aeration of 0.5 L/min. The produced enzyme is then applied to fruit juices. The optimum parameters for oligosaccharides synthesis have been reported as 30 °C and pH 5.2 [57]–[61]. Dextransucrase from *Weissella cibaria* was used for IMO production in a juice sugar, following two different strategies. In the first addressed strategy, the enzyme was produced by incubating the microorganism for 12 h at 20 °C and pH 6.9 with 180 rpm of agitation [64]. After enzyme purification, 200 µL of the pure enzyme was added to the mango and pineapple juice concentrates with pH adjusted to 5.4, followed by incubation for 24 h at 30 °C. On a second strategy, IMO was synthesized by 10 % (v/v) *W. cibaria* (10 M) inoculated in juice containing malt extract and GF, at 35 °C and pH 5.5, for 24 h [69].

The treatments with dextransucrase generally achieved a conversion of up to 60 % (w/w) with very low concentration of residual sugars after enzymatic action due to sugar consumption for oligosaccharide formation [57]–[59], [61]. An oligosaccharide concentration higher than 130 g/L was obtained in studies performed with 1 IU/mL of enzyme, 75 g/L of initial concentration of GF and 75 g/L of initial concentration of reduced sugars [57], [59]. The same initial concentration of GF and reduced sugars leads to lower concentrations of prebiotics, around 100 g/L, using the half amount of enzyme in lemon and orange juice [58], [61]. Other works have been reported lower conversion yields such as Almeida *et al.*, 2015 reported 7.77 % (w/w) in orange juice and Baruah *et al.* 2017 around 12.00 % (w/w) and 9.88 % (w/w) in mango and pineapple juice, respectively [60], [64]. Although these yields were significantly smaller, the final juice contained the necessary amount of prebiotics to be considered as a functional beverage. The low concentration found in orange juice might be justified by the degradation of prebiotics during atmospheric cold plasma and ozone treatments.

Significant differences in the prebiotic's concentrations were also observed for processes that use concentrated or non-concentrated juice. E.g., a higher concentration of GOS have been reported in concentrated juices [62], [63]. Since concentrated juices also have a higher initial sugar concentration, those results were expected.

IMO production was equally effective when Glucansucrase GTF180 was applied in apple and orange concentrates. Around 3 h of enzymatic treatment were enough to produce 103.30 g/L and 90.90 g/L of oligosaccharides in apple and orange matrix, respectively. More than 95 % of the intrinsic GF was converted and the main products were leucrose, isomaltose and isomaltotriose. The enzyme was inactivated during standard fruit juice pasteurization conditions, and the oligosaccharides showed stability during the process, presenting good potential for industrial applications [65].

The degree of polymerization (DP) of the oligosaccharides produced demonstrated a noticeable dependence on the initial concentration of sugars. It has been reported that an initial concentrations of 75 g/L of GF and 75 g/L of reducing sugar allowed the synthesis of 104.73 g/L of prebiotics containing DP below 5, while initial concentrations of 25 g/L of GF and 75 g/L of reducing sugar resulted in 53.49 g/L of oligosaccharides with DP up to 12 [61]. This fact suggests that a lower concentration of GF promotes a higher elongation of prebiotics. Nonetheless, Fontes *et al.*, 2015 observed the synthesis of oligosaccharides with different DP in melon, pineapple and orange juice when enzymatically treated under the same initial concentrations of sugars [59]. Thus, the chain length of the oligosaccharides formed is not only affected by the initial sugar concentration but also depends on the food matrix.

Some fruits are characterized by low sugar content and, to favour the oligosaccharide production, external GF and reducing sugars, mainly Glc and Fru, have been added to the juices [70]. This was described for acerola, lemon, pineapple, cantaloupe melon, orange and cashew apple juice, where the higher prebiotics formation was obtained when 75 g/L of reducing sugar and 75 g/L of GF were applied [58], [61], [71].

Overall, a successful *in situ* synthesis of prebiotics, behind allowing the synthesis of a sufficient amount of prebiotics for the juice to be considered a prebiotic formulation, must maintain the sensory characteristics and value of the original product. That mentioned can be achieved by having a good understanding of the interaction of the enzyme with the food matrix and the prebiotics produced with the matrix ingredients.

In contrast to what has been described for the fortification method, the synthesis *in situ* of fructans in fruit-based products has rarely been described. Possibly the reason is that fructans, namely FOS, is considered less stable than other oligosaccharides [35]. Nevertheless, an improvement of processing characteristics, namely yield, clarity, and TSS, as well as carbohydrate composition changes, were reported to a longan juice where FOS were enzymatically produced *in-situ*. A commercial enzyme from *Aspergillus. aculeatus*, Viscozyme L, containing a mixture of cellulases, glucanases, pectinases and β-fructofuranosidases (FFases) was applied in the juice pressing process. Significant amounts of two

different FOS, namely 1-kestose (GF_2) and nystose (GF_3), along with a significant decrease in GF content, around 80 %, was found after 5 h treatment with the enzyme, indicating that there was conversion of GF into FOS [56]. Regarding these results, the potential of enzymatically produce fructans in fruit-based products still has to be further investigated.

1.4 FRUCTANS

Fructans are carbohydrates characterized by containing chains of Fru units linked by β-glycosidic bonds. They are known to reach the colon without being hydrolysed by human digestive enzymes and are commonly metabolized by Bifidobacteria [72].

These non-digestible carbohydrates are widely present in nature, and depending on their source the molecular structure and weight can differ. According to the type of glycosidic bonds, fructans can be classified into three types: inulin, levan, and branched type. The inulin and levan types are characterized by containing mostly or exclusively (2→1) and (2→6) fructosyl-Fru linkages, respectively. The branched-type contained both linkages [73].

Natural sources have a small amount of fructans and their harvest is season limited, which is not suitable for industrial applications. Industrially, the biggest aim is to obtain greater yield and economic processes and, therefore, fructans are produced by the microbial source. The synthesis of fructans consists of the GF transfructosylation catalysed by microbial fructosyltransferases (FFase, EC 3.2.1.26 or β-D-fructosyltransferase (FTase), EC 2.4.1.9). The produced fructans are known as FOS and have been mainly assigned as inulin-type with a DP ranging between 2 and 4, namely GF_2, GF_3, and 1F-fructofuranosylnystose (GF_4), which chemical structure are represented in **Figure 3** [74], [75].

FTase has only transfructosylating activity and FFase contains both transfructosylating and hydrolytic activity. FFase liberates Glc from GF by cleaving the glycosidic bond β (2→1), and transferring the fructosyl group for a molecule of GF or FOS, as represented in **Figure 4** [76], [77].

Industrially, FOS have been produced via GF fermentation by purified enzymes in a two-step bioprocess. First step consists in microorganism growth and enzyme production, and the second step aims to synthesise FOS, with the purified enzymes, by transfructosylation reaction. The most studied microorganisms for FOS production are fungi belonging to the *Aspergillus*, *Penicillium* and *Aureobasidium* genera [78].

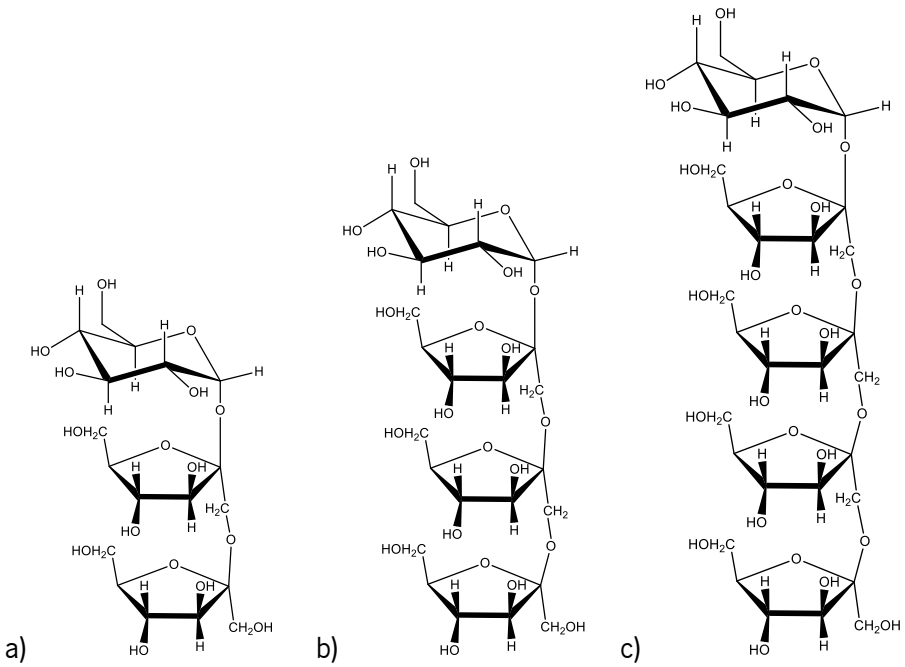


Figure 3. Chemical structure of (a) 1- kestose (GF_2), (b) nystose(GF_3), and (c) 1F-fructofuranosylnystose (GF_4) from ChemDraw 12.0.2. software.

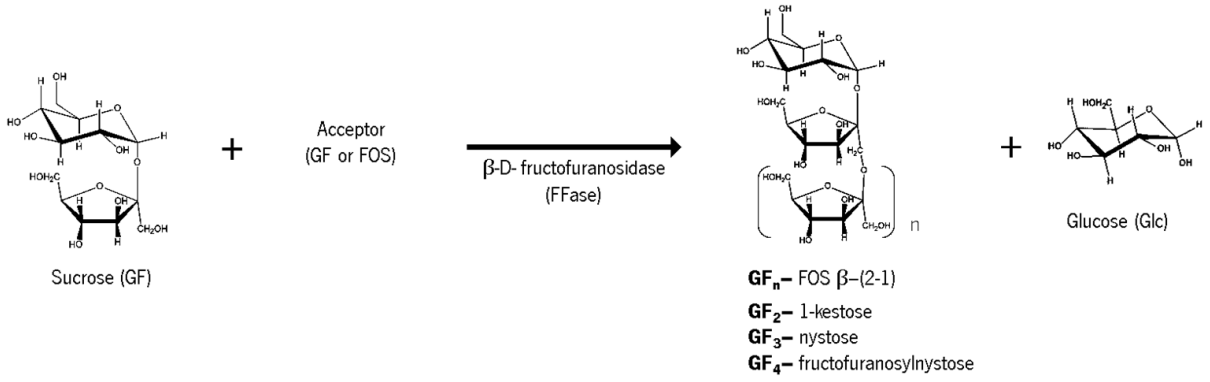


Figure 4. Scheme of transfructosylation reaction carried out by β-fructofuranosidase (FFase) enzymes to produce fructo-oligosaccharides (FOS) from ChemDraw 12.0.2. software.

The transfructosylating activity is inhibited by Glc, the main product generated during FOS synthesis. At the same time, FOS formed are hydrolysed back to their single monomer forms by the action of the same hydrolytic activity of the enzymes. The result is a maximum of 55 to 60 % (w/w) conversion yield of GF to FOS. Thus, final fermentative broth contains high quantity of non-prebiotic sugars, such as Glc, Fru and GF [78], [79].

Our research group has been applying different strategies to obtain FOS at higher yield and purity. Recently, a co-culture fermentation with *Aspergillus ibericus* and *Saccharomyces cerevisae* YIL162 W was

successfully used to produce FOS. FOS were simultaneously produced by the *A. ibericus* and purified by the yeast. *S. cerevisae* YIL162 W consumed both F and G released during FOS synthesis, increasing the final FOS purity and also the FOS yield (since lowering G concentration avoided enzyme inhibition). Moreover, the yeast used had a knockout of the gene responsible for GF hydrolysis, which avoided competition by the substrate within the *A. ibericus*. Maximum FOS production was reached in fermentations conducted under 30 °C, at initial pH 6.0, aeration of 0.8 vvm and with an added 17 g/L of yeast extract. FOS conversion yield achieved was of 0.70 ± 0.00 g_{FOS}/g_{GF} and the purity of the final FOS mixture was greater than 90 % (w/w) [80].

1.4.1 FFase production

Over the years, the screening and isolation of FFase microorganism producers, with high activity, stability and end suitable products for industrial conditions, have been one of the main focuses of the scientific community [81].

The characteristics of these enzymes may vary according to the microorganism and the culture medium used, essentially the carbon source, since it may be responsible for the induction of the reaction. Hence, the physicochemical properties of the enzymes produced depend on the source of each enzyme [37], [82].

These enzymes can be produced intra and/or extracellular by a diversity of microorganisms, including bacteria and fungi [37]. The preferred carbon source is GF. Bacterial FFases generally have molecular weights between 45 and 64 kDa and the fungal ones have molecular weights between 60 and 75 kDa. Other enzymes with higher molecular weight have also been reported [38].

The FFase production has been performed using either submerged or solid-state fermentation and a high transfructosylating activity has been found in fungi belonging the *Aureobasidium* [83], *Penicillium* [84], [85] and *Aspergillus* [86] genera.

Aspergillus spp. have been receiving particular attention and have been mentioned as good FOS producers, especially *Aspergillus oryzae* and *Aspergillus niger*, which are regularly used in biotechnological processes for the production of enzymes, since they have GRAS status [82]. The *A. ibericus* FFase enzyme has not yet been isolated or even characterized, despite the very promising results showed in previous studies in our research group as a FOS producer, as mentioned above.

The temperature used in the fermentation process has proved to be a crucial parameter, especially when using cultures of fungi because it influences the germination of spores, growth of the microorganism and

the formation of the product. An adverse effect is obtained on the metabolic activity of microorganisms when subjected to high temperatures [87]. Mussatto *et al.* 2013 verified an improvement of *Aspergillus japonicus* FFase production by increasing the temperature from 26 to 30 °C, but an opposite effect under temperatures above 30 °C. Maximum FFase activity of 64.12 U/mL was obtained at 30 °C [86]. Similar results were obtained by Nascimento *et al.*, 2016, that also observed a decrease in FFase production for higher temperatures. The authors achieved a high activity (227.56 U/mL) for *Penicillium citreonigrum* FFase, when produced at 30 °C under 150 rpm of agitation. After optimization they predicted an activity of 301.84 U/mL at 25.5 °C, pH 6.5 for 67.8 h [84].

1.4.2 Production of FOS by enzymatic synthesis

Several parameters and how they interact with each other should be considered when producing FOS by enzymatic treatment. **Table 4** summarizes the enzyme source, reaction temperature, pH, time of reaction, substrate concentration, amount of enzyme, and the resulting FOS yield identified from published scientific works on this subject [88].

The temperature and pH have a big impact in reaction rates and it is also important to mention that the pH used in the reaction has a strong impact on the activity of the enzyme, as it influences the ionization state of the amino acids, consequently affecting the primary and secondary structure of the enzyme [89].

The enzymatic reaction, as shown in **Table 4**, can be performed in a wide range of temperatures (35-70 °C) and pH values (3-7), depending on the enzyme source. Notwithstanding, more limited ranges can be defined since a large number of studies have placed the ideal temperature and pH to maximize the synthesis of FOS in the range of 40–60 °C and 4.5–6.5 respectively [90]. According to Vega *et al.*, 2012, which applied temperatures between 45 and 60 °C, the increase in the reaction temperature leads to an increase in the reaction rate [91]. Similar results have been obtained by other authors [92]–[95]. Lorenzoni *et al.*, 2014, also mentioned the importance of considering that using temperatures higher than 60 °C can promote a significant negative effect on enzyme activity due to thermal damage [95].

Regarding the substrate concentration, as shown in **Table 4**, it should ideally be greater than 400 g/L. According to Romano *et al.*, 2016, high initial concentration of GF promotes the production of shorter FOS, such as GF_2, GF_3 and GF_4, with low production of Glc. In contrast, lower substrate concentrations promotes the production of larger FOS and higher Glc concentration [110].

Most of the FOS yields obtained did not exceed 60 %. This is mainly due to the presence of Glc formed as a by-product of the FOS synthesis reaction, which inhibits the transfructosylation reaction as explained above.

At the commercial level, most enzyme preparations have a low price, versatility and high stability under conditions of industrial reaction processes. However, the non-prebiotic sugars continue to be produced as a result of their action [88].

Table 4. FOS synthesis by enzymatic treatment: conditions used and yields obtained (Adapted from Martins *et al.*, 2019) [88].

Enzyme source	T(°C)	pH	GF (g/L)	Enzyme amount	Reaction time (h)	$Y_{FOS\ max}$ (%, w/w) [a]	Reference
Microorganisms							
Aspergillus.	37	5.4	100,300,500	0.2,0.56,0.96 U/mL [d]	24	65-69	[96]
Aspergillus japonicus TIT-K J1							
A.japonicus	50	5	450-700	5.75 g.cell/100 mL	4	51-59	[97]
Aspergillus aculeatus	50-70	4.9-6.4	200-600	20-100 U/mL [d]	4-24	55 % DP3 [f];43 % DP4 [f]	[90]
Aspergillus niger	55	6	100,300,600	0.66 U/mL [d]	88	55-45	[98]
A. niger IMI 303386	39	6.5	500	0.4 U/mL [d]	72	62	[99]
A. niger AS0023	50	5.8	500	5×10^{6}KU	5	62 [f]	[100]
Aspergillus oryzae	55	5-6	600	0.14 (V/V) culture/GF	4-24	55	[101]
	60	5.5	750	275 U/gGF [e]	7	57	[102]
Saccharomyces cerevisiae (invertase)	40–55	5.5	210–850	0.5-0.8 U/mL [d]	8	10 %(d.b.)[c]	[77]
A. niger; Aspergillus awamori, S. cerevisiae	40	5	500	6 U/g GF [e]	9-72	50–37 [f]	[103]
A. niger, Aureobasidium pullulans.	50-65	4-8	700	1:9 (w/w) cell GF	8	35-38	[93]
Aureobasidium melanogenum 11-1	50	4.5	300	117 U/g GF [e]	7	66	[104]
Rhodotorula sp.	50	4.5	500-700	5 UFT/mL	96	50-59	[105]
	48	6	500	0.022 U/mL [d]	48-56-72-96	44-60	[106]
Cryptococcus sp.	50	4.5	500	1 FTA/mL	48	34	[107]
Bacillus subtilis natto CCT 7712	35-55	7.7	200-400 [g]	n.i [b]	12-36	388 mg/mL[h]	[108]
Levansucrase SacB of B. subtilis	37	6	90 [i]	1.47 U/mL [d]	24	54	[109]
Penicillium oxalium	50	5.5	500	2 U/mL [d]	6	45	[85]

Table 4. Continue

Enzyme source	T(°C)	pH	GF (g/L)	Enzyme amount	Reaction time (h)	$Y_{FOS\ max}$ (%, w/w) [a]	Reference
Commercial							
Rohapect CM (ABenzyme GmbH)	45-60	5.5-6.5	530-720	3.4-7.4 UT/mL [i]	3 and 5	41-64	[92]
Viscozyme L (Blumos SA)	55	5.5	100-720	56 FU/mL [j]	6	65-85	[110]
Viscozyme L (Novozyme)	50	5.5	600	1.230 UT/mL [i]	6	59-64	[95]
Pectinex Ultra SP-L and Rapidase TF	60	5.6	630	0.3 U/mL [d]	144	62 [f]	[111]
25 enzyme preparation from fungal strains	45-60	5-6	400-800	4.2-15 UT/mL [d]	6	59-64	[91]

(a) Y_{FOSmax}: Maximal yield of FOS conversion;

(b) n.i.: not informed;

(c) d.b.: dry basis;

(d) U/mL: One unit of enzyme activity; the amount that produces 1 μmol of reduced sugar per minute/ mL of a reaction volume;

(e) U/g: One unit of enzyme activity; the amount that produces lμmol of reduced sugar per minute/g of dry support;

(f) Informed yield: weight percentages of total sugar;

(g) Substrates: GF, sugarcane molasses and sugarcane juice;

(h) Yield informed as amount of DP4 produced;

(i) Substrates: GF and GF analogues;

(j) Substrates: maltose or GF;

(i) UT/mL: transfructosylation activity/ mL of reaction volume

(j)FU/g: FTase units/ mL of reaction volume

1.5 FOS SAFETY, DOSE AND HEALTH BENEFITS

FOS are present in the traditional diet as they are constituents of some foods such as fruits (e.g. bananas, tomato and apples), vegetables (e.g. garlic, onion, asparagus, lettuce, leek, artichoke and beetroot), cereals (e.g. wheat, rye, barley and oats), honey, beer, among others [34]. Therefore, their intake shouldn't be a problem. Moreover, several studies have been conducted in animals and humans to assess its effects *in-vivo*, and no evidence has been found regarding possible toxicological, carcinogenic or genotoxic effects. These oligosaccharides are officially recognized as natural food ingredients in most of the European countries and have got a GRAS status [112].

