Universidade do Minho
Escola de Engenharia

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Biotechnological processes for D-tagatose production

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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: Biotechnological processes for D-tagatose production

Society growing awareness of excessive sugar consumption impact in health has led to an increased attention towards several sugar substitutes. Despite the existing alternatives in the market, D-tagatose possesses unique features. The enzymatic isomerization of D-galactose has achieved more projection as it is a more environmentally friendly compared to chemical isomerization. On the other hand, the search of renewable sources of D-galactose is necessary to attain a sustainable growth. Nevertheless, D-galactose production from renewable resources requires a pre-treatment that includes a hydrolysis of polysaccharides or disaccharides to obtain monomer sugars. Thus, the main objective of this thesis was the production of D-tagatose from several renewable resources by enzymatic isomerization of D-galactose using a L-arabinose isomerase from *Bacillus subtilis* (BSAI). For that, production and purification of BSAI enzyme was performed. Moreover, *Gelidium amansii*, κ-carrageenan and cheese whey were evaluated as sources of D-galactose by acid and enzymatic hydrolysis of complex sugars.

BSAI was produced and purified with a yield of 34 mg/g cell. Moreover, isomerase activity for D-galactose substrate was verified by HPLC (High performance liquid chromatography). The BSAI enzyme was used for D-tagatose production from D-galactose obtained from several raw materials. Regards, red seaweed pre-treatment for D-galactose production, 12% (w/w) of biomass using a ratio of 0.125 g of sulphuric acid/g at 150 °C for 10 min was suitable for the extraction of galactan as galactose achieving a concentration of 22.28 g/L. On other hand, optimization of hydrolysis of κ-carrageenan was performed by an experimental design. Under selected conditions (sulphuric acid concentration of 2.25% (w/w) and a treatment time of 45 minutes), percentage of hydrocolloid was increased to 8% (w/w) in order to obtain a liquor with approximately 20 g/L of D-galactose. Finally, cheese whey powder was enzymatically hydrolysed using a lactase (5UI/g of lactose) achieving 26.67 g/L of D-galactose.

For liquors from *Gelidium amansii* there are no significant differences in the conversion between the use of detoxified and non-detoxified liquors. So, HMF did not inhibited the action of the BSAI enzyme. For the same BSAI concentration (7 mg/mL) conversions of 50.9, 52.0, 55.6 and 27.8% were obtained in the isomerization assays with *Gelidium amansii* detoxified and non-detoxified liquor, cheese whey hydrolysate and κ-carrageenan hydrolysate, respectively. The results obtained are very similar to those obtained in the group previously with pure galactose solutions. Thus, the use of liquors instead of pure solutions does not affect BSAI enzyme activity.

Keywords: D-galactose; D-tagatose production; Enzymatic isomerization; Renewable resources.

RESUMO: Processos biotecnológicos para a produção de D-tagatose

A crescente consciencialização da sociedade sobre o impacto do consumo excessivo de açúcar na saúde levou a uma atenção crescente para vários substitutos do açúcar. Apesar das alternativas existentes no mercado, D-tagatose possui características únicas. A isomerização enzimática de D-galactose alcançou mais projeção, uma vez que é menos prejudicial para o ambiente quando comparada com a isomerização química. Por outro lado, a busca de fontes renováveis de D-galactose é necessária para alcançar um crescimento sustentável. No entanto, a produção de D-galactose a partir de recursos renováveis requer um pré-tratamento que resulte na hidrólise de polissacarídeos ou dissacarídeos para obter monómeros de açúcares. Assim, o principal objetivo desta tese foi a produção de D-tagatose a partir de vários recursos renováveis através da isomerização enzimática de D-galactose, utilizando a L-arabinose isomerase de *Bacillus subtilis* (BSAI). Para isso, foi realizada a produção e purificação da enzima BSAI. Além disso, *Gelidium amansii*, κ-carragenano e o soro de queijo foram avaliados como fontes de D-galactose por hidrólise ácida e enzimática de açúcares complexos.

A enzima BSAI foi produzida e purificada com um rendimento de 34 mg/g de células e a sua atividade para o substrato D-galactose foi verificada por HPLC (*High performance liquid chromatography*). A enzima BSAI foi utilizada para a produção de D-tagatose a partir de D-galactose obtida a partir de várias matérias primas. O pré-tratamento de *Gelidium amansii* foi adequado atingindo uma concentração de 22,28 g/L de D-galactose, com 12% (p/p) de biomassa utilizando uma proporção de 0,125 g de ácido sulfúrico/g a 150 °C durante 10 min. Por outro lado, a otimização da hidrólise do κ-carragenano foi realizada por um desenho experimental. Sob condições selecionadas (concentração de ácido sulfúrico de 2,25% (p/p) e tempo de tratamento de 45 minutos), a percentagem do sólido foi aumentada para 8% (p/p), a fim de obter um licor com aproximadamente 20 g/L de D-galactose. Finalmente, o soro de queijo foi hidrolisado enzimaticamente usando uma lactase (5UI/g de lactose) atingindo 26,67 g/L de D-galactose.

Utilizando os licores de *Gelidium amansii,* destoxificado e o não destoxificado, não se verificaram diferenças significativas na conversão. Assim, o HMF não inibiu a ação da enzima BSAI. Para a mesma concentração de BSAI (7 mg/mL), conversões de 50,9; 52,0; 55,6 e 27,8% foram obtidas nos ensaios de isomerização com o licor destoxificado e não destoxificado (*Gelidium amansii*), o hidrolisado do soro de queijo e o hidrolisado de κ-carragenano, respetivamente. Os resultados obtidos são muito semelhantes aos obtidos no grupo anteriormente com soluções puras de D-galactose. Assim, o uso de licores em vez de soluções puras não afeta a atividade da enzima BSAI.

Palavras-chave: D-galactose; Produção de D-tagatose; Isomerização enzimática; Fontes Renováveis.

TABLE OF CONTENTS

TABLE OF FIGURES

TABLE OF TABLES

ABBREVIATION LIST

AS – Artificial Sweeteners

BSAI – *Bacillus subtilis* L-Arabinose Isomerase

CV – Column Volumes

E – Eluted

EFSA – European Food Safety Authority

FDA – Food and Drug Administration

FT – Flow-Through

FW – Final Wash

GRAS – Generally Recognized As Safe

HMF – Hydroxymethylfurfural

HPLC – High Performance Liquid Chromatography

IF – Insoluble Fraction

IMAC – Immobilized Metal Ion Affinity Chromatography

IPTG – Isopropyl-β-D-thiogalactopyranoside

IW – Initial Wash

Kan – Kanamycin

L-AI – L-Arabinose Isomerase

LB – Luria-Bertani

PES – Polyethersulfone

PMSF – Phenylmethylsulfonyl Fluoride

RSM – Response Surface Methodology

SDS-PAGE – Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SF – Soluble Fraction

INTRODUCTION

1. INTRODUCTION

1.1. Contextualization

The prevalence of obesity worldwide has been largely attributed to excessive consumption of added sugars. Recent guidelines call for limiting the consumption of simple sugars to less than 10% of daily caloric consumption [1].

Artificial Sweeteners (AS) were initially developed and recommended as sugar substitutes in order to decrease caloric intake, increase weight loss and decrease the incidence of diabetes mellitus [2]. However, artificial sweeteners have adverse health effects, including glucose intolerance and weight loss failure. [1].

Society growing awareness of excessive sugar and AS consumption impact in health has led to an increased attention towards natural, low- or zero-calorie and plant-derived sweeteners. Despite the existing alternatives in the market, D-tagatose possesses unique features like taste and texture like sucrose, health benefits and its compatibility with several food matrixes.

1.2. Sweeteners

AS, also known as non-caloric high-intensity sweeteners [1] or non-nutritive sweeteners [2], are recognized as food additives by the European Food Safety Authority (EFSA) [3] and by the Food and Drug Administration (FDA) unless they are generally recognized as safe (GRAS). FDA approved six AS as food additives, aspartame, acesulfame-k, neotame, saccharin, sucralose and advantame [1].

Saccharin is the oldest AS and is 200 to 700 times sweeter than sucrose and is commonly used in soft drinks, candies, chewing gum, toothpaste, oral elixirs and medications [2]. This AS has an unpleasant bitter or metallic off-taste and it is only moderately soluble in water [4]. Aspartame is about 200 times sweeter than sucrose and is usually used as a tabletop sweetener, in chewing gum and soft drinks like saccharin [4], and in coffees and yoghurts [2]. Upon ingestion aspartame will lead to the formation of formaldehyde, formic acid and diketopiperazine, therefore its safety has been called into question [1]. Acesulfame-k is about 300 times sweeter than sucrose, while sucralose is approximately 600 times sweeter [2].

Most AS are low in calories and are not metabolized by the body or activate the sweet taste receptors at such low concentrations that the calories can be ignored. This is the main characteristic to have

occurred as an exponential increase of the consumption of AS for the last two decades [2]. Studies have shown that the consumption of AS does not reduce the risk of developing certain chronic diseases such as hypertension, stroke, renal disease, coronary artery disease, diabetes or obesity. It may even represent an equivalent or greater risk of developing these diseases when compared with the consumption of sucrose [5]–[11].

Newer products on the market include plant-derived compounds such as stevia [2] and sugar alcohols, also called nutritional sweeteners such as sorbitol, xylitol, lactitol, mannitol, erythritol and maltitol, which occur naturally in fruits and vegetables [12].

Stevia is a natural plant (*Stevia rebaudiana*) that contains steviol glycosides, which are used as sweeteners. These calorie-free glycosides, are 200 to 350 times sweeter than sucrose [13] and cannot be metabolized by the body [4]. FDA recognizes certain high purity steviol glycosides as GRAS, however the plant leaf and raw plant extract are not recognized as GRAS nor approved as food [13]. EFSA recognizes these steviol glycosides as food additives for use in several food categories [14]. Studies have indicated that this sweetener tends to lower high blood pressure and significantly improve the nutritional status of diabetic patients [15]. However, these glycosides have a bitter aftertaste, which limited their widespread commercial development [16].

In the European Union, sugar alcohols are considered food additives and their use in foods are controlled by the regulation which determines the approved food additives and their conditions of use [3]. In the US, the FDA classifies some as GRAS and some as approved food additives [17]. The most commonly used sugar alcohols are sorbitol and xylitol, and in general these nutritive sweeteners are slightly lower in calories than sucrose and they are not fermentable by oral bacteria therefore do not promote tooth decay. However, these alcohols have a lower sweetening power that varies from 25% to 100% of the sweetness of sucrose besides having a caloric value only slightly lower, which may restrict the use of these sweeteners. Furthermore these sugar alcohols do not cause health concerns associated with AS but may cause gastrointestinal discomfort and diarrhoea when consumed in large quantities [1].

Thus, reducing or stopping the consumption of any sweetener would be ideal, however this can be difficult to practice since the desire for sweet taste is a genetically predetermined behaviour, at least in a substantial subgroup of people [18].

1.3. Rare sugars - An alternative

Rare sugars represent the class of monosaccharides and their derivatives that rarely exist in nature [19]. Most monosaccharides are designed as rare sugars, only seven are known to be common sugars that exist amply in nature, including D-glucose, D-fructose, D-galactose, D-mannose, D-ribose, D-xylose and L- Arabinose. The rare sugars have great potential for use in the food and pharmaceutical industries, therefore they are studied extensively for biological and functional uses [20].

These monosaccharides have advantages such as palatability, absence of unpleasant aftertaste and low calories, not being metabolized by the body or being less metabolized than sucrose. There are several rare sugars studied as sugar substitutes, such as D-alulose (formerly known as D-psicose), D-tagatose, D-sorbose and D-allose (**Figure 1**) [1]. FDA recently approved D-tagatose and D-psicose as GRAS, these promising sugar substitutes will probably change the future of the sweetener market [20].

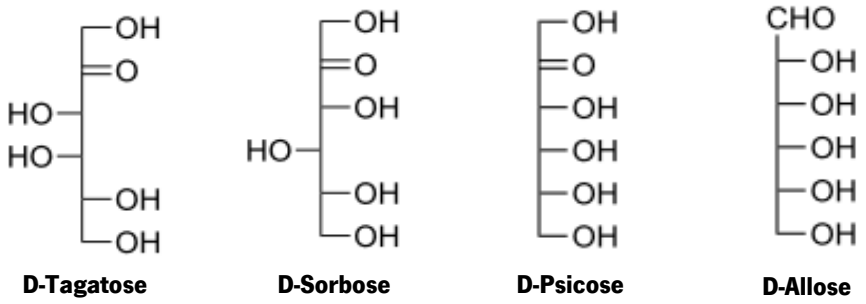

Figure 1. Rare sugars studied as sugar substitutes. Adapted from Z. Li et al [19].

Due to their scarcity in nature and expensive production methods, rare sugars are available only in small quantities and at a high cost [21].

1.3.1. D-tagatose

Tagatose, or more precisely, D-tagatose is an isomer of D-galactose and a stereoisomer of D-Fructose that occurs naturally in small amount in dairy products [22]. This rare sugar has several properties that make it an ideal sweetener (**Table 1**).

Table 1. Properties of D-tagatose.

Properties	Description
Molecular formula	$C_6H_{12}O_6$
Classification	Carbohydrate Monosaccharide Keto-hexose
Molecular weight	180 g/mol
Physical property	Anhydrous crystalline solid
Colour	White
Odour	None
Taste	Intensively sweet
Relative sweetness	90% of sucrose
Daily intake	4-5 g/day
Melting temperature	134 ºC
Solubility	High
Calorific value	1.5 kcal/g
Bulk sweetening	Yes
Caramel formation	Yes, it's a reducing sugar

This monosaccharide is a white anhydrous crystalline solid with a molecular weight of 180 g/mol, has no odour, your melting temperature is 134 °C and is highly soluble. For being a reducing sugar it can be used in browning reactions during baking or heating [23].

FDA recognized tagatose as GRAS for use in food in 2003 [23], since no toxic events have been reported [22]. In 2005, tagatose was formally approved as a novel food in the European Union, with the estimated daily intake for an average consumer of about 4-5 g/day, but larger intakes are conceivable [24].

In addition to its low calorie content (1.5 kcal/g), tagatose has a sweetening power (90%) taste and texture similar to sucrose [22], being used as a low-energy sweetener initially in products like chocolate candy, soft confectioneries, hard confectioneries, diet soft drinks, cereals, ice cream, frozen yogurt and diet chewing gum [25].

The metabolism of tagatose is identical as fructose but it is not completely absorbed by the body [4]. A study on the absorption of tagatose in humans which concluded that with the consumption of 15 g per day the apparent absorption of tagatose was 81% in the small intestine and that this sugar had only a minor influence on the apparent absorption of other nutrients [26]. Studies in humans, pigs and rats report urinary tagatose losses of about 1-5% of the ingested dose. Consumption of tagatose has a prebiotic effect, has been shown in animals that most, if not all, unabsorbed tagatose in the small intestine is metabolized by the large intestine microbiome [24], this metabolized tagatose fraction gives rise to short-chain fatty acids which are subsequently absorbed and metabolized [4].