A daily intake of 4 g of FOS per day is the amount necessary to increase the levels of Bifidobacteria in the human intestine [113]. However, since FOS are not digested by body enzymes, a high intake of FOS can cause a laxative effect similar to that in lactose intolerants. The laxative effects seem to be related and dependent on the daily intake regime. It should be noted that the intestinal acceptability of non-digestible fermentable carbohydrates is characteristic of each person [114], [115].

The use of FOS as food ingredient is increasingly growing due to their physicochemical and physiological properties that promote health benefits to consumers [116]. Among the health benefits promoted by FOS are an antipathogenic effect, improvement of intestinal health, immunomodulatory effect, reduction of cardiovascular diseases and absorption of minerals. The antipathogenic effect refers to the inhibition of human and animal weight pathogens after the competitive exclusion, reduction of ulcerative colitis damage and increment in short-chain fatty acids (SCFA) resultant from the fermentation of FOS in the large intestine, *e.g*, acetate, propionate and butyrate. The improvement of intestinal health is achieved by the stimulation of health-promoting bacteria, such as Lactobacilli and Bifidobacteria. In turn, the immune system can be modulated by ingesting FOS as it provides the modulation of immune responses in lymphoid tissue associated with the intestine. This leads to increased production of anti-inflammatory cytokines, increased activity of natural killer cells, and increased production of antibodies in peripheral blood. Moreover, cardiovascular diseases are reduced by increasing the plasma concentration of ferulic acid, thereby decreasing Glc and lipid levels and inducing satiety. There is also a reduction of low-density lipoproteins coupled with an increase in high-density lipoprotein [27], [117], [118]. Finally, the absorption of mineral ions is promoted by lowering the pH in the colon due to the intake of FOS. Mostly, calcium (Ca^{2+}) absorption is promoted, which can contribute to healthier bones and help prevent osteoporosis.

Magnesium absorption (Mg^{2+}) is also promoted and can positively influence cardiovascular health [101], [119].

2. GOALS

Overconsumption of sugar has proven to be a problem for public health, being one of the main contributors to obesity and to the increased risk of number diseases. To overcome the problem, several strategies have been applied, such as the functionalization of products. Within this sphere, the functionalization of fruit-based products has been applied, since they are daily consumed by people of all ages, and reducing their sugar content can be crucial.

The functionalization of fruit-based products can be accomplished through the incorporation of prebiotics. FOS are well-known prebiotics that can be incorporated into fruit-based preparations or juices, through *in situ* synthesis catalysed by FTase or FFase. This treatment involves the bioconversion of the sugars contained in the product into prebiotics. Thus, in addition to the reduction of sugars that occur naturally in food or that are added by the manufacturer, ingredients that promote beneficial health effects are also included.

Previous results obtained in our research group demonstrated the potential of *A. ibericus* as a FOS producer, but the potential of the pure *A. ibericus* enzyme (FFase) for FOS enzymatic synthesis has not yet been evaluated.

Thus, the main goal of this dissertation is the development of a new functional fruit-based product through the conversion of GF content of commercial fruit preparation into FOS by the application of FFase from *A. ibericus*.

In order to successfully achieve the main goal, the following specific aims were established:

- Production of *A. ibericus* FFase enzyme - optimization of the operational conditions, including fermentation time, temperature and pH.
- Characterization of the enzyme extract – identification of the enzyme as extra or intracellular.
- Production of FOS by *A. ibericus* FFase - optimization of operational conditions including reaction time, temperature, pH and ratio enzyme:GF solution to maximize FOS enzymatic synthesis;
- Synthesis *in situ* of FOS in a fruit preparation by *A. ibericus* FFase – optimization of operational conditions including reaction time, temperature and ratio enzyme:product to maximize FOS enzymatic synthesis and reduction of the caloric value of the product;
- Characterization of the fruit preparation – identification of processing effects in the fruit preparation properties including pH, a_w, TSS, sugars, colour and rheology

3. MATERIALS AND METHODS

3.1 FFASE PRODUCTION USING A *A. IBERICUS* CULTURE

3.1.1 MICRORGANISM AND CULTURE CONDITIONS

The fungus *A. ibericus* (MUM 03.49) was obtained from the *Micoteca da Universidade do Minho* (MUM) culture collection. Stock cultures were maintained at – 20 °C. The strain was revived from frozen glycerol stock solutions on Petri dishes containing malt extract agar (MEA) medium (g/L): malt extract (20), G (20), agar (20) and peptone (5) and grown at 30 °C. A concentrated spore suspension was prepared by scrapping the spores with a 0.1 % (w/v) solution of Tween 80 from a 7-day-old culture plate (**Figure 5**). The spore concentration of the suspension was determined using an improved Neubauer chamber and afterwards adjusted to 1×10^{7} spores per mL.

Figure 5. Culture plate of *Aspergillus ibericus* MUM 03.49.

3.1.2 FFASE PRODUCTION IN SHAKE FLASKS

The production of FFase pursued an adaptation of the method described by Nobre *et al.*, 2018. The inoculum of *A. ibericus* (MUM 03.49) was prepared in a 250 mL flask, containing 100 mL of fermentation medium with the following composition (g/L): $NaNO_3$ (5), KCL (0.5), K_2SO_4 (0.35), $MgSO_4$ (0.5), KH_2PO_4 (4), $FeSO_4.7H_20$ and GF (200). Culture medium was then seeded with 1 mL of the spore suspension solution (1×10^{7} spores per mL). Fermentation was conducted at 37 °C and 150 rpm for a maximum of 62 h. Samples were collected at 0, 14, 20, 25, 38, 43, 48 and 62 h [120]. Samples were filtered through a 0.2 µm cellulose acetate membrane before sugars analysis and FFase activity determination (sections 3.1.4 and 3.1.5, respectively).

A fermentation was then run to produce FFase. The fermentation was run until maximum FFase activity, the fermentation time was optimized. Then, the fermentative broth was filtered (0.2 µm), and the extract without cells was used as extracellular enzyme source.

3.1.3 Extraction of intracellular FFase enzyme by ultrasonication

The extraction of intracellular FFase was performed as described by Ganaie & Gupta, 2014 [121]. The final broth resulted of fermentation performed at optimized fermentation time was centrifuged at 6000 g for 20 min at 4 °C in Multifuge X3R centrifuge (Thermo Scientific). Compact pellet cells were resuspended in 50 mL of cold distilled water. Afterwards, the cell suspension was subject to ultrasonication using a high-intensity ultrasonic horn (20 KHz, Sonic& Materials Vibrocell CV33, 3 mm diameter titanium microtip) immersed at a depth of 3 cm of sample.

The experiments were carried out at 40 % amplitude, acoustic power of 20 W or 40 W for an irradiation period of 3, 6, 9 and 12 min. The amount of energy to be applied in each treatment was defined by following **Equation 1** where E, corresponds to energy (J), P to acoustic power (W) and Δt to irradiation time (min).

$$\textbf{(1)}\ E = P \times \Delta t$$

The cell suspension was kept in a salt ice bath during disruption to prevent overheating. The ultrasonic energy was pulsed 0.4 s active and passive intervals for reduction of free radical formation. At the end of ultrasonication, the cell-free lysate was centrifuged at the same conditions mentioned above. Samples of each treatment were collected for FFase activity determination. The extract with the maximum activity was used as intracellular FFase extract in further experiments.

3.1.4 Sugar analysis

Samples were analysed by high-performance liquid chromatography (HPLC) with refractive index (RI) detector for sugars quantification. The HPLC system was performed with a Nexera X2 UHPLC (Shimadzu Corporation, Japan) coupled with the RI detector K-2300 (Knauer, Germany). The chromatographic signal was recorded and further integrated using the software Lab Solutions (Shimadzu Corporation, Japan). The separation was performed with an Asahipak NH2P-50 4E (4.6 mmI.D. x 250 mm) column (Shodex, Japan) linked to an Asahipak NH2P-50G 4A (4.6 mm I.D. x 10 mm) pre-column. Samples were eluted with a mixture of acetonitrile in pure water, containing 0.04 % of ammonium hydroxide (68:32, v/v) (HPLC Grade, Sigma Aldrich, Germany). The elution was conducted at a flow rate of 1 mL/min and 30 °C. FOS standards were acquired from Wako (Japan). Standards of GF, Fru and Glc were obtained from VWR (Belgium).

3.1.5 FFase activity

The FFase activity was determined according to the method described by A. Flores-Maltos *et. al.*, 2019 [122]. The reaction mixture was composed of 100 µL of crude extract with 500 µL of 0.6 M GF solution in 0.1 M acetate buffer at pH 5 and 400 µL distilled water. Then, the mixture was incubated for 20 min in a water bath at 30 °C. At the end of incubation, the reaction was stopped by heating the mixture for 5 min in boiling water. The unit of FFase activity (U) was defined as the amount of enzyme required to release 1 mol of Glc per minute. The enzyme activity (U/mL) was determined using **Equation 2**, where C_G is the concentration of Glc (µg/mL), V_T is the total volume of reaction mixture (mL), MM_G is the molar mass of G (µg/µmol), t is the time of reaction(min), V_E the enzyme volume (mL) and df represents the enzyme dilution factor.

$$\textbf{(2)}\ Enzyme\ activity\ (U/mL) = \frac{C_G \times V_T}{MM_G \times t \times V_E} \times df$$

3.2 Optimization of enzymatic synthesis of FOS by A. ibericus FFase extract

3.2.1 Application of intracellular and extracellular FFase extract in GF solution

Experiments were performed at 50 °C and 150 rpm of agitation for 27 h with 300 g/L of initial GF concentration. Reaction mixture included 5 mL of the FFase extract and 10 mL of GF solution in 0.1 M acetate buffer (pH 5). At the end of incubation, the reaction was stopped by heating the samples for 10 min in boiling water. Samples were collected at 0, 3, 6, 9, 12, 24, 27 h, diluted and filtered at 0.2 µm for sugars analysis. Only the FFase extract that showed more promising results was used in the following experiments.

3.2.2 Effect of initial GF concentration

The effect of initial GF concentration in FOS enzymatic synthesis was determined for 100, 200, 300 and 400 g/L of GF. Simultaneously, the optimum reaction time was defined. Tests were carried out with 5 mL of FFase extract and 10 mL of GF solution in 0.1 M acetate buffer (pH 5), at 50 °C and 150 rpm of agitation for 27 h. At the end of incubation, the reaction was stopped by heating the samples for 10 min in boiling water. Samples were collected at 0, 3, 6, 9, 12, 24, 27 h and were further diluted, and filtered at 0.2 µm for sugars analysis.

3.2.3 Effect of Temperature

The effect of temperature in FOS enzymatic synthesis was studied at 40, 50 and 60 °C. Experiments were conducted with 150 rpm of agitation. Reaction mixture included 5 mL of the FFase extract and 10 mL of GF solution in 0.1 M acetate buffer (pH 5). The optimized initial GF concentration and reaction time was used. At the end of incubation, the reaction was stopped by heating the samples for 10 min in boiling water. Samples were collected and further diluted, when necessary, and filtered at 0.2 μm for sugars analysis.

3.2.4 Effect of pH

The effect of pH in FOS enzymatic synthesis was determined in a range between 3.6 and 6.4, with variations of 0.7 of unity per assay. For preparation of the reaction mixture, 5 mL of FFase crude extract and 10 mL of a GF solution in 0.1 M citrate buffer (pH 3.6), acetate buffer (pH 4.3 and 5) or sodium phosphate buffer (5.7 and 6.4) were added into a 50 mL shake flask. Tests were carried out at 150 rpm agitation, under the optimum conditions selected in the previous studies, such as initial GF concentration, temperature and reaction time. At the end, the reaction was stopped by heating the samples for 10 min in boiling water. Samples were collected and further diluted, when necessary, and filtered at 0.2 μm for sugars analysis.

3.2.5 Optimization of the enzyme:GF solution ratio

The enzyme:GF solution ratio was optimized to maximize FOS enzymatic synthesis. Experiments was performed with 150 rpm of agitation at optimum temperature, pH, initial GF concentration and reaction time. The reaction mixture consisted of the FFase crude extract and the GF solution in 0.1 M appropriated buffer, in proportions established in **Table 5**. Reaction was stopped by heating the samples for 10 min in boiling water. Samples were collected and further diluted and filtered at 0.2 μm for sugars analysis.

Table 5. Proportions of volumes of GF solution and FFase crude extract.

Volume of GF solution (mL)	Volume of FFase extract (mL)
13	2
12	3
11	4
10	5
9	6

3.3 ENZYMATIC SYNTHESIS OF FOS IN A FRUIT-BASED PRODUCT

3.3.1 FRUIT-BASED PRODUCT

Pasteurized strawberry preparation was provided by FRULACT, which is specialized in the production and development of stabilized preparations, based on fruits and vegetables, cereals and seeds, functional ingredients, savoury, among others. This is a competitive company that anticipates market trends and creates new products with added value in order to satisfy customer needs. Nowadays, the company is present on three continents, with nine business units in five countries (Portugal, Morocco, France, South Africa and Canada). Multinationals such as Nestlé, Danone, Yoplait and Unilever are some of the collaborators of this company [123].

The fruit preparation was divided into samples of 50 mL and refrigerated at -20 °C. Before application, the sample was refrigerated overnight at 4 °C.

The nutritional information per 100 g of fruit preparation was provided by FRULACT (**Annex II**) and are presented in **Table 6**.

Table 6 Nutritional information per 100 g of strawberry preparation provided by FRULACT.

Per 100 g of product	
Energy	298 kcal / 1267 kJ
Lipids	0.1 g
Saturated fat acid	< 0.1 g
Carbohydrate	75 g
Sugars	70 g
Protein	0.3 g
Salt	0.09 g

3.3.2 EFFECT OF TEMPERATURE ON THE FOS SYNTHESIS

The effect of temperature in FOS enzymatic synthesis was studied at 40, 50 and 60 °C. Experiments were conducted with 5 g of FFase crude extract and 10 g of fruit-based product, in a shake flask (50 mL), at 150 rpm of agitation for 30 h. At the end of incubation, the reaction was stopped by heating the samples for 10 min in boiling water. Samples were collected at 0, 6, 12, 24, 30 h and were further diluted and filtered at 0.2 µm for sugars analysis. Simultaneously, optimum reaction time was defined.

3.3.3 Effect of enzyme:product ratio on the FOS synthesis

The effect of enzyme:product proportion in FOS enzymatic synthesis was studied at optimum temperature and reaction time and 150 rpm of agitation, using the FFase extract and fruit-based product in proportions established in **Table 7**. At the end of incubation, the reaction was stopped by heating the samples for 10 min in boiling water. Samples were collected and further diluted and filtered at 0.2 μm for sugars analysis.

Table 7. Proportions of mass of strawberry preparation and enzyme extract volume used in experiments.

Mass of fruit preparation (g)	Volume of FFase extract (mL)
11	4
10	5
9	6

3.4 Characterization of the functional fruit preparation

A functional fruit-based product was produced by application of a FFase crude extract at optimal conditions. Samples before and after enzymatic treatment were analysed for several parameters, namely, pH, colour, a_w, sugars, TSS contents and rheology.

3.4.1 pH

The pH of samples was measured using a pH meter HI 2210 (Hanna Instruments). The pH measurements were done in triplicate using buffers with pH 4.0 and 7.0 as reference.

3.4.2 Water activity (A_w)

The a_w was analyzed, in triplicate, by an AquaLab 4TE Dew Point water activity meter (METER Group Inc, Pullman, WA, USA) after an equilibrium at 25 °C.

3.4.3 Total Soluble Solids (TSS)

TSS, expressed in °Brix (total soluble solids g/100 g) were determined, in triplicate, by a portable refractometer HI96801 (Hanna Instruments), using distilled water as the reference.

3.4.4 Sugars analysis

The content of FOS, Fru, Glc and GF were determined by HPLC as described in section 3.1.5.

3.4.5 COLOUR ANALYSIS

Colour of fruit preparation samples was measured according to Pathare *et al.*, 2013, using a colourimeter CR-400 (Konica Minolta) [48]. The colourimeter was calibrated against white calibration plate using the D65 illuminant (Y = 93.9; x = 0.3133; y = 0.3193). The colour measurements were done in triplicate using a low reflectance glass sample cup and rotating the sample at three different positions. Samples were measured in a dark background using CIE-Lab colour space. The parameters L* (whiteness or brightness/darkness), a* (redness/greenness), and b* (yellowness/blueness) were determined and total colour difference (ΔE) was calculated using **Equation 3**.

$$\textbf{(3)}\ \Delta E = \sqrt{\Delta a^{*2} + \Delta b^{*2} + \Delta L^{*2}}$$

Also, were calculated by **Equation 4 and 5** the quantitative and qualitative attributes of colour, chroma (c) and hue angle ($h°$), respectively. The $h°$ was expressed in degrees in which the 0° angle corresponds pure red, 90° pure yellow, 180° pure green and 270° pure blue.[48].

$$\textbf{(4)}\ c = \sqrt{a^{*2} + b^{*2}}$$

$$\textbf{(5)}\ h^{\circ} = tan^{-1}\left(\frac{b^*}{a^*}\right)$$

3.4.6 RHEOLOGY ANALYSIS

Rheological measurements were carried out in triplicate at 25 °C in a TA Instruments HR-1 rheometer equipped with a Peltier plate (TA Instruments, New Castle, DE). Flow curves were obtained for the fruit-based product samples using a cone-plate (60 mm, 2° angle, truncation 64 μm) and performing a three steps program (up-down-up) using a continuous ramp and shear rate range between 1 and 500 1/s.