Unlike fructose, which has a high glycosylation capacity and promotes lipogenesis, tagatose has a lower fat accumulation and lower glycosylation index [4]. Studies have also indicated that tagatose reduces postprandial glucose and insulin response, also stimulating weight loss [27]. These results have led to studies proposing tagatose as a possible antidiabetic drug [23]. Its mode of action is thought to be by inhibiting sucrose and maltose digestion, possibly by inhibiting sucrase and maltase, respectively, and/or by interfering with glucose transport [23]. Thus, substances such as glucose and especially fructose which promote lipogenesis and which have high glycosylation rates could be replaced by tagatose [4].

Tagatose is compatible with a wide variety of food products, and its consumption has health benefits [28], so an economical and viable industrial production of this rare sugar is necessary to compete with other commercial sweeteners.

1.4. D-tagatose production

There are different methods to produce D-tagatose, through oxidation of D-galactitol, bioconversion of D-psicose to D-tagatose and bioconversion of D-galactose to D-tagatose (chemical or enzymatic isomerization). **Figure 2** displays a scheme of different alternatives used for D-tagatose production, as well as, the substrates used for that. Chemical and enzymatic isomerization are currently employed for commercial scale production, but the cost is still an obstacle for the production of this rare sugar [29]. However, enzymatic isomerization has achieved more projection as it is a more environmentally friendly, since it does not use chemical catalysts and use milder conditions of temperature and pH.

The first biological production process to emerge was through the oxidation of D-galactitol using microorganisms such as *Arthrobacter globiformis* [30], *Mycobacterium smegmatis* [31], *Enterobacter agglomerans* [32] or *Gluconobacter oxydans* [33], [34]. The maximum yield in these biotransformation processes has been reported to be 92% [32]. Unfortunately, this process is difficult to be carried out in a large scale production due to the high cost and unavailability of D-galactitol as raw material [35].

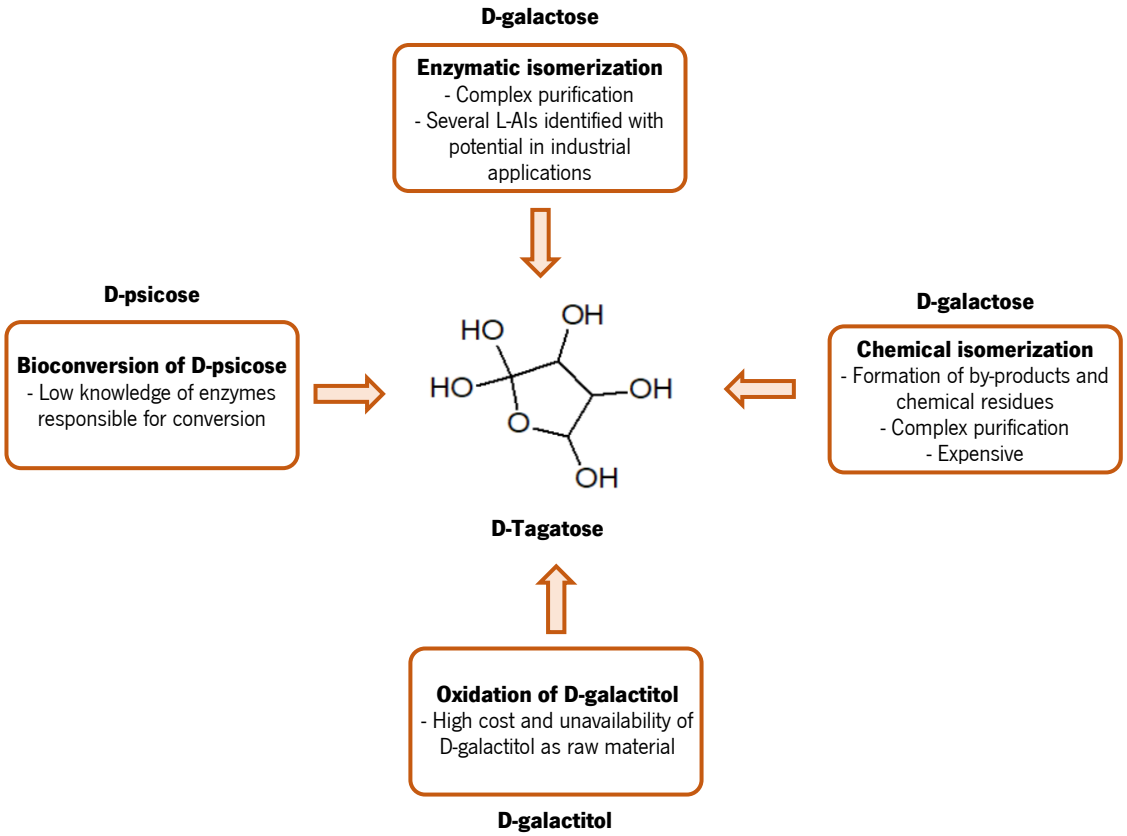

Figure 2. Methods to produce D-tagatose, as well as, the substrates used in each one. Characteristics of the different methods to produce D-tagatose. Chemical and enzymatic isomerization are currently employed for commercial scale production.

The method to produce D-tagatose by bioconversion of D-psicose uses various strains of *Mucoraceae* fungi. It is suggested that the *Mucoraceae* fungi probably converts D-psicose first to D-talitol by reduction and then to D-tagatose by oxidation. Enzymes such as oxidoreductases and/or dehydrogenases are supposed to be responsible for these redox reductions [36]. Recently, mass production of D-psicose has become economically viable using D-fructose as a raw material. D-psicose can be produced by epimerization of D-fructose using *Pseudomonas cichorii* D-tagatose-3-epimerase [37] or *Rhodobacter sphaeroides* [38], as well as *Agrobacterium tumefaciens* D-psicose 3-epimerase [39]. However, in order to make the conversion process economically viable, further investigation into the mechanisms of D-tagatose and D-talitol production, including the elucidation of enzymes responsible for their production, is required [36].

The production of D-tagatose based in the isomerization of D-galactose it is the most economical and viable option for large scale production. As referred above, D-galactose can be isomerized chemically or enzymatically [35].

The chemical method to produce D-tagatose was developed and patented by Biospherics Incorporated (USA). The patent describes that D-galactose can be isomerized to D-tagatose with calcium hydroxide in the presence of calcium chloride as a catalyst. Calcium hydroxide forms an insoluble complex with D-tagatose under a relatively low temperature but strongly alkaline conditions. The insoluble complex is treated with acid, normally carbon dioxide, in order to release the D-tagatose from the complex. The resultant insoluble calcium salt is removed by filtration and the remaining ions are eliminated by ion-exchange chromatography. The fraction containing the released D-tagatose is then concentrated and the pure D-tagatose is recovered by crystallization [40]. However, this method has some disadvantages, such as the formation of by-products and chemical residues, besides the complexity of the purification steps [41]. This process is also expensive because it requires intense cooling of the reaction mixture [35].

The enzymatic method to produce D-tagatose from D-galactose uses L-arabinose isomerase (L-AI, EC 5.3.1.4) as a biocatalyst (**Figure 3**). L-AI catalyzes the conversion of D-galactose to D-tagatose as well as the conversion of L-arabinose to L-ribulose, due to the similar configurations of the substrates. Several microorganisms have been studied as a source of L-AI [35]. Some of the newly identified L-AI have remarkable potential in industrial applications. D-tagatose biological production processes using L-AI have been developed and improved in recent years [42].

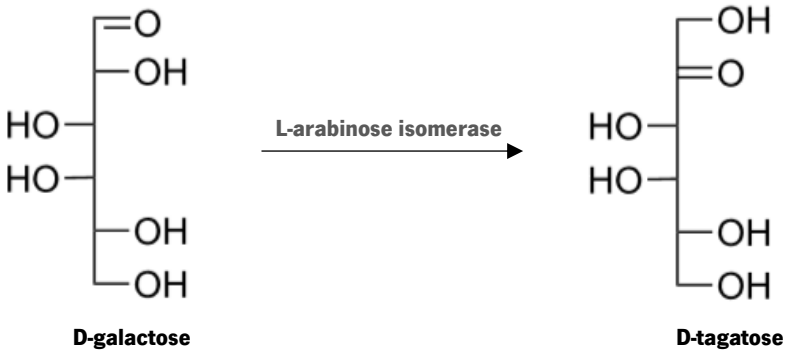

Figure 3. Enzymatic method to produce D-tagatose. Synthesis of D-tagatose from D-galactose using L-arabinose isomerase. Adapted from *Li et al.* [19].

1.4.1. Enzymatic method

Currently, several L-AI from different organisms have been identified. (**Table 2**). The major factors influencing the catalytic activity of L-AI are source organisms, pH, temperature and metal ion. The catalytic efficiency of L-AI varies from 0.4 to 10.3 (mM^{-1} min^{-1}) for isomerisation of D-galactose. The optimum

temperature for L-AI are 30 to 50°C for mesophilic bacteria, 60 to 80 °C for thermophilic bacteria and 85 to 90°C for hyperthermophilic bacteria. Several metal ions play a critical role on the functioning of L-AI.

Table 2. Sources and properties of several L-arabinose isomerase. Optimum temperature (°C), optimum pH, metal ion requirement, catalytic efficiency (mM^{-1} min^{-1}) and maximum bioconversion (%) of D-galactose to D-tagatose.

Microbial source	Optimum temperature (°C)	Optimum pH	Metal ion requirement	K_{cat}/K_M (mM^{-1} min^{-1}) (D-galactose)	Maximum Bioconversion (%)	Reference
Bacillus stearothermophilus US100	80	7.5-8.0	No requirement	8.5	48.0	Rhimi and Bejar [43]
Bacillus halodurans	50	7.5-8.0	No requirement	0.4	Negligible activity	Lee *et al.* [44]
Geobacillus stearothermophilus T6	70	7.0-7.5	Mn^{2+}, Mg^{2+}, Co^{2+}	4.3	38.0	Lee *et al.* [44]
Geobacillus stearothermophilus	60	8.0	Mn^{2+}	1.2	45.0	Kim *et al.* [45]
Geobacillus thermodenitrificans	70	8.5	Mn^{2+}, Co^{2+}	0.5	48.0	Kim and Oh [41]
Geobacillus thermodenitrificans C450S-N475K	70	8.5	No requirement	3.1	29.8	Kim *et al.* [46]
Geobacillus thermodenitrificans F280N-C450S-N475K	55	8.0	No requirement	4.7	54.6	Kim *et al.* [46]
Lactobacillus fermentum CGMCC2921	65	6.5	Mn^{2+}, Co^{2+}	9.0	55.0	Xu *et al.* [47]
Lactobacillus plantarum NC8	60	7.5	Mn^{2+}, Co^{2+}	1.6	30.0	Chouayekh *et al.* [48]
Lactobacillus sakei 23K	40	5.5	Mn^{2+}, Mg^{2+}	10.3	36.0	Rhimi M. *et al.* [49]
Thermoanaerobacterium saccharolyticum NTOU1	75	7.0	Mn^{2+}, Co^{2+}	2.4	NR	Lin *et al.* [50]
Alicyclobacillus hesperidum URH17-3-68	70	7.0	Mn^{2+}, Co^{2+}	1.2	43.0	Fan *et al.* [51]
Alicyclobacillus acidocaldarius	65	6.0	Mn^{2+}, Mg^{2+}, Co^{2+}	3.3	44.0	Lee *et al.* [52]
Acidothermus cellulolyticus ATCC 43068	75	7.5	Mn^{2+}, Co^{2+}	9.3	50.0	Cheng *et al.* [53]
Anoxybacillus flavithermus	95	9.5-10.5	Ni^{2+}	5.2	60.0	Li *et al.* [54]
Pediococcus pentosaceus PC-5	50	6.0	Mn^{2+}, Co^{2+}	2.9	52.0	Men *et al.* [55]

Bifidobacterium longum	55	6.0-6.5	Ca^{2+}, Mg^{2+}	0.7	36.0	Salonen *et al.* [56]
Thermotoga maritima	90	7.5	Mn^{2+}, Co^{2+}	8.4	56.0	Lee *et al.* [57]
Thermotoga neapolitana	85	7.0	Mn^{2+}, Co^{2+}	3.2	68.0	Kim *et al.* [58]
Bacillus coagulans NL01	60	7.5	No requirement	1.0	32.0	Mei *et al.* [59]
Shigella flexneri	40	8.0	Mn^{2+}, Co^{2+}	0.1	22.3	Patel *et al.* [60]
Lactobacillus brevis	65	7.0	Mn^{2+}, Co^{2+}	0.12	43.0	Du *et al.* [61]
Enterococcus faecium DBFIQ E36	50	5.5	Mn^{2+}	0.68	45.0	Manzo *et al.* [62]
Paenibacillus polymyxa	30	7.5	Mn^{2+}	Not reported	41.7	Kim *et al.* [63]

Mesophilic L-AI are believed to provide a good biological conversion of D-galactose to D-tagatose, however studies of thermophilic L-AI have shown that they have better conversion [41], [58], [64]. This is because the interconversion equilibrium between D-galactose and D-tagatose shifts toward D-tagatose at higher temperatures, so higher thermal stability biocatalysts are more efficient [19].

Most characterized L-AI require divalent metal ions such as Mn^{2+} or Co^{2+} for high activity and stability, however ideally the isomerization reaction should be performed without the addition of these ions, especially Co^{2+} ions, that are not accepted in food products [65]. L-AI which are independent of metal cofactors and are active at low temperatures have been of great interest [28]. Thus, an L-AI optimized for isomerization of D-galactose to D-tagatose at an industrial level should have high activity at acidic pH, be thermostable and have higher affinity for D-galactose [65].

The isomerization activity of L-AI for D-galactose is relatively low compared to the natural substrate (L-arabinose). The production of D-tagatose could be enhanced by protein engineering and immobilization of L-AI.

Enzymatic engineering tools, such as directed evolution and site directed mutagenesis, have been used to improve the efficiency of L-AI concerning the D-galactose substrate. Site directed mutagenesis of L-AI from *Geobacillus thermodenitrificans* [46] and *Bacillus stearothermophilus* [66], resulted into increase of the specific activity, catalytic efficiency, substrate affinity and change of optimum pH. Another example is the use of direct evolution approach in L-AI enzyme of *Geobacillus stearothermophilus* [45] that leads to higher enzyme activity.

Current methods to produce D-tagatose such as the use of packed-bed reactor containing immobilized recombinant *Escherichia coli* cells or immobilized L-AI enzymes attempt the establishment of efficient continuous production systems. This kind of methods improves the biochemical properties of recombinant cells and L-AI enzymes, such as the optimal temperature and thermostability. Ordinarily, the most widely used material for immobilization is the alginate because it is inexpensive and viable to operate [42].