The rheological behaviour of fruit preparation described by the flow curves was fitted to the Ostwald–de Waele model. The model is represented by the **Equation 6**, where τ is shear stress (Pa), $\dot{\gamma}$ is a shear rate ($1/s$), K is the consistency coefficient ($Pa.s^n$), and n is the flow behaviour index (dimensionless).

$$\textbf{(6)}\ \tau = \mathrm{K}(\dot{\gamma})^{\mathrm{n}}$$

3.5 STATISTICAL ANALYSIS

All the experiments are presented with the means and respective standard deviations. Data were compared using two-way ANOVA and one-way ANOVA followed by a Tukey's multiple comparison test using GraphPad Prism 6.0 software (GraphPad Software, California, USA) to estimate significant differences ($p<0.05$) among treatments with a confidence level of 95 %.

4. RESULTS AND DISCUSSION

4.1 CHARACTERIZATION OF FRUIT PREPARATION

This dissertation consisted of the development of a functional product by incorporation of prebiotics in a strawberry preparation. Therefore, it was essential to perform the nutritional, physicochemical and functional characterization of the initial product. Due to consumer acceptability, the occurrence of significant changes in the product is undesirable.

The parameters evaluated were pH, a_w, TSS, carbohydrates and colour and the results are summarized in **Table 8**.

Table 8. Summary of organoleptic characterization of strawberry preparation. Results correspond to the average of two independent assays ± standard deviation.

		Results
pH		3.77 ± 0.01
Water activity -a_w		0.687 ± 0.002
Total soluble solids - TSS (Brix°)		75.8 ± 0.2
Carbohydrates (g/L)	Glc	181 ± 17
	Fru	260 ± 10
	GF	467 ± 15
Colour	L*	29.42 ± 0.09
	a*	4.2 ± 0.2
	b*	5.85 ± 0.02
	c*	7.2 ± 0.1
	h°	0.95 ± 0.02

The pH value is crucial for food stability and preservation and depending on several factors such as cultivars, maturity, seasonal variations, geographical areas and processing conditions. This parameter can affect microbial growth, enzymes behaviour and some food properties such as colour, flavour, and texture [124]. Regarding the enzymes, each one has an ideal pH range and outside this range, activity is reduced. Extreme pH values can cause denaturation of the enzymes and therefore lead to activity losses [89].

The a_w is an essential parameter in preventing or limiting moisture migration. It is usually responsible for the stability of the food, modulating the microbial response and consequently determining the type of microorganisms found in food products [125].

TSS was expressed in °Brix, which is a scale based on the amount of light refract when passing through a liquid. High-density solutions promote greater light refraction and, consequently, a higher Brix value is obtained. Carbohydrates, minerals, amino acids, among others biological substances can increase the density of the solution [126].

The determination of the carbohydrates profile was imperative to monitor the enzymatic action of FFase throughout all procedures by changes in different sugars, including Glc, Fru, GF and FOS.

Colour is the first quality parameter evaluated by consumers, and it is critical to product acceptance [48]. Furthermore, most of the changes related to the modification of colour in processed fruits are associated with enzymatic and non-enzymatic reactions, as well as the degradation of pigments. Therefore, the colour determination by instrumental measurements is important not only for the description of colour change but also as a source of useful information for quality control of food products [127].

The sample specification sheet provided by FRULACT, presented in **Annex II**, informed that fruit preparation used in the present study was composed by 34 % of GF, 16.65 % of strawberry pulp, 15.4 % of Glc syrup, 4.4 % of glycerol (E422), pectin (E440), anthocyanin (E163), citric acid (E330), trissodic citrate (E5331) and enough water to reach 100 %. Furthermore, some physicochemical information was also provided. The sample was characterized by pH 3.6 ± 0.2, Brix° 76 ± 2 and density of 1.40 ± 0.02. The pH value, TSS and carbohydrates profile observed for original product corroborate the information provided by the company.

In the following experimental procedures, all these parameters will be taken into account.

4.2 FFase production by *A. ibericus*

4.2.1 Extracellular FFase

In a previous work performed by our research group, *A. ibericus* showed promising results as FOS producer. Hence, the production of FFase was tested for this strategy under optimal temperature and pH previously described for shake flasks, 37 °C and pH 6.2, respectively [120]. Sugar's profile obtained during fermentation is presented in **Figure 6**.

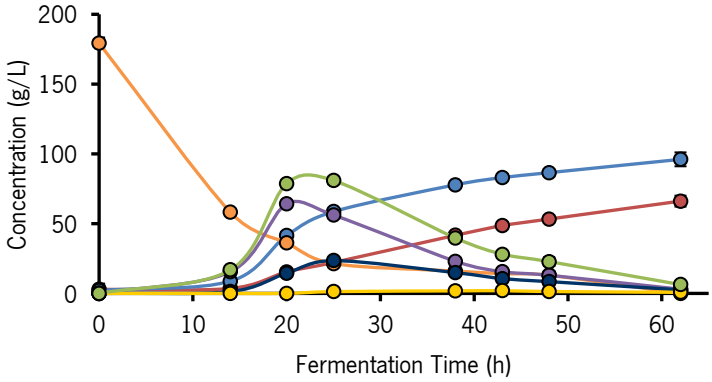


Figure 6. Shake flask fermentation of sucrose by an *A. ibericus* monoculture performed at 37 °C, pH 6.2 and 150 rpm: Fru (red), Glc (light blue); GF (orange); GF_2 (purple), GF_3 (dark blue) and GF_4 (yellow), total of FOS (green). Results correspond to the average of two independent experiments ± standard deviation.

The substrate, GF, was quickly converted to FOS until the maximum concentration of produced FOS was attained, at 25 h of fermentation. At this point, the concentration of total FOS obtained was 81 ± 3 g/L, where GF_2, GF_3, and GF_4 concentrations were, respectively, 56 ± 2, 23.5 ± 0.6, and 1.30 ± 0.04 g/L. A FOS conversion yield of 0.45 ± 0.01 g_{FOS}/g_{GF} was achieved with a purity of 44.2 ± 0.3 % and the productivity of the process was 3.2 ± 0.1 g_{FOS}/L.h.

For similar fermentation conditions, a maximum FOS production yield of 0.53 ± 0.03 g_{FOS}/g_{GF} was reported, with the content of 101 ± 8 g/L of FOS and a purity of 50.8 ± 0.9 % [120]. Results obtained in the present study were slightly lower because the fungus inoculum was seeded at half concentration of the spore suspension that was previously used. Nevertheless, results obtained were still in line with results obtained in the literature for other *Aspergillus* strains [128].

Until the end of fermentation, the high amount of G produced as a by-product of the transfructosylation reaction led to the consumption of GF at a considerably lower rate and, consequently to the promotion of FOS hydrolysis. Thus, Fru and Glc content in the medium increased significantly. At the end of the fermentation, all the GF was consumed, but only 6.3 ± 0.6 g/L of FOS was obtained associated with a concentration of monosaccharides corresponding to 96 % (w/w) of the total sugars. A fermentation profile identical was previously observed by our research group for the same microorganism [129].

Considering that one of the specific objectives of this work was to obtain the FFase extract from *A. ibericus*, the enzyme activity was determined for each point of fermentation in order to optimize fermentation time with maximum activity. The results are present in **Figure 7**.

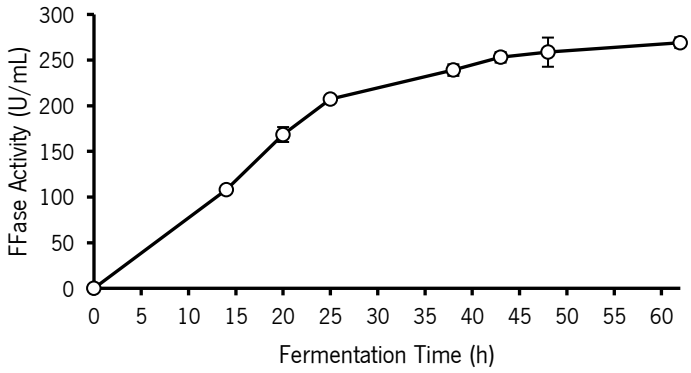


Figure 7. Activity profile of β-fructofuranosidase (FFase) from *A. ibericus* produced in shake flasks using a monoculture strategy. Results correspond to the average ± standard deviation of two independent experiments.

Analysing the results, it can be observed that enzyme activity increases at a considerable rate up to 25 h of fermentation, where the maximum FOS production was obtained. Thereafter, the activity increased at a slower rate until 38 h fermentation obtaining an activity of 239 ± 6 U/mL. Until the end of fermentation, the activity was approximately constant. There were no statistical differences between enzyme activity obtained for 38, 43, and 62 h of fermentation.

These results allowed to establish 38 h fermentation as the optimal fermentation time for the production of FFase of *A. ibericus* with maximum activity. Subsequently, a large volume of FFase was produced at the ideal fermentation time, obtaining FFase extracts with 234 ± 9 U/mL with the profile of the sugar presented in **Table 9**. Enough enzyme extract was produced for all of the following experiments, which allowed a better comparison among results.

Table 9. Sugar's profile of extracellular FFase extract from *A. ibericus* performed at 37 °C, pH 6.2 at optimum fermentation time (38 h). Results correspond to the average of thirteen independent experiments replicas ± standard deviation.

Sugars	Concentration (g/L)
Fru	25 ± 5
Glc	66 ± 3
GF	24 ± 2
GF_2	60 ± 8
GF_3	26 ± 2
GF_4	1,5 ± 0.1

Several studies have reported the production of FFase from the genera *Aspergillus* and *Penincilium* with maximum activity below 80 U/mL, at temperatures ranging between 25-30 °C [130]–[134].

On the other hand, Nascimento *et al*. 2016 found a higher FFase activity (313.33 U/mL) of *Penicillium citreonigrum* for 72 h of fermentation in shake flasks, using a temperature of 30 °C and pH 5.0 and with partial purification [84]. However, the FFase produced by *A. ibericus* becomes more appealing. Approximately 75 % of FFase activity from *P.citreonigrum* can be achieved in FFase crude extract from *A. ibericus* resulted from a fermentation carried out during much less time than *P.citreonigrum* and without any purification step.

4.2.2 Intracellular FFase

Microbial intracellular FFase production has also been reported [100], [121], [130], [135], [136]. Thus, since promising results were obtained using the extracellular approach, it was decided to verify if there was intracellular FFase in *A. ibericus* and to evaluate its potential.

The extraction of an intracellular enzyme involves a method of cell disintegration, such as sonication and small beads. Sonication is used due to its low operating cost and simplicity of operation, without sophisticated equipment or extensive technical training. Currently, ultrasonication is the most applied method but despite the mentioned advantages, it also has disadvantages. The cavitation phenomenon, responsible for the disruption of cells, promotes chemical changes that can be harmful to the labile molecules released from the cells. In addition, denaturation can occur due to the use of high intensities and the inactivation of released products can be promoted by mechanical effects such as shear stress [136].

The present study evaluated the effects of ultrasonication on the released intracellular FFase, by variation of acoustic power and irradiation time as described by Ganaie & Gupta, 2014 [121]. The FFase activity was determined for each treatment in order to optimize the procedure parameters and to maximize enzyme activity. The results are presented in **Table 10**.

Most of the treatments led to an enzyme activity statistically equal. A statistical difference was verified for extract number 7 versus all extracts subjected to an acoustic power of 20 W, and extract number 5 as compared to extracts numbers 2 and 4. It is important to mention that the high error associated with each treatment may be related to the broth volume not removed after the first centrifugation in order to avoid loss of cell mass, as well as to the negative effects promoted by this cell disintegration method as mentioned above.

Table 10. FFase activity determined for all intracellular extracts obtained by ultrasonication. The enzyme activity was measured in duplicate for each treatment and the present results are the average ± standard deviation.

Extract number	Acoustic power (W)	Irradiation Time (min)	Energy (J)	FFase Activity (U/mL)
1	20	3	3600	75 ± 9
2		6	7200	79 ± 11
3		9	10800	75 ± 10
4		12	14400	77 ± 10
5	40	3	7200	62 ± 6
6		6	14400	68 ± 8
7		9	21600	59 ± 5
8		12	28800	70 ± 8

Analysing at an absolute level, the acoustic power of 20 W was more effective than 40 W, suggesting that a higher power is harmful to the enzyme, probably due to the phenomenon of cavitation, as discussed above.

The highest enzyme activity (79 U/mL) was obtained by the treatment performed at 20 W for 6 min, which corresponded to extract 2. Similar results were reported for intracellular FFase enzyme extraction from *A. niger* [121].

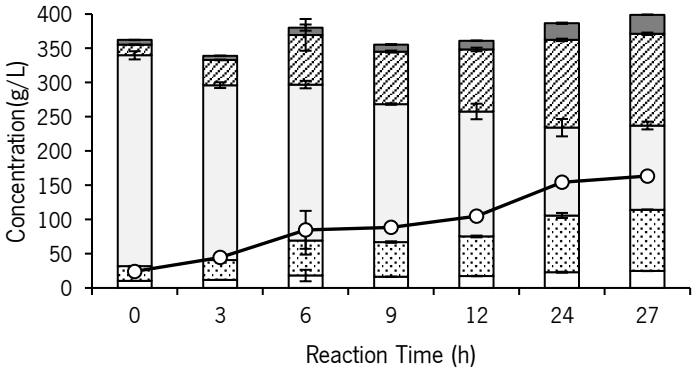
Considering the results, it was decided to use the extract 2 in the following experiments.

4.3 THE POTENTIAL OF FFASE EXTRACTS FOR THE SYNTHESIS OF FOS

The synthesis of FOS, catalysed by FFase from *A. ibericus* was evaluated. The potential of the intracellular and extracellular extract was evaluated under the same conditions. The composition of the reaction mixture, as well as the temperature (50 °C) and pH value (5) used, followed the same as the methodology previously used for the FFase of the *P. citreonigrum* [84]. An initial GF concentration of 300 g/L was used as initial carbon source considering the GF content of fruit preparation provided. The reaction profile obtained for extracellular and intracellular approaches is present in **Figure 8a)** and **8b)**, respectively.

The enzymatic treatment by extracellular extract (**Figure 8a**) showed an initial reaction mixture composed mostly of GF and also a small amount of Glc, Fru and FOS, which were present in the enzymatic extract used. Analysing the evolution of FOS production, in the first 6 h of reaction, the FOS synthesis was performed at a considerable rate.

a)



b)

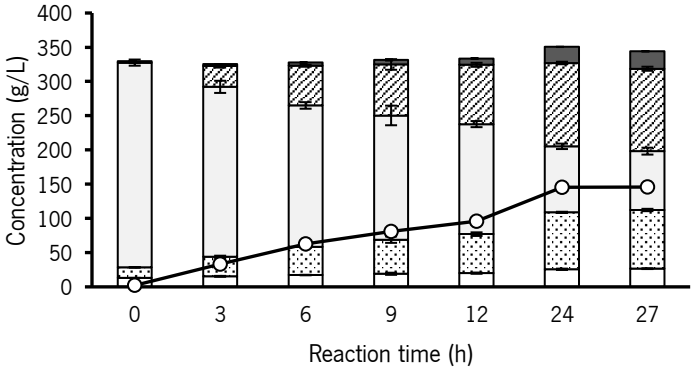


Figure 8. Sugars profile obtained for FOS enzymatic synthesis reaction performed at 50 °C, pH 5.0 and 150 rpm for 27 h by (a) extracellular and (b) intracellular FFase extract. Fru (white bars), Glc (dotted bars), GF (light grey bars), GF_2 (striped bars), GF_3 (dark grey bars), total FOS (○). Results correspond to the average of three independent assays ± standard deviation.

Then, between 6 and 24 h, the conversion rate was constant but much lower than initially, which may be the result of a possible inhibition by the Glc content produced by the transfructosylation reaction. A concentration of 54 ± 11 g/L of Glc was obtained after 6 h of enzymatic treatment. The FOS content was not statistically different after 27 h of reaction, which allowed to establish the 24 h as the optimum reaction time. After 24 h of reaction, 58 % (w/w) of the initial GF was consumed, with a formation of 153 ± 3 g/L of FOS, composed mainly of GF_2 (131 ± 2 g/L) with GF_3 (22 ± 1 g/L). A FOS conversion yield of 0.51 ± 0.03 g_{FOS}/g_{GF} was obtained. Also, a high concentration of non-prebiotic sugars (243 ± 17 g/L) was verified in the final mixture, which corresponds to a reduction of 26 % (w/w) of the initial non-prebiotic sugars.

On the other hand, in the enzymatic treatment by intracellular FFase (**Figure 8b**) the reaction mixture was initially composed of 90 % (w/w) of GF. At the first 9 h of reaction, the GF conversion into FOS was

carried out at a considerable rate. Afterwards, a reduction of the reaction velocity was observed, similar to the reduction observed after 6 h of the reaction catalysed by the extracellular FFase extract. Just as it was observed in the extracellular FFase catalysis, FOS concentration between 24 h and 27 h of reaction was not statistically significant. After 24 h, it was obtained a FOS concentration of 146 ± 2 g/L, consisting mainly of GF_2 (122 ± 2 g/L) with GF_3 (23.5 ± 0.2 g/L). A FOS conversion yield of 0.496 ± 0.002 g_{FOS}/g_{GF} was obtained. Regarding non-prebiotic sugars, a concentration of 207 ± 1 g/L was obtained in the final mixture, which corresponds to a reduction of 37 % (w/w).

In general, the sugar profile obtained by the two enzyme treatment methods was very similar. Such results would not be expected *a priori* due to the activity of the extracellular extract being three times higher than that shown for the intracellular extract.

Regarding the FOS conversion rate, an inhibitory effect was observed in both treatments, but in the intracellular case, the reduction was observed only 3 h later. This result may be due to the fact that the intracellular extract does not contain an initial Glc concentration as high as that of the extracellular extract, allowing the reaction mixture in 6 h to reach 41 ± 1 g/L of Glc compared to 54 ± 11 g/L of Glc obtained with the extracellular. Furthermore, for both cases it was observed an inhibitory effect on the transfructosylation reaction responsible for the production of FOS when a concentration of approximately 50 g/L of Glc was achieved.

Moreover, it is important to mention that there is a difference between the composition of non-prebiotic sugars in the initial and final reaction mixture in both experiments. At the beginning of the reaction, most non-prebiotic sugars are GF, and after 24 h of reaction, Glc is the main component followed by Fru and lastly GF. This is due to the fact that Glc is a by-product of the transfructosylation reaction responsible for the synthesis of FOS.

In addition, it is possible to infer that the *A. ibericus* FFase does not present a good elongation capacity of the glycosidic chain through the use of GF_3 synthesised as a donor of fructosyl residue. However, the ability to produce GF_4 was seen above in the fermentation of the fungus *A. ibericus*, which suggests that the FFase reaction time has not been sufficient for the synthesized GF_3 to react as a donor.

Based on all the results obtained, it was selected the extracellular FFase extract for the following tests, aimed to maximize the production of FOS. The production of an extracellular extract is a simpler method than the intracellular one, since the latter involved an extra ultrasonication procedure and achieved similar results. The increase in the difficulty of the procedure to obtain the FFase extract, and consequently the increase in the production cost, was not offset by the results obtained.

4.4 Optimization of FOS enzymatic synthesis

4.4.1 Effect of initial GF concentration

The reaction rate is clearly influenced by the initial substrate content in the enzyme mixture. Consequently, the production of FOS was also dependent on the initial substrate concentration.

An enzyme depends on the bond with the substrate to act thus, small concentrations of substrate hinder the interaction with the enzyme, leading to a low reaction rate. In contrast, high amounts of substrate increase the chances of interaction with the enzyme molecules present in the reaction mixture and consequently also increases the reaction rate. Notwithstanding, the substrate concentration influences the reaction rate up to a maximum point. At this point, all enzyme will be linked to the substrate, catalysing the reaction at the maximum possible rate. Thus, the increase of substrate concentration will not increase the reaction rate [89].

Previously, it has been demonstrated for FFases from other microorganisms, that a concentration higher than 400 g/L of GF promotes a greater performance of the enzyme leading to an increase of synthesis of FOS [85], [93], [97], [99]–[103], [105]–[107], [137]. However, it must be considered that the main objective of the production of this enzyme is its application in a fruit-based product. Therefore, it became important to verify the potential of applying *A. ibericus* FFase in lower initial concentrations of GF since such a high concentration of GF is not always present in this type of products. Taking that in consideration, it was decided to study a range of concentrations between 100 and 400 g/L of initial GF. The obtained results are presented in **Figure 9**.

Analysing the FOS profile (**Figure 9a**) obtained for the various initial substrate concentrations, it is possible to infer that as the initial substrate concentration increase the FOS production rate also increases, as expected. A reduction in the rate of production of FOS is noticeable throughout the reaction, which leads to a constant concentration of FOS after some time of reaction. This is due to the inhibitory effect on the reaction by the concentration of Glc present in the mixture, as explained above. The FOS concentration demonstrated to be not vary significantly between 24 h and 27 h of reaction for all concentrations evaluated, indicating once again that 24 h is the optimal time for the enzymatic treatment. At 24 h reaction, it was obtained 65 ± 1, 105 ± 5, 154 ± 3 and 193 ± 4 g/L of FOS with a purity of 35.52 ± 0.05, 42.34 ± 0.07, 40 ± 1 and 40.5 ± 0.5 % for initial GF concentrations of 100, 200, 300, 400 g/L, respectively.

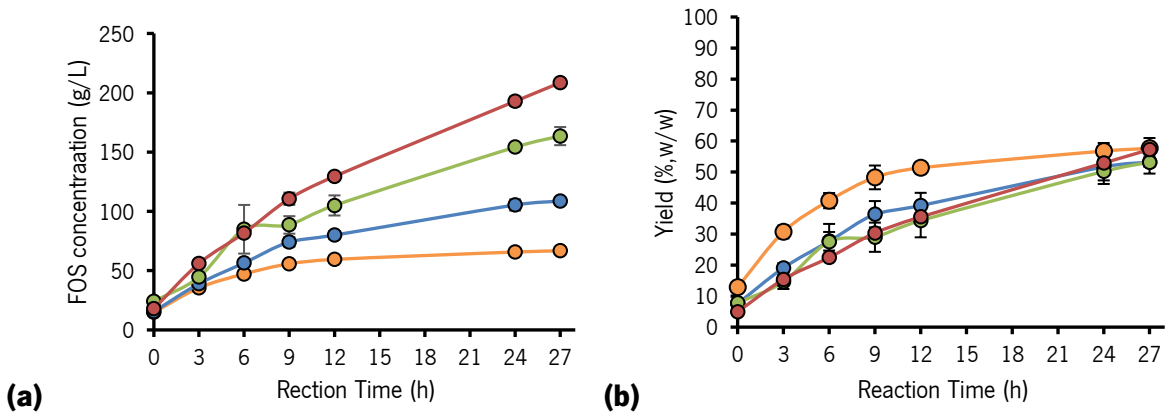


Figure 9. Effect of initial GF concentration in FOS enzymatic synthesis. Experiments carried out at 50 °C, pH 5.0 and 150 rpm for 27 h. (a) FOS production and (b) conversion yield obtained for 100 g/L (orange), 200 g/L (blue), 300 g/L (green) and 400 g/L (red) of initial GF concentration. Results correspond to the average of two independent assays ± standard deviation.

Regarding the FOS conversion yield, similar behaviour was observed between the different initial concentrations. Initial GF concentrations of 100, 200, 300 and 400 g/L resulted in a yield of 0.57 ± 0.02, 0.52 ± 0.06, 0.50 ± 0.03 and 0.529 ± 0.005 g_{FOS}/g_{GF} which suggested that the FFase of *A. ibericus* allows a FOS conversion of about 0.53 ± 0.03 g_{FOS}/g_{GF} regardless of the initial substrate concentration.

Following tests were performed at 300 g/L of initial GF concentration due to the GF content of fruit preparation provided.

4.4.2 Effect of Temperature

The temperature is a crucial factor in an enzymatic reaction. Very low temperatures can hinder the movement and interaction of molecules, and high temperatures can promote the breakdown of weaker bonds, causing the enzyme to lose its quaternary, tertiary and secondary structures and consequently loss of its activity [89].

There is an optimal temperature in which its activity is maximum, meaning that the enzyme operates with a maximum acceleration of the reaction, and the formation of the product occurs in the shortest possible time. According to the literature, a temperature range between 40–60 °C is optimal for maximizing the production of FOS [85], [90], [93], [96]–[108], [137]. Thus, this range of temperature was selected for studies aiming at FFase production by the *A. ibericus*. Reaction was run until 24 h as previously defined. Results obtained are shown in **Figure 10**.

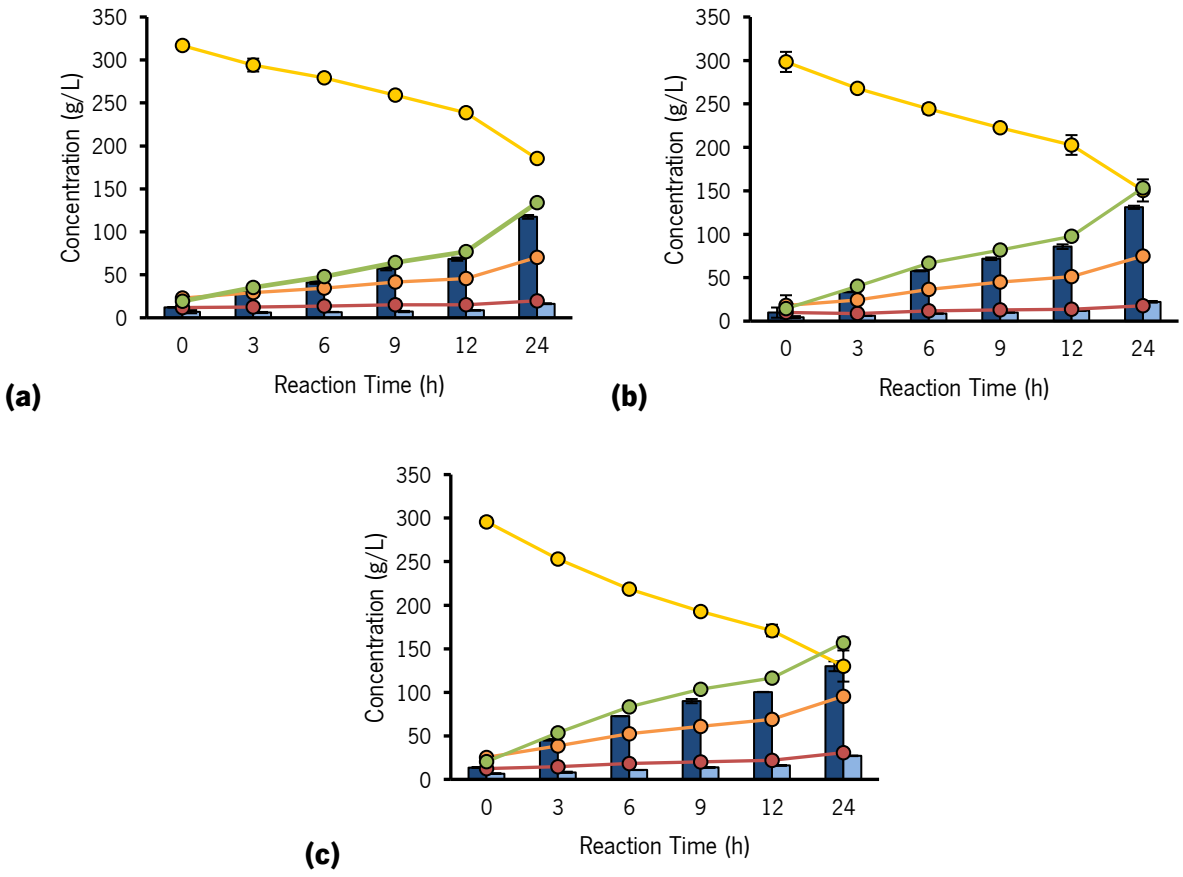


Figure 10. Effect of temperature in FOS enzymatic treatment by addition of 5 g of FFase extract. Experiments carried out at pH 5 and 150 rpm for 24 h. Effect of (a) 40, (b) 50 and (c) 60 °C in the sugar profile. Fru (red), Glc (orange); GF (yellow); GF_2 (dark blue bar), GF_3 (light blue bar) and total FOS (green). Results correspond to the average of two independent experiments ± standard deviation.

Analysing the FOS synthesis profile, similar initial rate of synthesis of FOS was obtained for 50 and 60 °C, which was significantly higher when compared to the treatment conducted at 40 °C. After 24 h of reaction, a maximum FOS concentration of 134 ± 3, 153 ± 3 and 157 ± 6 g/L with a purity of 32.7 ± 0.3, 39 ± 1 and 38 ± 1 % and a FOS conversion yield of 0.42 ± 0.01, 0.51 ± 0.03 and 0.53 ± 0.01 g_{FOS}/g_{GF} was attained for experiments carried out at 40, 50 and 60 °C, respectively. There was a reduction of 76, 83 and 77 g/L of the non-prebiotic sugars (GF, Fru and Glc) after the enzymatic treatment at 40, 50 and 60 °C, respectively.

Comparing the total FOS concentration and conversion yield of each temperature, temperature of 40 °C revealed to be statistically different from other temperatures, and 50 and 60 °C was statistically equal ($p<0.05$).

Thus, in order to optimize the FOS production process, the temperature of 50 °C was established as optimal because, while it requires a smaller amount of energy expended than the process performed at 60 °C.

4.4.3 Effect of pH

As described above the pH value is another parameter that significantly influences the activity of an enzyme.

Based on what has already been reported for several microbial FFases, the ideal pH range is usually between 4.5 and 6.5 [77], [85], [90], [93], [96]–[109], [137]. Considering that the fruit-based product used later in the study case has a pH of approximately 3.8, a range between 3.6 and 6.4 was selected for evaluation. This study was carried out at the ideal temperature previously defined and over time until the optimized reaction time. The results are present in **Figure 11**.

A negative effect in FOS production was clearly observed to the lowest pH value (3.6), which resulted in 25.96 ± 0.03 g/L with a purity of 6.6 ± 0.3 % and a FOS conversion yield of 0.0997 ± 0.0002 g_{FOS}/g_{GF} at the end of the reaction. The pH 4.3 promoted a higher increase in the synthesis of FOS, 120 ± 3 g/L with a purity of a 29.9 ± 0.1 % and a FOS conversion yield of 0.3758 ± 0.0009 g_{FOS}/g_{GF}. The remaining pH values led to a much higher FOS concentration, which were statistically equal ($p<0.05$) after 24 h of reaction for those pH values. The enzymatic treatments concentrations performed at pH 5.0, 5.7 and 6.4 resulted in a FOS concentration of 153 ± 3, 167 ± 14 and 156 ± 11 g/L with a purity of 39 ± 1, 39.8 ±0.1 and 40.2 ± 0.5 % and a FOS conversion yield of 0.4777 ± 0.0004, 0.53 ±0.02 and 0.49 ± 0.03 g_{FOS}/g_{GF}, respectively.

These results may be indicative that the application of this enzyme in acidic products, such as several fruit-based products, will lead to low FOS production. However, it is necessary to keep in mind that even if there is only a small percentage of conversion of GF into FOS, there will always be a reduction in the non-prebiotic sugars content in the product and consequently a reduction of the caloric value, which is the main objective of the study. As well as the inclusion of prebiotics in the product, adding functional value to the food.

Since pH 5 was used in all previous experiments and belongs to the optimal pH range, this pH was selected for the following experiments.

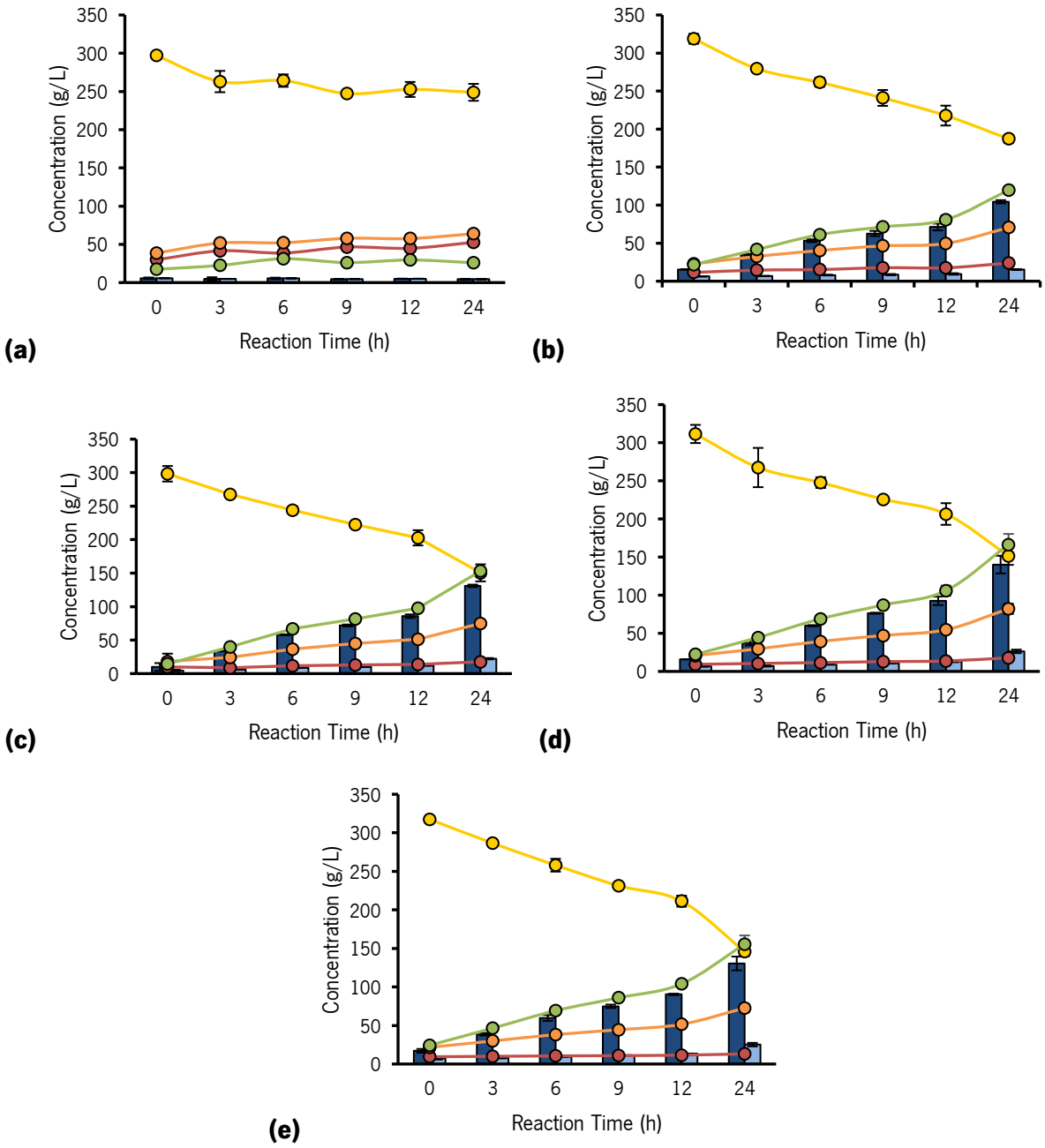


Figure 11. Effect of pH on FOS enzymatic synthesis. Experiments carried out at 50 °C and 150 rpm for 24 h. Sugar's profile under different pH values: (a) 3.6, (b) 4.3, (c) 5.0, (d) 5.7 and (e) 6.4. Fru (red), Glc (orange); GF (yellow); GF_2 (dark blue bar), GF_3 (light blue bar), total FOS (green). Results correspond to the average of two independent experiments ± standard deviation.

4.4.4 Effect of enzyme:GF ratio

Considering a constant initial concentration of substrate, it is expected that the increase in the amount of available enzyme will lead to an increase of the initial rate. Furthermore, since there is consumption of substrate over time, the yield and velocity of the reaction will gradually decrease.

Volumes ranging between 2 and 6 mL of FFase from *A. ibericus* were applied in a total volume of 15 mL in order to optimize the enzyme:GF ratio. Sugar's profile obtained are presented in **Figure 12**.