Mostly immobilized L-AI leads to higher productivity when compared to cell immobilization, due to that no other proteins could impede biocatalysis [42]. A study in which a packed-bed reactor loading alginate immobilized *Geobacillus stearothermophilus* L-AI obtains a productivity of 54 g L^{-1} h^{-1} for D-tagatose, being considered the highest to date [67]. Although, cell immobilization is more viable and economical due to the elimination of enzyme purification steps which naturally decrease the cost of production. These two types of immobilization are at a comparable level because both require large amounts of cells or enzymes, so a culture technique is required to obtain high cell densities [42].

The purification of D-tagatose is another challenge in the enzymatic process due to the similar properties between D-tagatose and D-galactose [19]. However, a method for the purification of D-tagatose has already been described using a strain of *Saccharomyces cerevisiae* which selectively metabolizes D-galactose thus D-tagatose could be obtained at above 95% purity [68].

The industrial process of isomerization of D-galactose should focus on the use of L-AI enzymes, since enzymatic isomerization offers several advantages compared to chemical isomerization, namely milder temperature and pH conditions, absence of by-product formation and the possibility of improving the biocatalyst through molecular techniques [65]. Also, a GRAS host should be used for L-AI expression to avoid possible food safety problems [21]. Thus, an efficient and cost-effective strategy is yet to be developed for mass production of D-tagatose.

1.5. *Bacillus subtilis* L-arabinose isomerase

Kim *et al*. characterized the L-AI enzyme from *Bacillus subtilis* (BSAI), so the araA gene was identified and cloned from *Bacillus subtilis* and expressed in *Escherichia coli*, confirming that the product of this gene exhibited L-AI activity. The araA gene encodes a 496 amino acid polypeptide with a calculated molecular weight of 56 kDa, and results obtained later by performing an SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) were following the predicted molecular mass. This L-AI occurs

mainly as homodimers with a subunit molecular mass of 53 to 57 kDa, the results obtained in this study indicated that the BSAI enzyme also migrates as a homodimer (approximately 115 kDa of molecular weight) and was present and active in this form in solution [21].

According to the same study the optimum pH of BSAI for isomerization of the natural substrate L-arabinose is 7.5, this optimum alkaline pH is common to similar enzymes isolated from other microorganisms. The isoelectric point of this enzyme is 4.9, which meets the estimated value of the amino acid sequence. The optimal temperature for L-arabinose isomerization is 32°C [21]. BSAI activity is not stimulated by Mg^{2+}, Ca^{2+}, Zn^{2+}, Fe^{2+} or Ni^{2+} cofactors, whereas Cu^{2+} significantly inhibits enzymatic activity. The metal ion Mn^{2+} increases enzyme activity 42-fold and appears to be necessary for catalytic activity, at least for L-arabinose substrate.

Kim *et al.* reported that BSAI has no activity for D-galactose substrate, suggesting that this L-AI has unique substrate specificity for L-arabinose [21]. However, an earlier study reported by Roh *et al.* [69] proved that this enzyme has activity for the D-galactose substrate. Furthermore, preliminary results obtained in the research group where I develop this project also show that BSAI has activity for the D-galactose substrate, being able to isomerize D-galactose to D-tagatose, from pure D-galactose solutions.

Roh *et al.* [69] obtained a final concentration of tagatose of 0.56 g/L from colonies expressing araA from *Bacillus subtilis.* The conversion medium used contained 10 g of D-galactose in 3 mL of medium and the cells resuspended are maintained at 37°C for 72 h. So, there is a need to improve the purification and characterization of araA and the optimization of the enzymatic process for the high production of D-tagatose in order to get more concluding results.

1.6. Renewable Raw Materials for a Sustainable Production of D-tagatose

The use of renewable resources can provide environmental, economic and strategic benefits. The latest progress in biotechnology has permitted the economical use of agro-industrial residues, agro-food residues, as well as, marine biomass. In most cases these materials are already used being normally discarded, sometimes causing some environmental impact [70]. For the use of these renewable sources, a pre-treatment is usually required in order to hydrolyse the complex sugars into monosaccharide sugars that are used as substrates.

Lately, the production of rare sugars like tagatose from various monosaccharides has increased, as these sugars have biological functions that are suitable for industrial applications, including the food, cosmetic, and pharmaceutical industries. Therefore, as described above, galactose is the most sustainable substrate for tagatose production. In this sense, there are several renewable sources of galactose (**Table 3**) that can be used to produce tagatose or other high value-added products.

Table 3. Some renewable sources of D-galactose. These renewable sources can be used to produce tagatose or other high value-added products.

Renewable Sources of D-galactose	Reference
Watermelon rinds	Romdhane *et al.* [71]
Potatoes peels	Jeddou *et al.* [72]
Soybean residue	Nguyen *et al.* [73]
Onion juice residue	Kim *et al.* [63]
Spent coffee ground	Jooste *et al.* [74]
Pea pod	Belghith-fendri *et al.* [75]
Cheese whey	Zheng *et al.* [76]
Gelidium amansii (red seaweed)	Jang *et al.* [77]

A galactose content of 3.84, 26.2, 2.5, 18.0 and 49.32% has been reported for potatoes peels [72], onion juice residue [63], spent coffee ground [74], pea pod [75] and *Gelidium amansii* [77], respectively. Nguyen *et al.* [73] reported that soybean residue has in its composition 39.3 g/L of galactose, while Romdhane *et al.* [71] reported that in the watermelon rinds galactose is the dominant sugar in the extracted polysaccharides. Cheese whey is one of the best sources of galactose, since its composition is mostly lactose (69%) [76] and lactose can be easily hydrolysed to galactose and glucose.

Interestly, onion juice residue was used for the production of rare sugars and bioethanol following the biorefinery concept achieving a tagatose bioconversion yield of 41.7% [63]. However, red seaweed have recently received much attention as a source of renewable biomass due to its abundant carbohydrate content, low lignin content and lack of conflicts for food production [77], [78], [79]. There is a growing interest in red seaweed compounds with biomedical properties, and after extraction of these compounds, red seaweed residues have no value and can be used as substrates for chemical and/or microbial processes to obtain high value-added products, like tagatose.

The main processes to produce monosaccharides sugars (namely, galactose) will be described in the next sections, since the methodology used is dependent of renewable resources and structure.

1.6.1. Red seaweed

Seaweed is generally categorized into three groups: red algae (*Rhodophyta*), green algae (*Chlorophyta* and *Charophyta*), and brown algae (*Phaeophyceae*) [80]. Red algae are still classified into agarophytes and carrageenophytes based on their main carbohydrate types, agar and carrageenans, respectively [80]. Compared to brown algae, red algae have a higher carbohydrate content, in addition to their simple carbohydrate composition, since red algae are composed of polysaccharides with galactose units (agar and carrageenan) and glucose units (cellulose). Thus, red algae have advantages regarding saccharification and fermentation [79].

Pre-treatment of red algae is essential to increase substrate accessibility to enzymes through fibre fractionation, increasing substrate solubility in water. One of the advantages of red algae is that it has no lignin or only a small amount, resulting in easier and cheaper pre-treatment. Currently, the most commonly used pre-treatment methods for these algae fractionation are physical (e.g., microwave assisted extraction), hydrothermal (e.g., hot water extraction) and chemical processes (e.g., alkaline modification). Hot water extraction is the most commonly used hydrothermal pre-treatment method for extracting macroalgae agar and carrageenan due to its simplicity and increased solubility in high temperature agar and carrageenan water (above 85 °C) [81].