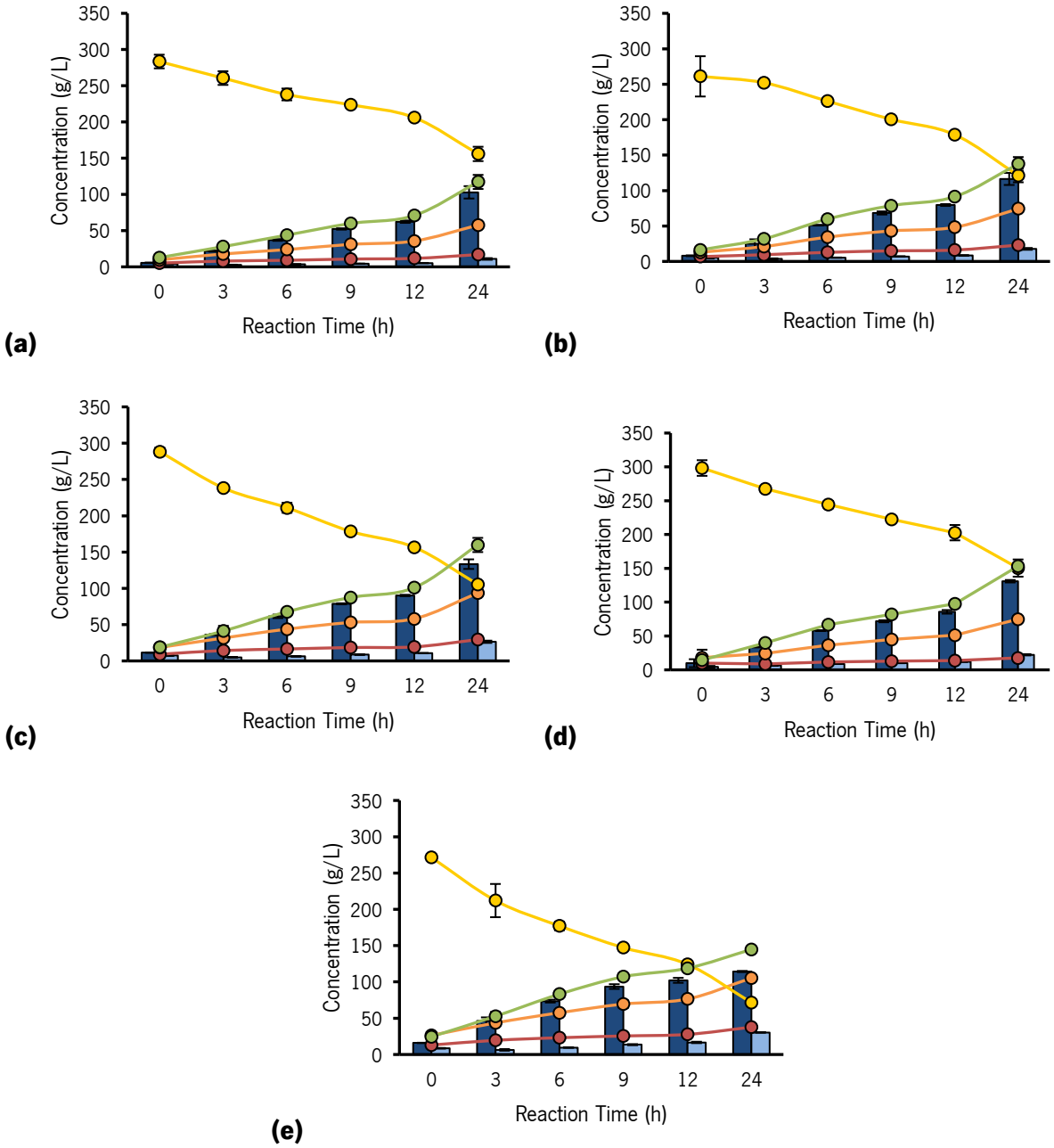


Figure 12. Effect of the enzyme:GF ratio on FOS enzymatic production. Sugars profile obtained in assays with total volume of 15 mL and (a) 2, (b) 3, (c) 4, (d) 5 and (e) 6 mL of FFase from *A.ibericus*. Experiments carried out at 50 °C, pH 5 and 150 rpm for 24 h. Fru (red), Glc (orange); GF (yellow); GF_2 (dark blue bar), GF_3 (light blue bar), total FOS (green). Results correspond to the average of two independent experiments ± standard deviation.

The application of 2 mL of FFase resulted in the lowest concentration of FOS obtained (117.2 ± 9.7 g/L) with a purity of 33.7 ± 0.4 %. Also, this experiment showed the lowest FOS conversion yield, about 0.41 ± 0.02 g_{FOS}/g_{GF}, corresponds to a reduction less than 70 g/L of non-prebiotic sugars in the mixture.

On other hand, FOS production revealed to be statistically similar for the remaining experiments. The application of 3, 4, 5 and 6 mL of FFase resulted in the production of 1383 ± 10, 160 ± 8, 153 ± 3 and 144.7 ± 0.7 g/L of FOS with a purity of 38.6 ± 0.1, 41.1 ± 0.5, 40 ± 1 and 40.29 ± 0.07 % and a FOS conversion yield of 0.53 ± 0.04, 0.55 ± 0.04, 0.4969 ± 0.0004, 0.533 ± 0.002 g_{FOS}/g_{GF}, respectively.

The results showed that the supplementation of an increasing amount of FFase to the same GF concentration did not correspond to an increasingly FOS production. This fact could be explained once again by the inhibitory behaviour of glucose in the transfructosylation reaction.

Therefore, the most suitable proportion may be the addition of 3 mL of the enzyme in a total volume of 15 mL since it has achieved similar results with less enzyme addition.

Overall, the optimized conditions established for FFase from *A. ibericus* enzymatic treatment in GF solution were 50 °C, pH 5 with an enzyme:GF ratio composed with 3 mL of enzyme and 12 mL of buffer.

4.5 PRODUCTION OF THE FUNCTIONAL FRUIT-BASED PRODUCT

The main goal of the production of the FFase of *A. ibericus* was its further application in a fruit-based product. This enzymatic treatment aimed to reduce its caloric value at the same time that prebiotics are synthesised directly in the product, which provides benefits to the health of its consumers.

It is important to mention that the high consistency of the fruit preparation does not allow a good initial homogenization, even with heating in boiling water for 3 min before adding the enzyme. Consequently, a high experimental error is associated with the starting point of the reaction. In order to overcome this error, the initial point was estimated for each treatment. The sugar concentrations determined for the original product, as well as for the FFase extract were taken into account. This estimate allowed to obtain a yield and caloric value closer to reality.

4.5.1 EFFECT OF TEMPERATURE

As discussed above, the temperature is a very important parameter in an enzymatic reaction.

Previously it was found that within the temperature range between 40 and 60 °C. The lowest temperature was not the most suitable for the activity of the FFase from *A. ibericus* and both 50 and 60 °C leading to the highest concentration of FOS.

Thus, its effect was also evaluated in the same range (40 - 60 °C) for the enzymatic treatment of the fruit preparation and the same behaviour was expected. The reaction time for the enzymatic treatment was also optimized. The results are present in **Figure 13**.

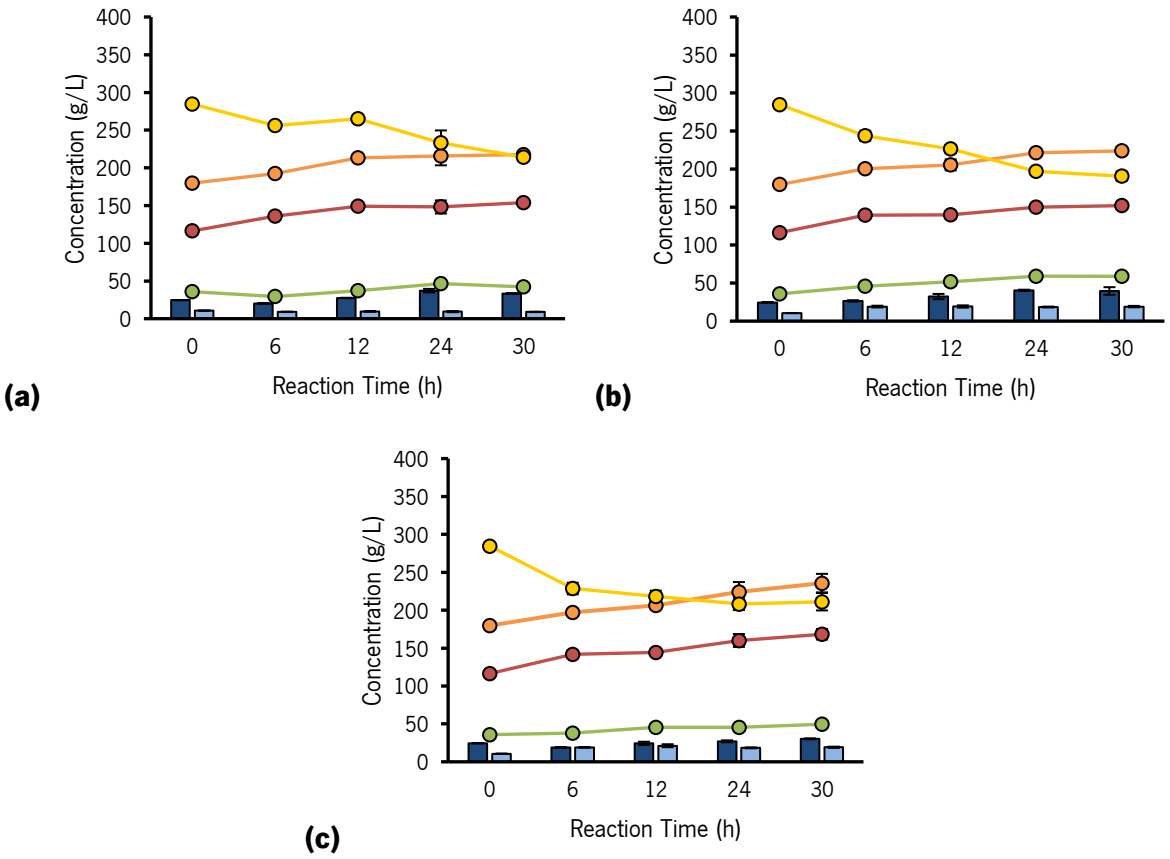


Figure 13. Effect of temperature in the enzymatic treatment of a fruit preparation, by addition of 5 mL of FFase extract. Experiments carried out for 30 h with 150 rpm of agitation at (a) 40, (b) 50 and (c) 60 °C. Fru (red), Glc (orange); GF (yellow); GF_2 (dark blue bar), GF_3 (light blue bar), total FOS (green). Results correspond to the average of two independent experiments ± standard deviation.

Analysing the results, it is evident that the range of FOS synthesised in the reaction is much lower than what was expected. Values above 100 g/L had previously been achieved for reactions in GF solution. However, it is necessary to take into account that the original product is characterized by having a pH value of 3.77 ± 0.01. As demonstrated previously, this lower pH was not the most suitable for the application of FFase from *A. ibericus*. The pH 3.6 promoted 0.0874 ± 0.0002 g_{FOS}/g_{GF} of FOS conversion yield after 24 h of reaction. Also, the original product contains a high amount of Glc in its composition, which has an inhibitory effect on the reaction. These two factors were responsible for the low synthesis of FOS in the strawberry preparation.

Simultaneously with the synthesis of FOS, a decrease in GF and an increase in the content of Glc and Fru were observed. The GF was hydrolysed, but only a small part of Fru was used as a donor of the fructosyl group for the synthesis of FOS, indicating the inhibition of the transfructosylation reaction by the Glc content.

The maximum concentration of FOS was observed at 24 h in experiments performed at 40 and 50 °C (47 ± 3 and 59.2 ± 0.2 g/L, respectively) and at 30 h in experiments performed at 60 °C (45.4 ± 0.7 g/L).

Although the highest FOS concentration obtained at 60 °C is after 30 h of reaction, the increase in FOS between 24 and 30 h was not statistically significant. Hence, results suggested 24 h as the optimum reaction time.

The optimum temperature was established by comparison of the results obtained at each temperature after 24 h enzymatic treatment, as presented in **Figure 14**.

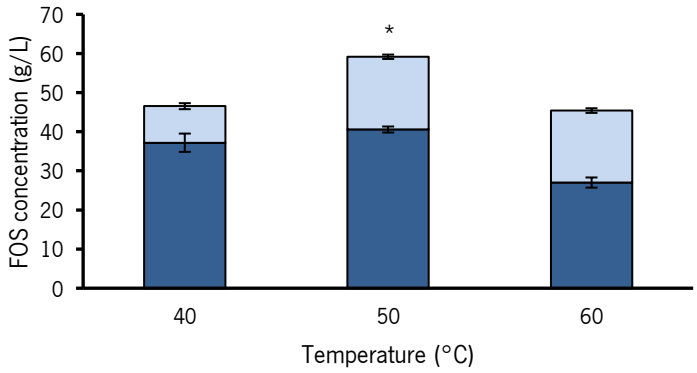


Figure 14. Total FOS produced by enzymatic treatment for 24 h, 150 rpm at 40, 50 and 60 °C by addition of 5 mL of FFase extract. GF_2 (dark blue bar) and GF_3 (light blue bar). Results correspond to the average of duplicate experiments ± standard deviation. Statistical analysis was performed by one-way ANOVA. *p<0.05

The experiments submitted for 24 h to 40, 50 and 60 °C obtained 47 ± 3, 59.2 ± 0.2 and 45.4 ± 0.7 g/L of FOS with purity of 7.23 ± 0.03, 9.4 ± 0.2 and 7.12 ± 0.04 % and a FOS conversion yield of 0.16 ± 0.01,0.208 ± 0.001and 0.160 ± 0.002 g_{FOS}/g_{GF}, respectively.

Considering the results, there were statistically significant differences between the 50 °C and other temperatures. Thus, the ideal temperature for the treatment, that is, the one that promotes a greater amount of FOS, was 50 °C at the pH of strawberry.

Higher temperatures demonstrated to promote the elongation of the glycosidic chain in higher quantities, through the use of GF_2 as fructosyl donor, but also a decrease in the total amount of FOS.

The synthesis *in situ* of prebiotics in fruit-based products, by microbial enzymes, was mostly described for IMO. For example, Fontes *et al.*, 2015 described the synthesis of 130.17, 141.10 and 138.12 g/L of IMO in orange juice, pineapple and melon pulps, respectively, using a partially purified dextransucrase at 30 °C and pH 5.2 for 24 h. The initial pH of pineapple and orange products was around 3.4 and authors

adjusted pH to 5.2 before enzymatic treatment, considered the optimum pH for enzyme activity [59]. The pH adjustment of the product might be the key to obtain better results.

4.5.2 Effect of ratio enzyme:fruit-based product

The ration enzyme:GF applied in the GF solution showed relevance in the optimization of the process. The addition of 3 mL of the enzyme in a total volume of 15 mL was sufficient to achieve the same yield as ratios in which a greater amount of enzyme was applied. Although, this ratio did not promote a good homogenization between fruit preparation and enzyme. Therefore, for the fruit preparation, it was evaluated the effect of using 4, 5 and 6 mL of the enzyme in a total mass of 15 g using the optimum temperature (50 °C) for 24 h. The sugars profile obtained for each treatment are present in **Figure 15**.

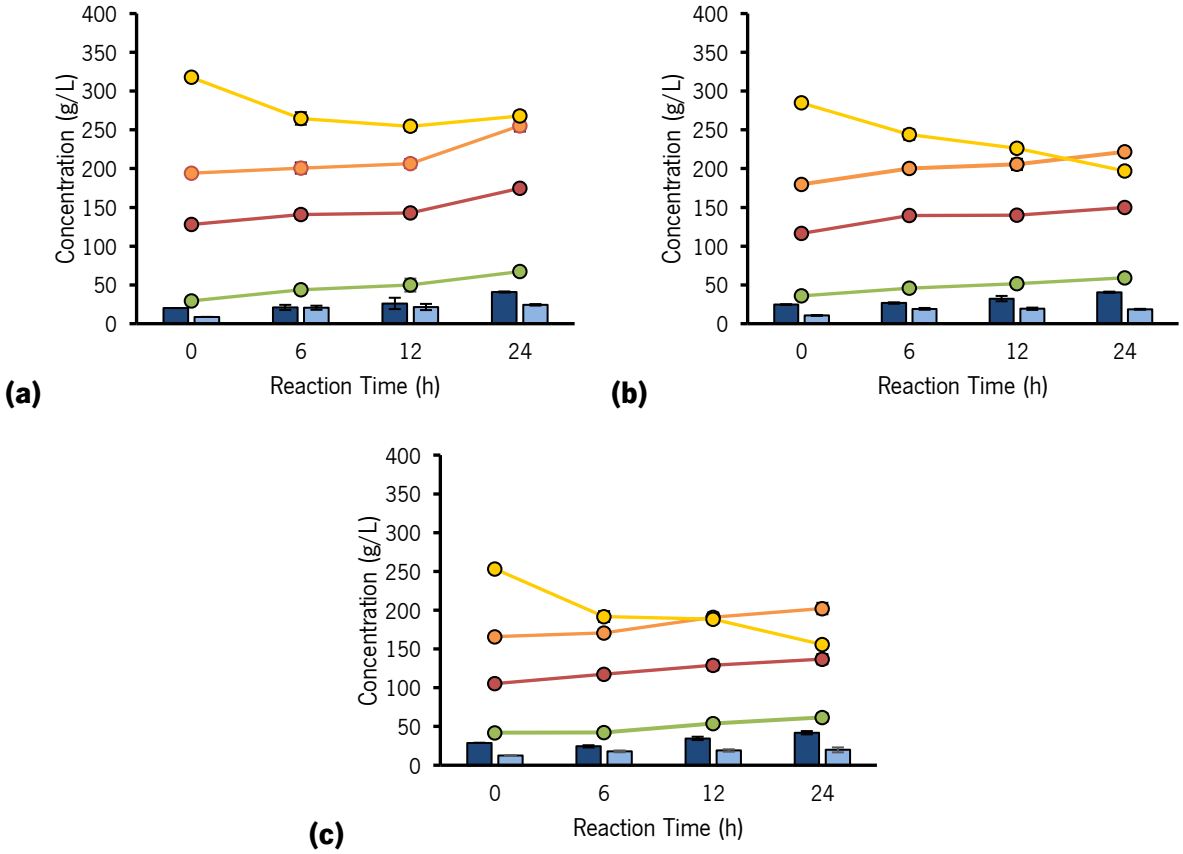


Figure 15. Effect of ratio enzyme:fruit preparation on FOS enzymatic synthesis performed at 50 °C and 150 rpm for 24 h. The volume (mL) of FFase extract addition was evaluated for a total mass of 15 g: (a) 4, (b) 5 and (c) 6. Fru (red), Glc (orange); GF (yellow); GF_2 (dark blue bar), GF_3 (light blue bar), total FOS (green). Results correspond to the average of two independent experiments ± standard deviation.

After 24 h of reaction by addition of 4,5 and 6 mL of FFase were obtained 67 ± 5, 59.2 ± 0.2 and 61 ± 5 g/L of FOS with a purity of 8.8 ± 0.8, 9.4 ± 0.2 and 11.1 ± 0.5 % and a FOS conversion yield of 0.21 ± 0.01, 0.2078 ± 0.0007 and 0.24 ± 0.02 g_{FOS}/g_{GF}, respectively.

Analysing the results, there was no statistical difference between treatments concerning the total FOS and yield obtained. Thus, the results suggested the addition of 4 mL of the enzyme in a total mass of 15 g as the most suitable proportion since it has achieved similar results with less enzyme addition. However, in order to balance a good homogenization of the product and production of FOS, the proportion composed by 5 mLof FFase was considered the best option.

4.6 CHARACTERIZATION OF FUNCTIONAL FRUIT-BASED PRODUCT

A good impact of the addition of prebiotics on the organoleptic and functional characteristics of the fruit juices is a key goal to obtain a successful product. Significant changes that impair nutritional, textural, sensory, and rheological parameters compared to the original product are undesirable to guarantee consumer acceptance [22], [46].

The organoleptic and functional characterization of the product diluted with the enzyme at the initial point of reaction and in the final product after enzymatic treatment using the previously optimized conditions (50 °C, 24 h,5 mL of FFase) was evaluated. **Table 11** summarizes the results observed.

There was a slightly acidification of the product with the addition of enzyme (before treatment), but during the treatment the pH value increased to original pH. Thus, no significant differences were observed between pH of the initial and the final product.

During the treatment, a slight increase in Brix was obtained due to the fact that, despite the reduction in non-prebiotic sugars, FOS was produced. Some authors described the same increase in Brix after the incorporation of prebiotics in other fruit-based products [53], [57].

The initial reaction mixture showed a higher a_w compared to original strawberry preparation which was expected due to the addition of enzyme that diluted the original product. Before and after enzymatic treatment no significant changes was observed for this parameter.

The carbohydrate profile showed a reduction of approximately 10 g/L of non-prebiotic sugars at the same time that approximately 24 g/L of FOS were produced by the use of the product's native sugars. The final strawberry preparation contained 59 ± 3 g/L of FOS because the FFase extract added to the product also contained FOS. As described above a daily intake of 4 g of FOS per day is the amount necessary to increase the levels of Bifidobacteria in the human intestine [113]. Considering the FOS concentration produced, it is suggestive that the final product has enough FOS to be considered functional. Furthermore, the claims that a food is a source of fibre or rich in fibre , or any claim that may have the same meaning for the consumer, can be made when the product contains at least 3 or 6 g fibre per 100 g of product,

respectively [138]. Since the final product have approximately 6 g of fibre per 100 mL and the density of product was not determined, we cannot affirm that the product have enough fibre to be considered rich in fibre.

Table 11. Summary of organoleptic and functional characterization of strawberry preparation before and after enzymatic treatment performed at 50 °C, 150 rpm for 24 h by addition of 5 g of FFase. Results correspond to the average of two independent assays ± standard deviation.

		Before treatment	After treatment
pH		3.27 ± 0.04	3.57 ± 0.02
a_w		0.887 ± 0.001	0.872 ± 0.009
TSS (Brix°)		54.6 ± 0.4	58.3 ± 0.1
Carbohydrate (g/L)	Glc	179.67*	220 ± 1
	Fru	116.33*	153.8 ± 0.5
	GF	284.66*	197 ± 2
	GF_2	24.61*	38 ± 1
	GF_3	10.60*	21 ± 1
	GF_4	0.63*	0
Colour	L*	27.4 ± 0.2	25.96 ± 0.04
	a*	1.21 ± 0.05	1.24 ± 0.05
	b*	1.17 ± 0.01	1.30 ± 0.06
	c*	1.69 ± 0.03	1.79 ±0.07
	h°	0.77 ± 0.02	0.81 ± 0.01

*Estimated sugars content for initial point of reaction, considering the FFase extract and fruit preparation composition.

After enzymatic treatment with FFase from *A. ibericus*, it was observed a decrease of brightness and a slight increase of redness and yellowness. The c increased indicating that the treated sample is more vivid than the non-treated sample. The *h*° also increase indicating that the colour moved slightly away from pure red towards yellow. The ΔE value was 1.45, and considering that any value below 3 corresponds to an imperceptible colour change for the human eye, then the enzymatic treatment did not significantly alter the colour of the sample [139].

The rheology behaviour of non-treated and treated samples was also evaluated. Results of influence of shear rate on the flow curve and viscosity are present in **Figure 16**.

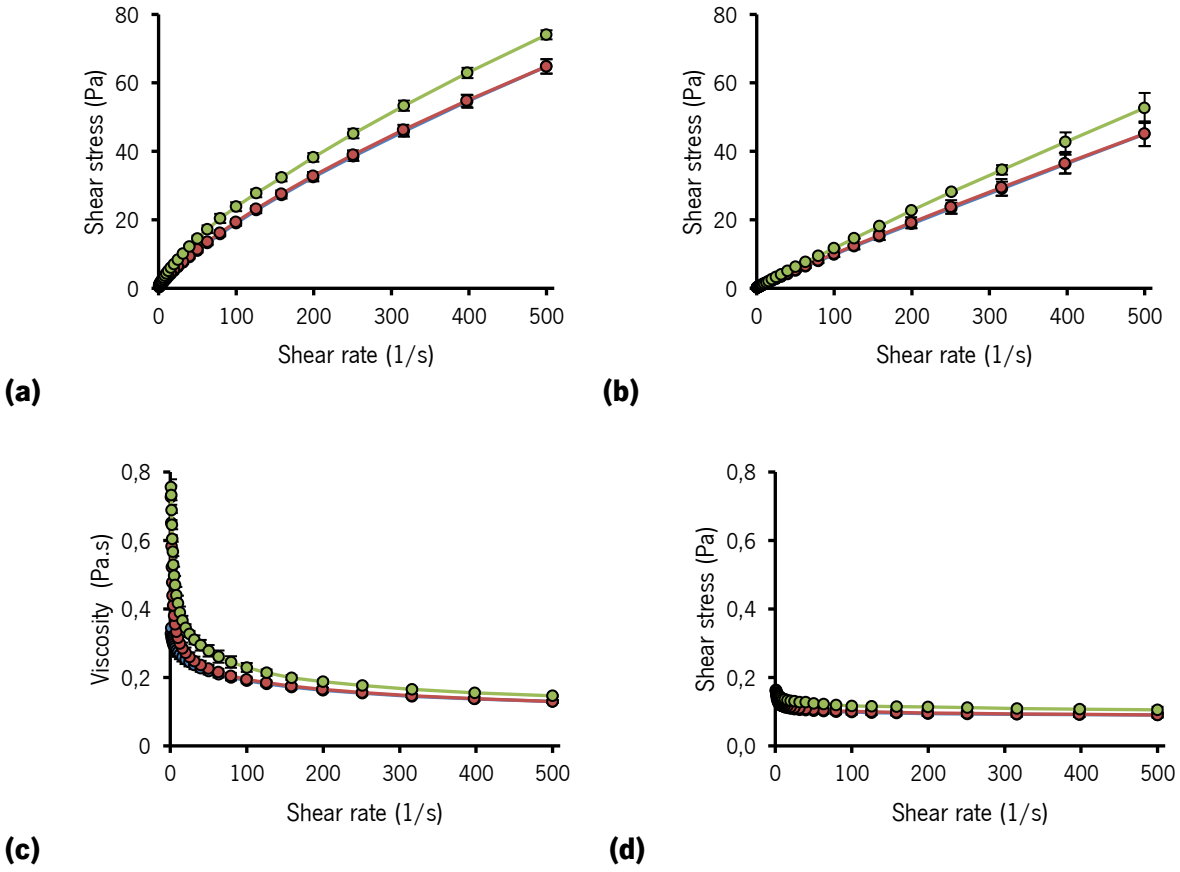


Figure 16. Influence of shear rate on the flow curve of strawberry preparation (a) non-treated and (b) treated and the influence of shear rate on the viscosity of strawberry preparation (c) non-treated and (d) treated. A three steps program (up-down-up) are performed, which corresponds to blue, red and green curves, respectively. Results correspond to the average of two independent experiments ± standard deviation.

Analysing the results, it is clear that the structure of preparation of the fruits (non-treated and treated) changes after the application of the shear rate. Thus, fruit preparation structure did not show regeneration, since the third flow (green) presented deviation in relation to the first flow (blue).

The rheological behaviour was fitted to the Ostwald–de Waele model. The parameters obtained for the non-treated samples were 0.68 ± 0.07 and 0.73 ± 0.01 to K and n, respectively, and for treated samples were 0.17 ± 0.05 to K and 0.93 ± 0.06 to n.

The K, which is the consistency index, was lower to the treated samples, indicating a weaker and more fluid structure, which is corroborated with the lower viscosity range obtained to the treated sample compared to the non-treated one.

The n, which is the exponential factor, showed a value close to 1 for the treated sample, which indicates an almost linear behaviour and, consequently, a Newtonian fluid. In addition, the viscosity was apparently independent of the shear stress for the treated sample, which again refers to a Newtonian fluid. A lower

value of n was obtained for the non-treated sample, which suggests that the sample presented a more pseudoplastic behaviour.

Thus, rheological behaviour was affected by enzymatic treatment. There was a reduction of more than 80 g/L of GF and an increase of approximately 40 and 37 g/L of Glc and Fru, respectively, which could lead to a reduction in viscosity. Moreover, it should also be noted that the enzymatic treatment places the sample at a temperature of 50 °C for 24 h, which could lead to a reduction in viscosity as well. On the other hand, the formation of FOS may have had a negative impact on the rheological properties of the product as well as it was reported to FOS addition in Greek yogurt which resulted in a less consistent, elastic, viscous, and firm product [140].

5. CONCLUSIONS

The fungus *A. ibericus* is known to be a good producer of FOS, although the production of the FFase enzyme, responsible for FOS synthesis, has never been studied. This work demonstrated that the crude FFase extract from this fungus has high activity and a good ability to synthesize FOS. However, reaction time may not be sufficient for the enzyme show the capacity of chain elongation using GF_3 as a fructosyl donor to produce GF_4.

The application of the enzyme in a GF solution allowed to establish the optimum reaction time, temperature, pH and ratio enzyme:GF solution for the enzymatic treatment. The conversion yield of FOS in all tests was negatively affected by the G present in the reaction mixture resulting from the transfructosylation reaction.

Regarding the application of the FFase in the fruit preparation, it was possible to establish the optimum reaction time, temperature and enzyme: fruit preparation ratio despite homogenization difficulties due to the consistency of the product.

The enzymatic treatment of the fruit preparation at optimized conditions produced FOS, even if in small quantities, by the use of the product's native sugars. No significant changes in pH, a_w and colour were obtained and a slight increase of TSS and decrease of viscosity of the fruit preparation were observed.

Overall, this work demonstrated the high potential of FFase from *A. ibericus* as FOS producer. These results also address the potential of the enzyme as sugar replacer and FOS enricher of fruit-based products.

6. FUTURE WORK PERSPECTIVES

FFase from *A. ibericus* demonstrated promising enzyme activity when produced by fungus fermentation seeded with half the concentration of spore suspension used in the previous studies. Thus, future work should verify if FFase production with higher inoculum concentration led to a FFase with higher activity. Furthermore, various studies reported a higher enzyme activity after applying some purification steps. For this reason, the purification of FFase extract can also be evaluated to further increase the enzyme activity.

The inhibitory effect of Glc content was the main contributor to reduce the FOS conversion yield. So, it is necessary to control this effect in order to obtain a final mixture with a low amount of non-prebiotic sugars and a much higher FOS concentration. The addition of an enzyme that consumes the Glc content, such as glucose oxidase, simultaneously with FFase can be a good methodology to be applied in the future.

Regarding the enzymatic treatment of fruit preparation, future studies should be aiming to increase FOS conversion yield, in order to develop a functional fruit product with much lower calories, and a higher FOS concentration, thus meeting the industrial search for alternative healthy food products.
The consistency of the product difficult the experiments. To overcome it, the addition of the enzyme can be evaluated during the production process of the fruit preparation. Instead of being applied in the final product, it can be applied at the time of mixing all ingredients. On other hand, the enzyme application in different fruit-based products as fruit juice and concentrate should be performed since they do not contain such high Glc content and more promising results might be obtained.

Some authors adjusted the pH of the fruit-based products before the enzyme treatment, and the same should be tested since the lower pH demonstrated to not be suitable for the FOS enzymatic synthesis.

Overall, it is important to keep in mind that the physicochemical, functional and organoleptic properties must be analysed in all tests that englobe the food product to guarantee acceptance by the consumer.

7. REFERENCES

[1] C. Sanchez-Moreno, S. De Pascual-Teresa, B. De Ancos, and M. P. Cano, "Nutritional Values of Fruits," in *Handbook of Fruits and Fruit Processing*, Y. H. Hui, Ed. Ames, Iowa, USA: Blackwell Publishing, 2006, pp. 29–43.

[2] M.-J. Rodrigo., B. Alquézar, F. Alférez, and L. Zacarías, "Biochemistry of Fruits and Fruit Products," in *Handbook of Fruits and Fruit Processing*, 2nd ed., John Wiley & Sons, Ltd, 2012, pp. 13–34.

[3] R. H. Matthews, P. R. Pehrsson, and M. Farhat-Sabet, *Sugar Content of Selected Foods: Individual and Total Sugars*. Washington DC: Deptartment of Agriculture, Human Nutrition Information Service, 1987.

[4] S. Yamaki, "Metabolism and accumulation of sugars translocated to fruit and their regulation," *Journal of the Japanese Society for Horticultural Science*, vol. 79, no. 1. Japanese Society for Horticultural Science, pp. 1–15, 2010.

[5] A. R. Vincente, G. A. Manganaris, C. M. Ortiz, G. O. Sozzi, and C. H. Crisosto, "Nutritional Quality of Fruits and Vegetables," in *Postharvest Handling*, Elsevier, 2014, pp. 69–122.

[6] D. Dhingra, M. Michael, H. Rajput, and R. T. Patil, "Dietary fibre in foods: a review," *J. Food Sci. Technol.*, vol. 49, no. 3, pp. 255–266, 2012.

[7] G. Ptkai, "Fruit as an Ingredient in A Fruit Product," in *Handbook of Fruits and Fruit Processing*, Ames, Iowa, USA: Blackwell Publishing, 2006, pp. 217–230.

[8] J. Barta, "Fruit Processing Plants and Equipments," in *Handbook of Fruits and Fruit Processing*, Oxford, UK: Wiley-Blackwell, 2012, pp. 297–313.

[9] A. Bearth, M. E. Cousin, and M. Siegrist, "The consumer's perception of artificial food additives: Influences on acceptance, risk and benefit perceptions," *Food Qual. Prefer.*, vol. 38, pp. 14–23, 2014.

[10] P. S. Raju and A. S. Bawa, "Food Additives in Fruit Processing," in *Handbook of Fruits and Fruit Processing*, Y. H. Hui, Ed. Ames, Iowa, USA: Blackwell Publishing, 2006, pp. 145–170.

[11] WHO, "Obesity and overweight fact sheet," 2020.

[12] V. S. Malik, A. Pan, W. C. Willett, and F. B. Hu, "Sugar-sweetened beverages and weight gain in children and adults : a systematic review and meta-analysis," *Am. J. Clin. Nutr.*, pp. 1084–1102, 2013.

[13] E. E. Noble *et al.*, "Early life sugar consumption has long-term negative effects on memory function

in male rats," *Nutr. Neurosci.*, vol. 22, , pp. 273–283, 2020.

[14] S. Kim, J. Shou, S. Abera, and E. B. Ziff, "Sucrose withdrawal induces depression and anxiety-like behavior by Kir2.1 upregulation in the nucleus accumbens," *Neuropharmacology*, vol. 130, pp. 10–17, 2018.

[15] N. Makarem, E. V. Bandera, Y. Lin, P. F. Jacques, R. B. Hayes, and N. Parekh, "Consumption of sugars, sugary foods, and sugary beverages in relation to adiposity-related cancer risk in the framingham offspring cohort (1991–2013)," *Cancer Prev. Res.*, vol. 11, pp. 347–358, 2018.

[16] J. N. Barnes and M. J. Joyner, "Sugar highs and lows: The impact of diet on cognitive function," *J. Physiol.*, vol. 590, pp. 2831–2831, 2012.

[17] A. Jacques, N. Chaaya, K. Beecher, S. A. Ali, A. Belmer, and S. Bartlett, "The impact of sugar consumption on stress driven, emotional and addictive behaviors," *Neuroscience and Biobehavioral Reviews*, vol. 103. Elsevier Ltd, pp. 178–199, 2019.

[18] World Health Organization, "Guideline: Sugars intake for adults and children.," Geneva, 2015.

[19] C. H. C. Yeung *et al.*, "Modelling of the impact of universal added sugar reduction through food reformulation," *Sci. Rep.*, vol. 7, no. 1, pp. 1–8, 2017.

[20] J. A. Grieger, B. J. Johnson, T. P. Wycherley, and R. K. Golley, "Comparing the nutritional impact of dietary strategies to reduce discretionary choice intake in the australian adult population: A simulation modelling study," *Nutrients*, vol. 9, no. 5, 2017.

[21] G. Scrinis, "Reformulation, fortification and functionalization: Big Food corporations' nutritional engineering and marketing strategies," *J. Peasant Stud.*, vol. 43, no. 1, pp. 17–37, 2016.

[22] M. R. Corbo, A. Bevilacqua, L. Petruzzi, F. P. Casanova, and M. Sinigaglia, "Functional Beverages: The Emerging Side of Functional Foods - Commercial Trends, Research, and Health Implications," *Compr. Rev. Food Sci. Food Saf.*, vol. 13, no. 6, pp. 1192–1206, 2014.

[23] L. Donato-Capel *et al.*, "Technological Means to Modulate Food Digestion and Physiological Response," in *Food Structures, Digestion and Health*, M. Boland, M. Golding, and H. Singh, Eds. Academic Press, pp. 389–422, 2014.

[24] W. Kopp, "How western diet and lifestyle drive the pandemic of obesity and civilization diseases," *Diabetes, Metab. Syndr. Obes. Targets Ther.*, vol. 12, pp. 2221–2236, 2019.

[25] C. S. Birch and G. A. Bonwick, "Ensuring the future of functional foods," *Int. J. Food Sci. Technol.*, vol. 54, no. 5, pp. 1467–1485, 2019.

[26] C. Hill *et al.*, "Expert consensus document: The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic," *Nat.*

Rev. Gastroenterol. Hepatol., vol. 11, no. 8, pp. 506–514, 2014.

[27] G. R. Gibson *et al.*, "Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics," *Nat. Rev. Gastroenterol. Hepatol.*, vol. 14, no. 8, pp. 491–502, 2017.

[28] S. H. Al-Sheraji, A. Ismail, M. Y. Manap, S. Mustafa, R. M. Yusof, and F. A. Hassan, "Prebiotics as functional foods: A review," *J. Funct. Foods*, vol. 5, no. 4, pp. 1542–1553, 2013.

[29] D. Mohanty, S. Misra, S. Mohapatra, and P. S. Sahu, "Prebiotics and synbiotics: Recent concepts in nutrition," *Food Biosci.*, vol. 26, no. October, pp. 152–160, 2018.

[30] L. K. Sarao and M. Arora, "Probiotics, prebiotics, and microencapsulation: A review," *Crit. Rev. Food Sci. Nutr.*, vol. 57, no. 2, pp. 344–371, 2017.

[31] V. Sangwan, S. K. Tomar, R. R. B. Singh, A. K. Singh, and B. Ali, "Galactooligosaccharides: Novel Components of Designer Foods," *J. Food Sci.*, vol. 76, no. 4, pp. R103–R111, 2011.

[32] A. Homayoni Rad, F. Akbarzadeh, and E. V. Mehrabany, "Which are more important: Prebiotics or probiotics?," *Nutrition*, vol. 28, no. 11–12. pp. 1196–1197, 2012.

[33] C. Nobre *et al.*, "In vitro digestibility and fermentability of fructo-oligosaccharides produced by *Aspergillus ibericus*," *J. Funct. Foods*, vol. 46, pp. 278–287, 2018.

[34] V. M. C. de Sousa, E. F. dos Santos, and V. C. Sgarbieri, "The Importance of Prebiotics in Functional Foods and Clinical Practice," *Food Nutr. Sci.*, vol. 02, no. 02, pp. 133–144, 2011.

[35] E. G. Alves Filho *et al.*, "Evaluation of plasma, high-pressure and ultrasound processing on the stability of fructo-oligosaccharides," *Int. J. Food Sci. Technol.*, vol. 51, no. 9, pp. 2034–2040, 2016.