The first saccharification process developed for red macroalgae was chemical hydrolysis using preferably acid catalysts such as sulphuric acid, hydrochloric acid and acetic acid. There are other saccharification processes such as enzymatic hydrolysis or even the use of acid and enzymatic hydrolysis together [81]. However, enzymatic hydrolysis has some disadvantages as is high cost and complexity of the saccharification process, as well as the high cost of commercial enzymes available [77].

Acid hydrolysis is the most attractive method because it is simple, rapid, and cheap to hydrolyse red seaweed into monosaccharides. Usually, hydrolysis is done with dilute acid in an autoclave at 121 °C for 60 min. Acid hydrolysis depends on acid type, acid concentration, temperature and pressure. However, hyper thermal acid hydrolysis has been used to increase fermentable sugars and require short processing time [82].

1.6.1.1. *Gelidium amansii*

One of the most abundantly available red seaweed species is *Gelidium amansii,* appearing along the warm and shallow coastal area of many sub-tropical countries being an excellent renewable source [80]. The *Gelidium amansii* mainly consists of polysaccharide complexes of cellulose and agar (galactan) whose monomer is glucose and galactose, respectively [83].

Agar that is the main carbohydrate of agarophytes, like *Gelidium amansii*, is composed of neutral agarose as the major component and charged agaropectin as the minor component. Agarose is a polysaccharide composed of equal molar amounts of D-galactose and 3,6-anhydro-L-galactose. Agaropectin is a polysaccharide consisting of alternating units of D-galactose and 3,6-anhydro-L-galactose, which are highly substituted by ester-sulfates, methyl groups, or pyruvates. Depending on the agarophyte genus, origin, and harvesting season, the ratio of agarose and agaropectin in the composition varies. The agarose content in agar is much higher in species like *Gelidium* and *Pterocladiathan* than *Gracilaria* species [81].

The main products obtain with dilute-acid hydrolysis of *Gelidium amansii* in a batch-type autoclave are D-galactose, 3,6-anhydro-L-galactose, and D-glucose [80]. Galactose and 3,6-anhydro-L-galactose are to be firstly released at relatively mild hydrolysis conditions due to the weak structure of agar comparatively to cellulose. For economic reasons, acid hydrolysis (**Figure 4**) is usually used to hydrolyse seaweed and enhance cellulose accessibility for subsequent enzymatic saccharification to overcome the low yield of glucose production [83].

Sulphuric acid is mainly used for acid hydrolysis due to its advantages of short reaction times and low cost. However, it has the disadvantages of requiring neutralization due to the acid used, and the production of sediment during neutralization [84]. Furthermore, the 3,6-anhydro-L-galactose is so acid-labile that it is very subject to be decomposed into hydroxymethylfurfural (HMF) and, consequently, into organic acids such as levulinic acid and formic acid that act as inhibitors in the fermentation process [80].

Several authors have evaluated the acid hydrolysis for galactose production [82], [85], [83], [80], using different reaction temperature (121 to 170 ºC), sulphuric acid concentration (0.5 to 6.0 %), and reaction time (2.5 to 45 min). Most of these works studied this pre-treatment to obtain fermentable sugars for bioethanol production. Nevertheless, there are no studies on the use of any seaweed to produce sweeteners or rare sugars like tagatose.

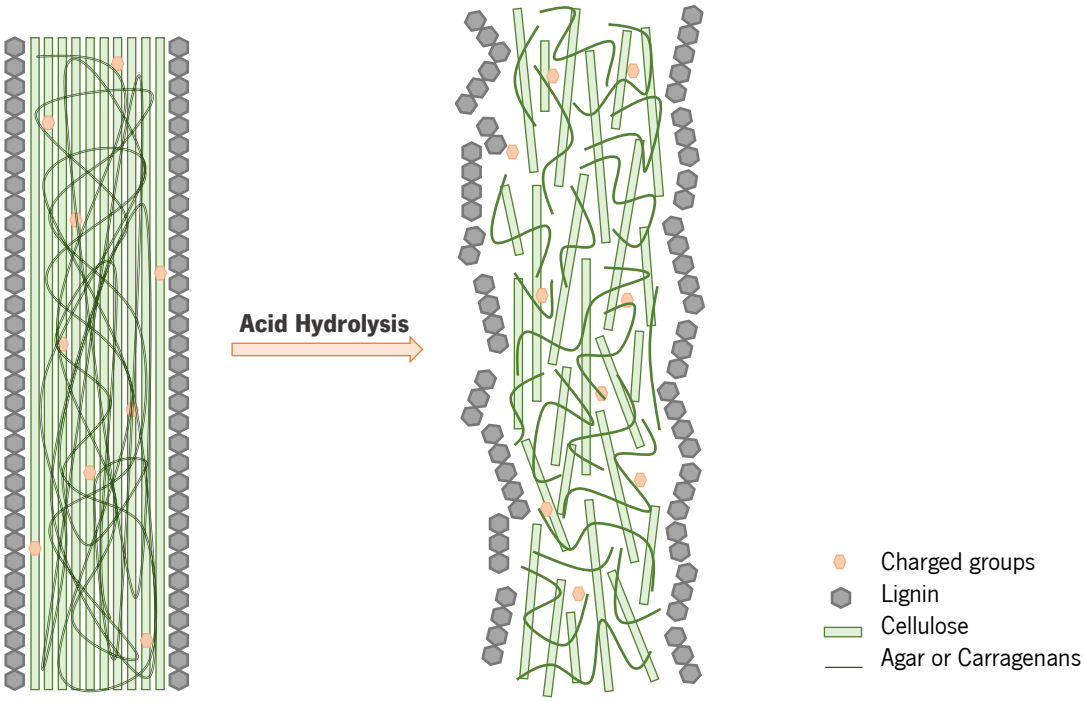

Figure 4. Acid hydrolysis of red seaweed to produce monosaccharides. Effect of pre-treatment on lignin, cellulose and agar or carragenans structures. Adapted from Yun *et al.* [82].

It is known that the sugar yields and the concentration of inhibitor essentially depend on the three major factors, reaction temperature, acid concentration, and reaction time. Usually, with more severe hydrolysis conditions a higher yield of sugars is obtained, however the amount of inhibitor also increases. Thus, the dilute-acid hydrolysis reaction should be designed toward increasing the yield of sugars and decreasing that of inhibitors [80].

The HMF has been known as the main inhibitor produced in red seaweed hydrolysates. Hydrolysate detoxification is required in some cases, activated carbon is widely used for that purpose as it is a cost-effective method with high capacity to absorb inhibitors without affecting hydrolysate monosaccharides levels [86].

1.6.1.2. κ-carrageenan

Carrageenans are isolated from red algae, and exploited on a commercial scale for use in food [87] preparation for its gelling, thickening, and emulsifying properties having none nutritional value [88]. These carbohydrates are sulfated polygalactans composed by alternate units of D-galactose and 3,6-anhydro-D-galactose (**Figure 5**) with 15 to 40% of ester-sulfate [81]. D-galactose is a fermentable sugar that can be

used by microorganisms, and most studies have focused on its production from carrageenan by acid hydrolysis. However, 3,6-anhydro-D-galactose is a rare, non-fermentable sugar and its production and metabolism have not yet been studied [89].

They are classified as κ, ι, and λ types based on their solubility in potassium chloride and their gel-forming ability [87]. Among the different types of carrageenans, κ-carrageenan (**Figure 5**), which forms a strong and rigid gel, contains the lowest content of ester-sulfates with 25 to 30% and the highest content of 3,6-anhydro-D-galactose with 28 to 35% [81].

These polygalactans are water soluble forming highly viscous aqueous solutions. However, viscosity depends on concentration, temperature, the presence of other solutes, and the type of carrageenan and its molecular weight. The viscosity of the aqueous solutions increases with concentration and decreases with temperature. Thus, carrageenans are susceptible to be hydrolysed through acid-catalysed hydrolysis at high temperatures and low pH [88]. κ-carrageenan hydrolysis results in a monomeric sugar-rich hydrolysate (**Figure 5**) and can therefore be used in bioprocesses that need simple sugars that are used as substrates.

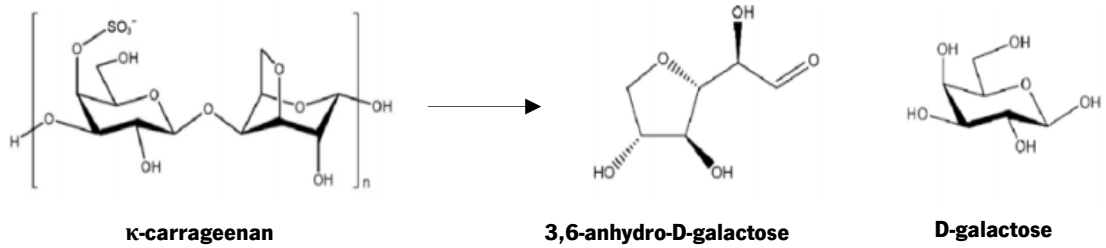

Figure 5. κ-carrageenan hydrolysis. Monomeric sugar-rich hydrolysate with 3,6-anhydro-D-galactose and D-galactose. Adapted from Kim and Lee [85].

1.6.2. Cheese whey

Cheese whey is an abundant residue generated during cheese production. After the cheese curds, only about 10% of the milk used is converted to cheese, the remaining liquid is a by-product called cheese whey, which still contains about 55% of the milk's nutritional load. Whey composition depends on several factors (e.g. milk quality, animal breed and feed), however high concentrations of lactose (about 45 g/L) and protein (about 6-10 g/L) are generally present. These amounts correspond to the total amount of lactose contained in milk and about 20% of milk proteins [90].

The current total worldwide production of whey is estimated at about 180 to 190 million tons/year [91], this waste cannot be disposed of in water systems without pre-treatment mainly due to its high lactose content, thus representing an environmental problem. However, this residue can be recovered rather than discarded, for example, by recovering the protein fraction which is separated by ultrafiltration or diafiltration to protein concentrates that can be used in the food, cosmetic and pharmaceutical industries. After this process, the remaining liquid, designated whey permeate, has the same whey lactose concentration and is still a very polluting residue [90]. The future trend for cheese factories is to move towards zero discharge, away from the high disposal costs and to find a friendly and more cost-effective lactose applications. Drying is one of the current solutions implemented in the industry to valorize whey [92].

Whey maybe directly used for fermentation using microorganism that metabolizes lactose for its bioconversion. However, in several cases lactose hydrolysis is performed before fermentation, when microorganisms are unable to hydrolyse lactose. The number of microorganisms capable of metabolizing lactose as a source of carbon is smaller than the number of microorganisms capable of metabolizing glucose and galactose. Hydrolysis of lactose into monosaccharides (glucose and galactose) is performed either with the enzyme β-galactosidase (**Figure 6**) or by acid hydrolysis [91]. β-Galactosidases may have an animal, vegetable, or microbial origins, but the microbial enzymes are more widely used due to this higher productivity, resulting in cost reduction. The β-galactosidases used in industrial-scale must come from GRAS microorganisms. The choice of the β-galactosidase used usually depends on the hydrolysis reaction conditions [93].

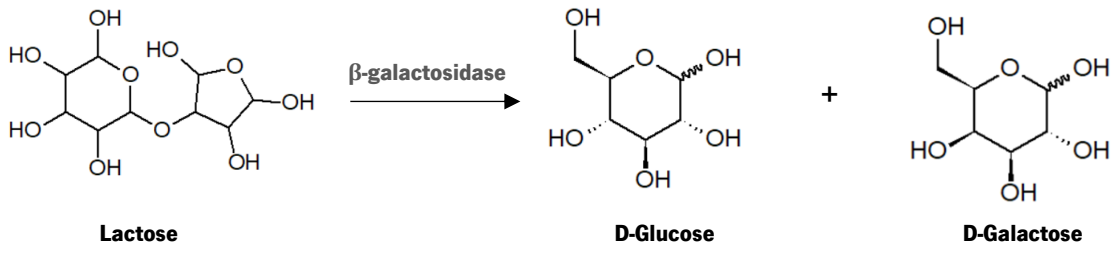

Figure 6. Hydrolysis of lactose into monosaccharides by β-galactosidase. Monomeric sugar-rich hydrolysate with glucose and galactose.

Cheese whey is undoubtedly the most widely used and reported renewable source to produce tagatose [77],[28]. Jayamuthunagai *et al.* [28] produced tagatose by direct addition of alginate immobilized *Lactobacillus plantarum* cells expressing L-AI to lactose hydrolysed whey permeate. They

obtained the maximum conversion of 38% with successive lactose hydrolysis by β-galactosidase from *Escherichia coli* and galactose isomerization using L-AI from *Lactobacillus plantarum*. Zheng *et al*. obtained a total of 23.5 g/L of tagatose from cheese whey powder containing 100 g/L lactose.

OBJECTIVES

2. OBJECTIVES

An industrial economic and sustainable production of tagatose is required to compete with the other commercial sweeteners. The industrial process of isomerization of galactose should focus on the use of L-AI enzymes, since enzymatic isomerization offers several advantages compared to chemical isomerization. Preliminary results obtained in the research group where I developed this project show that BSAI enzyme has activity towards the galactose substrate, being able to isomerize galactose to tagatose, from pure galactose solutions.

Therefore, the main objective of this thesis was the production of tagatose from several renewable resources by enzymatic isomerization of galactose using a BSAI enzyme. For that, the following specific objectives were proposed:

- Production and purification of BSAI enzyme to be used in the bioconversion of galactose into tagatose;
- Characterization of red seaweed (*Gelidium amansii*) as sustainable source of galactose;
- Hydrolysis of *Gelidium amansii*, κ-carrageenan and cheese whey to obtain enriched liquor in galactose;
- Evaluation of enzymatic isomerization for tagatose production using several galactose streams.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Recombinant L-arabinose isomerase expression, production and purification

3.1.1. Sterilization procedures

All materials and culture media used for bacteria were sterilized by autoclaving at 121 °C during 20 min at a pressure of 1 bar. Thermolabile solutions were sterilized by filtration with 0.20 μm sterile Polyethersulfone (PES).

3.1.2. Strain and culture medium

Escherichia coli BL21 (DE3) previously transformed with PETM10_*ara*A vector was used as protein expression system. Briefly, the *ara*A gene from *Bacillus subtilis* str. 168 which encodes for L-arabinose isomerase was cloned into the PETM10 expression vector. This vector carries the kanamycin-resistance gene, T7lac promoter and includes a C-terminal His6-tag.

The bacteria *Escherichia coli* (*E. coli*) cells were cultured in Luria-Bertani (LB) medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) sodium chloride (NaCl), at pH 7.5 supplemented with 50 μg/mL kanamycin (LB+kan). The strain was also grown in the corresponding solid media, supplemented with 2% (w/v) agar.

3.1.3. Recombinant L-arabinose isomerase expression and purification

E. coli BL21 (DE3) cells expressing the *ara*A gene were cultivated overnight at 37 °C and 150 rpm in Erlenmeyer flasks filled with 20 mL of LB+kan medium. The cell suspension was 100-fold diluted with fresh LB+kan, to a final volume of 250 mL, and grown again to an OD600 of 0.5-0.6. At this point, the protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM and the culture was incubated at 37 °C for 16h. The *E. coli* cells were harvested by centrifugation (at 4 °C for 20 min at 10 000 rpm) from 125 mL culture fractions and cell pellets were stored at -20 °C.