[36] M. J. Sánchez-Martínez, S. Soto-Jover, V. Antolinos, G. B. Martínez-Hernández, and A. López-Gómez, "Manufacturing of Short-Chain Fructo-oligosaccharides: from Laboratory to Industrial Scale," *Food Eng. Rev.*, vol. 12, no. 2, pp. 149–172, 2020.

[37] M. R. Michel, R. M. Rodríguez-Jasso, C. N. Aguilar, S. M. Gonzalez-Herrera, A. C. Flores-Gallegos, and R. Rodríguez-Herrera, "Fructosyltransferase Sources, Production, and Applications for Prebiotics Production," *Probiotics Prebiotics Hum. Nutr. Heal.*, pp. 169–189,2016.

[38] D. A. Flores-Maltos, S. I. Mussatto, J. C. Contreras-Esquivel, R. Rodríguez-Herrera, J. A. Teixeira, and C. N. Aguilar, "Biotechnological production and application of fructo-oligosaccharides," *Crit. Rev. Biotechnol.*, vol. 36, no. 2, pp. 259–267, 2016.

[39] A. Fara, C. Sabater, J. Palacios, T. Requena, A. Montilla, and G. Zárate, "Prebiotic galacto-oligosaccharides production from lactose and lactulose by *Lactobacillus delbrueckii subsp.*

bulgaricus CRL450," *Food Funct.*, vol. 11, no. 7, pp. 5875–5886, 2020.

[40] D. Charalampopoulos and R. A. Rastall, "Prebiotics in foods," *Curr. Opin. Biotechnol.*, vol. 23, no. 2, pp. 187–191, 2012.

[41] R. Klewicki, "The stability of gal-polyols and oligosaccharides during pasteurization at a low pH," *LWT - Food Sci. Technol.*, vol. 40, no. 7, pp. 1259–1265, 2007.

[42] E. van Leusen *et al.*, "Industrial Applications of Galactooligosaccharides," in *Food Oligosaccharides*, F. J. Moreno and M. L. Sanz, Eds. Chichester, UK: John Wiley & Sons, Ltd, 2014, pp. 470–491.

[43] W. Sorndech, K. N. Nakorn, S. Tongta, and A. Blennow, "Isomalto-oligosaccharides: Recent insights in production technology and their use for food and medical applications," *LWT*, vol. 95, pp. 135–142, 2018.

[44] J. Y. Song, Y.-M. Kim, B.-H. Lee, and S.-H. Yoo, "Increasing the dietary fiber contents in isomaltooligosaccharides by dextransucrase reaction with sucrose as a glucosyl donor," *Carbohydr. Polym.*, vol. 230, pp. 1–6, 2020.

[45] H.-W. Park, M.-J. Kim, S. Seo, S. Yoo, and J.-H. Hong, "Relative sweetness and sweetness quality of Xylobiose," *Food Sci. Biotechnol.*, vol. 26, no. 3, pp. 689–696, 2017.

[46] T. V. Fonteles and S. Rodrigues, "Prebiotic in fruit juice: processing challenges, advances, and perspectives," *Curr. Opin. Food Sci.*, vol. 22, pp. 55–61, 2018.

[47] L. Cassani, B. Tomadoni, and M. del Rosario Moreira, "Green ultrasound-assisted processing for extending the shelf-life of prebiotic-rich strawberry juices," *J. Sci. Food Agric.*, pp. 1–6, 2020.

[48] P. B. Pathare, U. L. Opara, and F. A. J. Al-Said, "Colour Measurement and Analysis in Fresh and Processed Foods: A Review," *Food Bioprocess Technol.*, vol. 6, no. 1, pp. 36–60, 2013.

[49] R. Perestrelo, C. Silva, P. Silva, S. Medina, and J. S. Câmara, "Differentiation of Fresh and Processed Fruit Juices Using Volatile Composition," *Molecules*, vol. 24, no. 5, pp. 1–20, 2019.

[50] B. Renuka, S. G. Kulkarni, P. Vijayanand, and S. G. Prapulla, "Fructooligosaccharide fortification of selected fruit juice beverages: Effect on the quality characteristics," *LWT - Food Sci. Technol.*, vol. 42, no. 5, pp. 1031–1033, 2009.

[51] H. F. Braga and A. C. Conti-Silva, "Papaya nectar formulated with prebiotics: Chemical characterization and sensory acceptability," *LWT - Food Sci. Technol.*, vol. 62, no. 1, pp. 854–860, 2015.

[52] R. A. Ghavidel, M. Karimi, M. Davoodi, R. Jahanbani, and A. F. A. Asl, "Effect of fructo-oligosaccharide fortification on quality characteristic of some fruit juice beverages," pp. 141–146,

2014.

[53] A. Alizadeh, A. S. Oskuyi, and S. Amjadi, “The optimization of prebiotic sucrose-free mango nectar by response surface methodology: The effect of stevia and inulin on physicochemical and rheological properties,” *Food Sci. Technol. Int.*, vol. 25, no. 3, pp. 243–251, 2019.

[54] N. J. Gonzalez, K. Adhikari, and M. F. Sancho-Madriz, “Sensory characteristics of peach-flavored yogurt drinks containing prebiotics and synbiotics,” *LWT - Food Sci. Technol.*, vol. 44, no. 1, pp. 158–163, 2011.

[55] H. Dai, C. E. Leung, M. G. Corradini, H. Xiao, and A. J. Kinchla, “Increasing the nutritional value of strawberry puree by adding xylo-oligosaccharides,” *Heliyon*, vol. 6, no. 4, 2020.

[56] H. Lan, Z. Hu, Y. Cheng, L. Zhao, and K. Wang, “Characterization and Prebiotic Potential of Longan Juice Obtained by Enzymatic Conversion of Constituent Sucrose into Fructo-Oligosaccharides,” *Molecules*, vol. 23, no. 10, pp. 1–13, 2018.

[57] A. D. A. Araújo, R. M. D. Coelho, C. P. M. L. Fontes, A. R. A. Silva, J. Maria, and S. Rodrigues, “Production and spouted bed drying of acerola juice containing oligosaccharides,” *Food Bioprod. Process.*, vol. 94, pp. 565–571, 2014.

[58] R. M. D. Coelho, A. D. A. Araújo, C. P. M. L. Fontes, A. R. A. da Silva, J. M. C. da Costa, and S. Rodrigues, “Powder lemon juice containing oligosaccharides obtained by dextransucrase acceptor reaction synthesis and dehydrated in sprouted bed,” *J. Food Sci. Technol.*, vol. 52, no. 9, pp. 5961–5967, 2015.

[59] C. P. M. L. Fontes, J. L. A. da Silva, M. C. Rabelo, and S. Rodrigues, “Development of low caloric prebiotic fruit juices by dexransucrase acceptor reaction,” *J. Food Sci. Technol.*, vol. 52, no. 11, pp. 7272–7280, 2015.

[60] F. D. L. Almeida *et al.*, “Effects of atmospheric cold plasma and ozone on prebiotic orange juice,” *Innov. Food Sci. Emerg. Technol.*, vol. 32, pp. 127–135, 2015.

[61] I. M. da Silva, M. C. Rabelo, and S. Rodrigues, “Cashew juice containing prebiotic oligosaccharides,” *J. Food Sci. Technol.*, vol. 51, no. 9, pp. 2078–2084, 2014.

[62] T. T. H. Nguyen *et al.*, “Production of a low calorie mandarin juice by enzymatic conversion of constituent sugars to oligosaccharides and prevention of insoluble glucan formation,” *Biotechnol. Lett.*, vol. 37, no. 3, pp. 711–716, 2015.

[63] T. T. H. Nguyen *et al.*, “Synthesis of oligosaccharide-containing orange juice using glucansucrase,” *Biotechnol. Bioprocess Eng.*, vol. 20, no. 3, pp. 447–452, 2015.

[64] R. Baruah, B. Deka, and A. Goyal, “Purification and characterization of dextransucrase from

Weissella cibaria RBA12 and its application in in vitro synthesis of prebiotic oligosaccharides in mango and pineapple juices," *LWT - Food Sci. Technol.*, vol. 84, pp. 449–456, 2017.

[65] S. Johansson, B. Diehl, P. Christakopoulos, S. Austin, and C. Vafiadi, "Oligosaccharide Synthesis in Fruit Juice Concentrates Using a Glucansucrase from *Lactobacillus reuteri* 180," *Food Bioprod. Process.*, vol. 98, pp. 201–209, 2016.

[66] S.-X. Huang *et al.*, "Enzymatic synthesis of non-digestible oligosaccharide catalyzed by dextransucrase and dextranase from maltose acceptor reaction," *Biochem. Biophys. Res. Commun.*, vol. 523, no. 3, pp. 651–657, Mar. 2020.

[67] W. Lan and S. Chen, "Chemical kinetics, thermodynamics and inactivation kinetics of dextransucrase activity by ultrasound treatment," *React. Kinet. Mech. Catal.*, vol. 129, no. 2, pp. 843–864, 2020.

[68] J. M. R. Tingirikari, W. F. Gomes, and S. Rodrigues, "Efficient Production of Prebiotic Gluco-oligosaccharides in Orange Juice Using Immobilized and Co-immobilized Dextransucrase," *Appl. Biochem. Biotechnol.*, vol. 183, no. 4, pp. 1265–1281, Dec. 2017.

[69] P. M. Rolim, Y. Hu, and M. G. Gänzle, "Sensory analysis of juice blend containing isomalto-oligosaccharides produced by fermentation with *Weissella cibaria*," *Food Res. Int.*, vol. 124, no. April 2018, pp. 86–92, 2018.

[70] E. G. Alves Filho *et al.*, "Evaluation of thermal and non-thermal processing effect on non-prebiotic and prebiotic acerola juices using 1H qNMR and GC–MS coupled to chemometrics," *Food Chem.*, vol. 265, pp. 23–31, 2018.

[71] C. P. M. L. Fontes, J. L. A. Silva, N. A. Sampaio-Neta, J. M. C. da Costa, and S. Rodrigues, "Dehydration of Prebiotic Fruit Drinks by Spray Drying: Operating Conditions and Powder Characterization," *Food Bioprocess Technol.*, vol. 7, no. 10, pp. 2942–2950, 2014.

[72] K. P. P. K. K. P. Scott *et al.*, "Developments in understanding and applying prebiotics in research and practice—an ISAPP conference paper," *J. Appl. Microbiol.*, vol. 128, no. 4, pp. 934–949, 2020.

[73] N. Benkeblia, "Fructo-oligosaccharides and fructans analysis in plants and food crops," *J. Chromatogr. A*, vol. 1313, pp. 54–61, 2013.

[74] C. Nobre *et al.*, "In vitro digestibility and fermentability of fructo-oligosaccharides produced by *Aspergillus ibericus*," *J. Funct. Foods*, vol. 46, pp. 278–287, 2018.

[75] P. Zambelli *et al.*, "Production of fructo-oligosaccharides by mycelium-bound transfructosylation activity present in *Cladosporium cladosporioides* and *Penicillium sizovae*," *Process Biochem.*, vol.

49, no. 12, pp. 2174–2180, 2014.

[76] V. Bali, P. S. Panesar, M. B. Bera, and R. Panesar, "Fructo-oligosaccharides: Production, Purification and Potential Applications," *Crit. Rev. Food Sci. Nutr.*, vol. 55, no. 11, pp. 1475–1490, 2015.

[77] D. C. Khandekar, T. Palai, A. Agarwal, and P. K. Bhattacharya, "Kinetics of sucrose conversion to fructo-oligosaccharides using enzyme (invertase) under free condition," *Bioprocess Biosyst. Eng.*, vol. 37, no. 12, pp. 2529–2537, 2014.

[78] C. Nobre *et al.*, "Production of fructo-oligosaccharides by *Aspergillus ibericus* and their chemical characterization," *LWT*, vol. 89, pp. 58–64, 2018.

[79] S. I. Mussatto, G. Dragone, L. R. M. Rodrigues, and J. a Teixeira, "Fructo-oligosacharides production using immobilized cells of *Aspergillus japonicus*," pp. 1279–1284, 2008.

[80] C. Nobre, D. A. Gonçalves, J. A. Teixeira, and L. R. Rodrigues, "One-step co-culture fermentation strategy to produce high-content fructo-oligosaccharides," *Carbohydr. Polym.*, vol. 201, pp. 31–38, 2018.

[81] M. A. Ganaie, U. S. Gupta, and N. Kango, "Screening of biocatalysts for transformation of sucrose to fructo-oligosaccharides," *J. Mol. Catal. B Enzym.*, vol. 97, pp. 12–17, 2013.

[82] A. E. Maiorano, R. M. Piccoli, E. S. Da Silva, and M. F. De Andrade Rodrigues, "Microbial production of fructosyltransferases for synthesis of prebiotics," *Biotechnol. Lett.*, vol. 30, no. 11, pp. 1867–1877, 2008.

[83] J. Yoshikawa, S. Amachi, H. Shinoyama, and T. Fujii, "Multiple β-fructofuranosidases by *Aureobasidium pullulans* DSM2404 and their roles in fructooligosaccharide production," *FEMS Microbiol. Lett.*, vol. 265, no. 2, pp. 159–163, 2006.

[84] A. K. C. Nascimento, C. Nobre, M. T. H. Cavalcanti, J. A. Teixeira, and A. L. F. Porto, "Screening of fungi from the genus *Penicillium* for production of β- fructofuranosidase and enzymatic synthesis of fructo-oligosaccharides," *J. Mol. Catal. B Enzym.*, vol. 134, pp. 70–78, 2016.

[85] Q. Xu *et al.*, "Purification and biochemical characterization of a novel β-fructofuranosidase from *Penicillium oxalicum* with transfructosylating activity producing neokestose," *Process Biochem.*, vol. 50, no. 8, pp. 1237–1246, 2015.

[86] S. I. Mussatto, L. F. Ballesteros, S. Martins, D. A. F. Maltos, C. N. Aguilar, and J. A. Teixeira, "Maximization of Fructo-oligosaccharides and β-Fructofuranosidase Production by *Aspergillus japonicus* under Solid-State Fermentation Conditions," *Food Bioprocess Technol.*, vol. 6, no. 8, pp. 2128–2134, 2013.

[87] G. K. Arumugam, V. Selvaraj, D. Gopal, and K. Ramalingam, "Solid-State Fermentation of Agricultural Residues for the Production of Antibiotics," in *Biotransformation of Waste Biomass into High Value Biochemicals*, New York: Springer, 2014, pp. 139–162.

[88] G. N. Martins, M. M. Ureta, E. E. Tymczyszyn, P. C. Castilho, and A. Gomez-Zavaglia, "Technological aspects of the production of fructo and galacto-oligosaccharides. Enzymatic synthesis and hydrolysis," *Front. Nutr.*, vol. 6, no. 78, pp. 1–24, 2019.

[89] A. Cornish-Bowden, *Fundamentals of enzyme kinetics*, 4th ed. Wiley-Blackwell, 2012.

[90] A. Nemukula, T. Mutanda, B. S. Wilhelmi, and C. G. Whiteley, "Response surface methodology: synthesis of short chain fructo-oligosaccharides with a fructosyltransferase from *Aspergillus aculeatus*," *Bioresour. Technol.*, vol. 100, no. 6, pp. 2040–2045, 2009.

[91] R. J. Vega-Paulino and M. E. Zúniga-Hansen, "Potential application of commercial enzyme preparations for industrial production of short-chain fructo-oligosaccharides," *J. Mol. Catal. B Enzym.*, vol. 76, pp. 44–51, 2012.

[92] R. Vega and M. E. Zúniga-Hansen, "Enzymatic synthesis of fructo-oligosaccharides with high 1-kestose concentrations using response surface methodology," *Bioresour. Technol.*, vol. 102, no. 22, pp. 10180–10186, 2011.

[93] A. Madlová, M. Antosová, M. Baráthová, M. Pplakovic, V. Štefuca, and V. Báles, "Screening of Microorganisms for Transfructosylating Activity and Optimization of Biotransformation of Sucrose to Fructo-oligosaccharides," *Chem. Pap.*, vol. 53, no. 6, pp. 366–369, 1999.

[94] J. A. M. van Balken, T. J. G. M. van Dooren, W. J. J. van den Tweel, J. Kamphuis, and E. M. Meijer, "Production of 1-kestose with intact mycelium of *Aspergillus phoenicis* containing sucrose-1F-fructosyltransferase," *Appl. Microbiol. Biotechnol.*, vol. 35, no. 2, pp. 216–221, 1991.

[95] A. S. G. G. Lorenzoni, L. F. Aydos, M. P. Klein, R. C. Rodrigues, and P. F. Hertz, "Fructo-oligosaccharides synthesis by highly stable immobilized β-fructofuranosidase from *Aspergillus aculeatus*," *Carbohydr. Polym.*, vol. 103, no. 1, pp. 193–197, 2014.

[96] K. J. Duan, D. C. Sheu, and J. S. Chen, "Purification and Characterization of β-Fructofuranosidase from *Aspergillus Japonicus* TIT-KJ1," *Biosci. Biotechnol. Biochem.*, vol. 57, no. 11, pp. 1811–1815, 1993.

[97] R. Cruz, V. D. Cruz, M. Z. Belini, J. G. Belote, and C. R. Vieira, "Production of fructo-oligosaccharides by the mycelia of *Aspergillus japonicus* immobilized in calcium alginate," *Bioresour. Technol.*, vol. 65, no. 1–2, pp. 139–143, 1998.

[98] Y. K. Park and M. M. Almeida, "Production of fructo-oligosaccharides from sucrose by a

transfructosylase from *Aspergillus niger*," *World J. Microbiol. Biotechnol.*, vol. 7, no. 3, pp. 331–334, 1991.

[99] Q. D. Nguyen, F. Mattes, Á. Hoschke, J. Rezessy-Szabó, and M. K. Bhat, "Production, purification and identification of fructo-oligosaccharides produced by β-fructofuranosidase from *Aspergillus niger* IMI 303386," *Biotechnol. Lett.*, vol. 21, no. 3, pp. 183–186, 1999.

[100] L. L'Hocine, Z. Wang, B. Jiang, and S. Xu, "Purification and partial characterization of fructosyltransferase and invertase from *Aspergillus niger* AS0023," *J. Biotechnol.*, vol. 81, no. 1, pp. 73–84, 2000.

[101] P. T. Sangeetha, M. N. Ramesh, and S. G. Prapulla, "Recent trends in the microbial production, analysis and application of fructo-oligosaccharides," *Trends Food Sci. Technol.*, vol. 16, no. 10, pp. 442–457, 2005.

[102] F. Guio, L. D. Rugeles, S. E. Rojas, M. P. Palomino, M. C. Camargo, and O. F. Sánchez, "Kinetic modeling fructo-oligosaccharide production using *Aspergillus oryzae* N74," *Appl. Biochem. Biotechnol.*, vol. 167, no. 1, pp. 142–163, 2012.

[103] H. Hidaka, M. Hirayama, and N. Sumi, "A Fructo-oligosaccharide-producing Enzyme from *Aspergillus niger* ATCC 20611," *Agric. Biol. Chem.*, vol. 52, no. 5, pp. 1181–1187, 1988.

[104] T. Aung, H. Jiang, G. L. Liu, Z. Chi, Z. Hu, and Z. M. Chi, "Overproduction of a β-fructofuranosidase1 with a high FOS synthesis activity for efficient biosynthesis of fructo-oligosaccharides," *Int. J. Biol. Macromol.*, vol. 130, pp. 988–996, 2019.

[105] M. B. Alvarado-Huallanco and F. Maugeri Filho, "Kinetic studies and modelling of the production of fructo-oligosaccharides by fructosyltransferase from Rhodotorula sp," *Catal. Sci. Technol.*, vol. 1, no. 6, pp. 1043–1050, 2011.

[106] M. R. Detofol, E. Aguiar-Oliveira, C. E. Bustamante-Vargas, A. B. de J. Soares, M. B. A. Soares, and F. Maugeri, "Modeling and simulation of fructo-oligosaccharides synthesis in a batch basket reactor," *J. Biotechnol.*, vol. 210, pp. 44–51, 2015.

[107] S. Hernalsteens and F. Maugeri, "Purification and characterisation of a fructosyltransferase from *Rhodotorula sp.*," *Appl. Microbiol. Biotechnol.*, vol. 79, no. 4, pp. 589–596, Jun. 2008.

[108] D. A. dos Santos, C. Baldo, D. Borsato, and M. A. P. C. Celligoi, "Utilização de substratos de baixo custo para produção de nistose por Bacillus subtilis natto CCT 7712," *Acta Sci. - Technol.*, vol. 38, no. 4, pp. 391–397, 2016.

[109] R. Beine *et al.*, "Synthesis of novel fructo-oligosaccharides by substrate and enzyme engineering," *J. Biotechnol.*, vol. 138, no. 1–2, pp. 33–41, 2008.

[110] N. Romano, M. Santos, P. Mobili, R. Vega, and A. Gómez-Zavaglia, "Effect of sucrose concentration on the composition of enzymatically synthesized short-chain fructo-oligosaccharides as determined by FTIR and multivariate analysis," *Food Chem.*, vol. 202, pp. 467–475, 2016.

[111] I. Ghazi, L. Fernandez-Arrojo, A. Gomez De Segura, M. Alcalde, F. J. Plou, and A. Ballesteros, "Beet sugar syrup and molasses as low-cost feedstock for the enzymatic production of fructo-oligosaccharides," *J. Agric. Food Chem.*, vol. 54, no. 8, pp. 2964–2968, 2006.

[112] M. Antosová and M. Polakovc, "Fructosyltransferases: The Enzymes Catalyzing Production of Fructo-oligosaccharides," *Chem. Pap.*, vol. 55, no. 6, pp. 350–358, 2001.

[113] T. S. Manning and G. R. Gibson, "Prebiotics," *Best Pract. Res. Clin. Gastroenterol.*, vol. 18, no. 2, pp. 287–298, 2004.

[114] T. Oku and S. Nakamura, "Fructo-oligosaccharide: Metabolism through Gut Microbiota and Prebiotic Effect," *Food Nutr. J.*, vol. 3, no. 1, 2017.

[115] P. A. A. Coussement, "Inulin and Oligofructose: Safe Intakes and Legal Status," *J. Nutr.*, vol. 129, no. 7, pp. 1412S-1417S, 1999.

[116] X. Qiang, C. YongLie, and W. QianBing, "Health benefit application of functional oligosaccharides," *Carbohydr. Polym.*, vol. 77, no. 3, pp. 435–441, 2009.

[117] A. G. Colantonio, S. L. Werner, and M. Brown, "The Effects of Prebiotics and Substances with Prebiotic Properties on Metabolic and Inflammatory Biomarkers in Individuals with Type 2 Diabetes Mellitus: A Systematic Review," *J. Acad. Nutr. Diet.*, 2019.

[118] A. S. Althubiani *et al.*, *Plant-Derived Prebiotics and Its Health Benefits*. Elsevier Inc., 2019.

[119] B. C. Tungland, "Fructo-oligosaccharides and Other Fructans: Structures and Occurrence, Production, Regulatory Aspects, Food Applications, and Nutritional Health Significance," pp. 135–152, 2003.

[120] C. Nobre *et al.*, "Production of fructo-oligosaccharides by *Aspergillus ibericus* and their chemical characterization," *LWT - Food Sci. Technol.*, vol. 89, pp. 58–64, 2018.

[121] M. A. Ganaie and U. S. Gupta, "Recycling of cell culture and efficient release of intracellular fructosyltransferase by ultrasonication for the production of fructo-oligosaccharides," *Carbohydr. Polym.*, vol. 110, pp. 253–258, 2014.

[122] A. Flores-Maltos, S. I. Mussatto, J. C. Contreras-Esquivel, R. Rodríguez, J. A. Teixeira, and C. N. Aguilar, "Production of a Transfructosylating Enzymatic Activity Associated to Fructo-oligosaccharides," in *Green Bio-processes, Energy, Environment, and Sustainability*, Singapore: Springer, 2019, pp. 245–265.

[123] "Frulact." [Online]. Available: https://frulact.com/. [Accessed: 22-Jan-2021].

[124] Mohammad Shafiur Rahman and M. R. T. Rahman, "pH in Food Preservation," in *Handbook of Food Preservation*, 3rd ed., M. S. Rahman, Ed. Taylor & Francis Group, pp. 323–332, 2020.

[125] M. S. Tapia, S. M. Alzamora, and J. Chirife, "Effects of Water Activity on Microbial Stability as a Hurdle in Food Preservation," in *Water Activity in Foods*, Wiley, pp. 323–355, 2020.

[126] A. F. Omar and M. Z. MatJafri, "Principles, methodologies and technologies of fresh fruit quality assurance," *Qual. Assur. Saf. Crop. Foods*, vol. 5, pp. 257–271, 2013.

[127] J. A. Ulloa, H. Escalona, and L. Díaz, "Colour behaviour on mango (*Mangifera indica*) slices self stabilized in glass jars by hurdle technology during storage," *African J. Biotechnol.*, vol. 7, no. 4, pp. 487–494, 2008.

[128] A. L. Dominguez, L. R. Rodrigues, N. M. Lima, and J. A. Teixeira, "An overview of the recent developments on fructo-oligosaccharide production and applications," *Food Bioprocess Technol.*, vol. 7, no. 2, pp. 324–337, 2014.

[129] D. A. Gonçalves, "Production of prebiotics using new culture techniques," University of Minho, 2017.

[130] L. M. Wang and H. M. Zhou, "Isolation and identification of a novel *Aspergillus japonicus* JN19 producing β-fructofuranosidade and characterization of the enzyme," *J. Food Biochem.*, vol. 30, no. 6, pp. 641–658, 2006.

[131] R. L. de Oliveira, M. F. da Silva, A. Converti, and T. S. Porto, "Production of β-fructofuranosidase with transfructosylating activity by *Aspergillus tamarii* URM4634 Solid-State Fermentation on agroindustrial by-products," *Int. J. Biol. Macromol.*, vol. 144, pp. 343–350, 2020.

[132] B. D. Taiomara *et al.*, "β-Fructofuranosidase production by *Aspergillus versicolor* isolated from Atlantic forest and grown on apple pomace," *African J. Microbiol. Res.*, vol. 10, no. 25, pp. 938–948, 2016.

[133] S. I. Mussatto, L. R. Rodrigues, and J. A. Teixeira, "β-Fructofuranosidase production by repeated batch fermentation with immobilized *Aspergillus japonicus*," *J. Ind. Microbiol. Biotechnol.*, vol. 36, no. 7, pp. 923–928, 2009.

[134] M. B. Prata *et al.*, "Fructooligosaccharide production by *Penicillium expansum*," *Biotechnol. Lett.*, vol. 32, no. 6, pp. 837–840, 2010.

[135] Q. D. Nguyen, J. M. Rezessy-Szabó, M. K. Bhat, and Á. Hoschke, "Purification and some properties of β-fructofuranosidase from *Aspergillus niger* IMI303386," *Process Biochem.*, vol. 40, no. 7, pp. 2461–2466, 2005.

[136] A. Lateef, J. K. Oloke, and S. G. Prapulla, "The effect of ultrasonication on the release of fructosyltransferase from *Aureobasidium pullulans* CFR 77," *Enzyme Microb. Technol.*, vol. 40, no. 5, pp. 1067–1070, 2007.

[137] S. Han *et al.*, "Purification and biochemical characteristics of a novel fructosyltransferase with a high FOS transfructosylation activity from *Aspergillus oryzae* S719," *Protein Expr. Purif.*, vol. 167, p. 105549, 2020.

[138] Comissão Europeia, "Regulamento (CE) nº 1924/2006 do Parlamento Europeu e do Conselho de 20 de dezembro de 2006 relativo às alegações nutricionais e de saúde sobre os alimentos," *J. Of. da União Eur.*, 2006.

[139] F. D. L. Almeida *et al.*, "Fructo-oligosaccharides integrity after atmospheric cold plasma and high-pressure processing of a functional orange juice," *Food Res. Int.*, vol. 102 pp. 282–290, 2017.

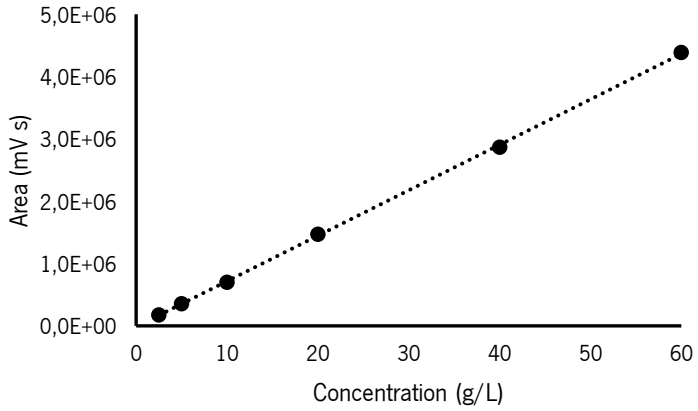
[140] M. F. Costa *et al.*, "Impact of prebiotics on the rheological characteristics and volatile compounds of Greek yogurt," *LWT*, vol. 105, pp. 371–376, 2019.

ANNEXES

ANNEX I– SUGARS CALIBRATION FOR HPLC ANALYSIS

Several calibration curves were determined along this work to calculate small sugars (Fru, Glc and GF) and FOS (GF_2, GF_3 and GF_4) concentrations after HPLC analysis. An example of the curves used for each sugar is represented, since at every time that this method was performed, a new calibration curve was made.

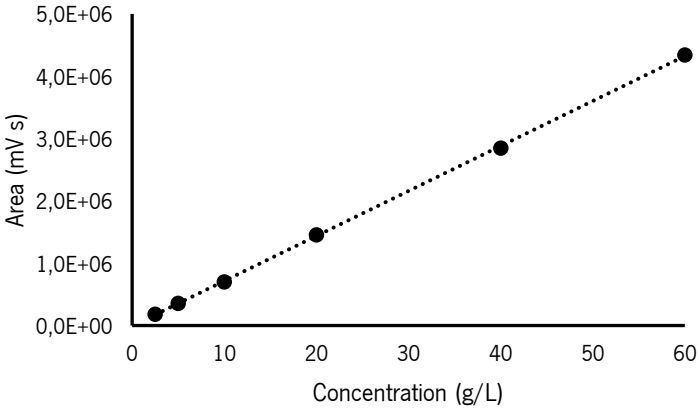
AI.1 FRUCTOSE (FRU)



[F] (g/L) = (72169 ± 45371) * Area (mV s) +(- 6590 ± 1468) (confidence level 95 %)

LOD (g/L) = 1 LOQ (g/L) = 4 R^2= 0.9998

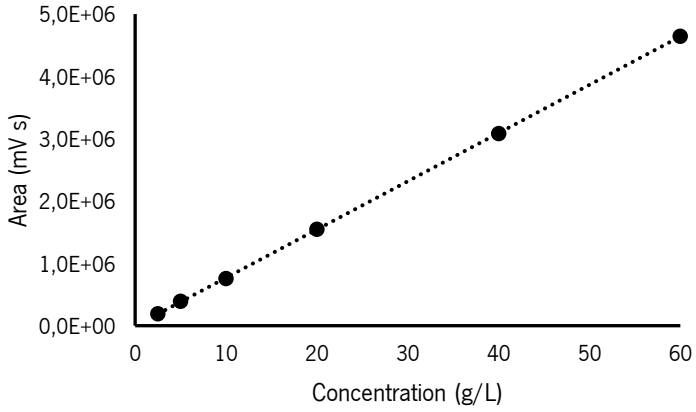
AI.2 GLUCOSE (GLC)



[G] (g/L) = (72948 ± 39600) * Area (mV s) + (-13064± 1281) (confidence level 95 %)

LOD (g/L) = 1 LOQ (g/L) = 3 R^2= 0.99991

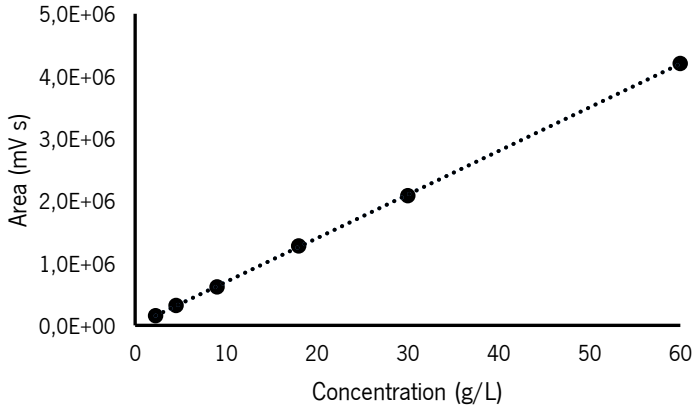
AI.3 SUCROSE (GF)



[GF] (g/L) = (77418 ± 15542) * Area (mV s) + (- 5768 ± 503) (confidence level 95 %)

LOD (g/L) = 0.4 LOQ (g/L) = 1 R^2= 0.99998

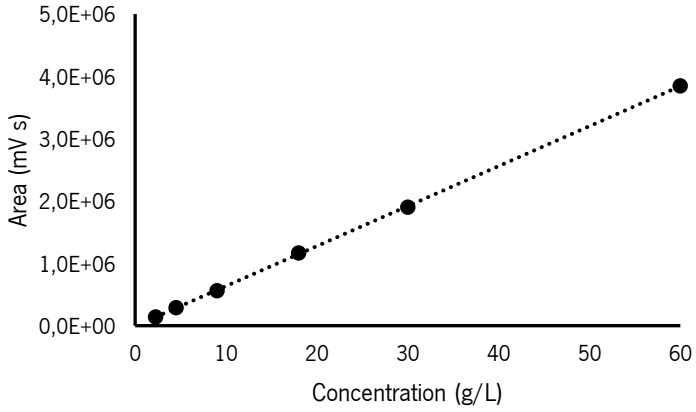
AI.4 1-KESTOSE (GF_2)



[GF_2] (g/L) = (69993 ± 20958) * Area (mV s) + (-2697 ± 731) (confidence level 95 %)

LOD (g/L) = 1 LOQ (g/L) = 3 R^2 = 0.99997

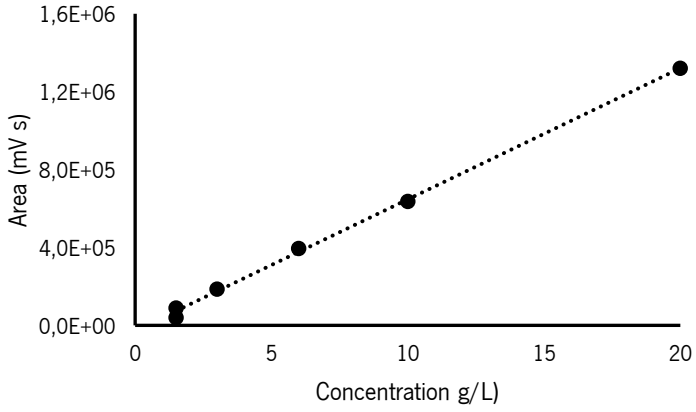
AI.5 Nystose (GF_3)



$[GF_3]$ (g/L) = (64148 ± 21726) * Area (mV s) + (-5805 ± 758) (confidence level 95 %)

LOD (g/L) = 1 LOQ (g/L) = 2 R^2= 0.99997

AI.6 1F-fructofuranosylnystose (GF_4)



$[GF_4]$ (g/L) = (67377 ± 35960) * Area (mV s) + (- 27125 ± 3758) (confidence level 95 %)

LOD (g/L) = 1 LOQ (g/L) = 3 R^2= 0.9992

ANNEX II– SPECIFICATION SHEET OF STRAWBERRY PREPARATION

Ficha de Especificações de amostra

Produto: Preparado de Morango

Cliente: FRULACT - INDÚSTRIA AGRO-ALIMENTAR,

Refª.: Ref.17348B

1 - DESCRIÇÃO DO PRODUTO

Preparado pasteurizado

3 - COMPOSIÇÃO (ORDEM DECRESCENTE)

SACAROSE		34	%
POLME MORANGO (à base de concentrado)		16,65	%
XAROPE DE GLUCOSE		15,4	%
GLICEROL	E422	4,4	%
PECTINA	E440(ii)		
ANTOCIANINA	E163		
ACIDO CITRICO	E330		
AROMA			
CITRATO TRISSODICO	E331(iii)		
ÁGUA		*q.s.p.* 100%	%

Nota:

Devem ser consideradas tolerâncias devido à variabilidade natural das matérias-primas.

Podem ser feitos ajustes a nível industrial de modo a assegurar as especificações do produto.

4 – Informação Nutricional por 100g

Energia			298 kcal / 1267 kJ
Lípidos		0,1	g
dos quais:			
Ácidos	Gordos	<0,1	g
Saturados			
Hidratos de carbono		75	g
dos quais:			
Açúcares		70	g
Proteínas		0,3	g
Sal		0,09	g

Souci Fachman Kraut (edição 07)

Ficha técnica da matéria-prima

USDA National Nutrient Database for Standard Reference

5 - Aplicação

- Produto: Pastelaria - Cobertura

6 - Características físico – químicas

Brix	º	76 ± 2
pH		3,6 ± 0,2
Densidade		1,40 ± 0,02

7- Informação Relativa a Segurança alimentar

7.1. Contaminantes/ Pesticidas / OGM /Radiação ionizante: Conforme Legislação Europeia em vigor.

7.2 - Corpos estranhos: Apesar de todas as medidas implementados no sentido de garantir a ausência de corpos estranhos, é possível, devido à natureza dos produtos que se verifique a presença de caroços, sementes e outros corpos estranhos de origem vegetal.

7.3 – Alergénios presentes: Nenhum.

Nota: Esta amostra foi elaborada num laboratório onde se utilizam alergénios

8 - Condições de conservação e Durabilidade mínima da amostra

- Pelo menos 30 dias em embalagem de amostra em refrigeração.