3.1.3.1. Cellular disruption of *Escherichia coli*

Cell-pelleted culture was resuspended in lysis buffer (20 mM sodium phosphate, 20 mM NaCl, pH 7.4) supplemented with 1mM of a protease inhibitor, phenylmethylsulfonyl fluoride (PMSF) and mixed by vortexing. Cells were disrupted by sonication (Cole-Parmer 750-Watt Ultrasonic Homogenizer, 230 VAC) on ice at 38% amplitude (30S ON, 30S OFF) for 8 cycles. After 30 min of centrifugation at 4°C and 10000 rpm to remove insoluble cell debris, the supernatants (soluble fraction, SF) were firstly separated from the pellet (insoluble fraction, IF) for purification by immobilized metal ion affinity chromatography (IMAC).

3.1.3.2. IMAC protein purification

The recombinant L-arabinose isomerase in the SF was purified by IMAC, using 5 ml Nickel His-Trap column (GE Healthcare).

Initially, SF was adjusted to a final concentration of 20 mM, to enhance the purification efficiency, and filtered through a 0.22 μm filter.

Before starting the IMAC purification, using a peristaltic pump (ISMATEC®), the column of purification was charged with the nickel solution (0.1 M $NiSO_4$) and washed with approximately 10 column volumes (CV) of filtered distilled water to remove the excess of nickel. The IMAC purification begins with the equilibration of the column with 6 CV of binding buffer. All solutions used for enzyme purification and applied to the column were filtered a 0.22 μm filter. The composition of IMAC solutions is described in **Table 4**.

The SF was then slowly loaded on the column (2 mL/min flow rate) and the unbounded proteins were washed out from the column with a binding buffer using 12 CV. The BSAI protein was eluted from the column with an elution buffer.

Finally, the stripping buffer was loaded to the column and the storage was performed under the manufacter's instructions. Aliquots of FS, flow-through (FT), washing and eluted (E) were taken during the procedure for SDS-PAGE analysis.

After purification, in order to remove imidazole from each eluted, buffer was exchanged using PD-10 Desalting Columns contain Sephadex G-25 (GE Healthcare). Briefly, the column was filled up with 25 mL of equilibration buffer (50 mM potassium phosphate, 0.05 mM $MnCl_2$, pH 7.5) and was allowed the equilibration buffer to enter the packed bed completely. The eluted fractions were applied one at a time.

Table 4. Composition of IMAC solutions for BSAI enzyme purification. Binding buffer, elution buffer and stripping buffer. All solutions that are applied to the column were filtered and the pH adjusted to 7.4.

Solution	Composition
Binding buffer	20 mM sodium phosphate
	0.5 M NaCl
	40 mM imidazole
Elution buffer	20 mM sodium phosphate
	0.5 M NaCl
	300 mM imidazole
Stripping buffer	20 mM sodium phosphate
	0.5 M NaCl
	50 mM EDTA

3.1.3.3. Protein quantification

Protein concentration of each elute was measured through the optical density at 280nm (NanoDrop One; Thermo Scientific) considering the extinction coefficient of this protein (ε=1,644).

3.1.3.4. SDS-PAGE analysis

Protein marker PageRuler™ Unstained Broad Range Protein Ladder (5-250 kDa; Thermo Scientific) was used as a molecular weight marker. The samples (16 μL) were resuspended in 4 μL of 5 x SDS-PAGE Sample Loading Buffer (NZYTech) and then incubated at 100 °C for 5 min. Then, 15 μL of the samples and 5 μL of protein marker were loaded into the gel. Gels were run in Tris-Glycine-SDS-PAGE buffer (0.025 M Tris, 0.192 M glycine and 0.10 % sodium dodecyl sulphate, SDS) at 15 mA. For staining, gels were incubated in BlueSafe (NZYTech) at room temperature with slow shaking until complete staining. Then, gels were washed with distilled water. The images of the gels were obtained using a transilluminator Gel Doc 2000 (BioRad).

The IF was completely resuspended in distilled water and then 16 μL were resuspended in 4 μL of 5 x SDS-PAGE Sample Loading Buffer (NZYTech) and treated as previously described.

3.2. Hydrolysis of several raw materials to produce liquors rich in D-galactose

3.2.1. Raw materials

The raw seaweed *Gelidium amansii* was purchased from Iberagar SA and the κ-carrageenan was purchased from Sigma-Aldrich. Cheese whey was kindly provided by Lactogal Produtos Alimentares S.A. The composition of cheese whey powder was previously analysed and was composed by (% w/w, dry weight) 58.5 % of lactose, 12.6 % of protein and less than 0.2 % of lipids [94].

3.2.2. Chemical characterization of *Gelidium amansii*

In order to determine the structural components of *Gelidium amansii*, a quantitative acid hydrolysis was performed. On the first stage of the process, polysaccharides were converted into oligosaccharides through the addition of 5 mL of sulphuric acid concentrated at 72 % (w/w) to a sample of approximately 0.5 g of raw material and placed for 60 min in a water bath at 30 °C. After this, distilled water was added to the tube in order to stop the reaction. The tube content was transferred to a 250 mL Schott flask and filled with water until final weight of 148.68 g was achieved. With this, sulphuric acid concentration dropped to 4 % (w/w), and the solution was introduced in autoclave for 60 min at 121 °C, beginning the second stage of this hydrolysis, where the oligosaccharides were converted into monomers. After cooling the flasks, these were dried and weighted to access the losses during the second stage.

Sample was filtered by vacuum in a Kitasato flask, using a glass Gooch crucible number 3, separating the liquor with the sugars from the lignin fraction (or acid insoluble solid) retained in the filter. The filter was kept for 24 h in an oven at 105 °C for posterior quantification. The liquor was analysed by High performance liquid chromatography (HPLC). Polysaccharides content was determined by glucose and galactose concentration in liquor after acid hydrolysis.

3.2.3. Hydrothermal pre-treatment and enzymatic saccharification of *Gelidium amansii*

Hydrothermal pre-treatment *of Gelidium amansii* was performed in order to hydrolyse the agar into oligosaccharides. The biomass was washed with distilled water to remove salinity and impurities, followed by drying at 50 °C in an incubator overnight. In the final volume of solution to be added, algae moisture

measured by dry weight was considered. In a reactor (Series 4520 Bench Top Reactor; Paar) of stainless steel, 4% (w/w) of dry biomass was packed with 400 mL of distilled water.

Approximately 5 min of preheating was necessary to reach the desired temperature (140 and 170 ^{0}C). Reaction temperature was controlled using temperature controller (Series 4848 Reactor Controller; Paar). Mixing was done at approximately 200 rpm. Once the reaction was complete, the reactor was cooled down to room temperature.

After hydrothermal pre-treatment, *Gelidium amansii* was submitted to enzymatic saccharification in order to hydrolyse the oligosaccharides into monomeric sugars. The enzymatic saccharification after hydrothermal pre-treatment was performed by adding 20 FPU/g substrate of Cellic CTec2 (Novozyme) or mixed enzymes by adding the same load of Cellic CTec2 and Viscozyme L (Novozyme) in proportion 1:2. The reaction was carried out in an orbital incubator at 50 ^{0}C, 150 rpm for 2 days. Samples were withdrawn at desired times and analysed by HPLC to determine the hydrolysis efficiency.

3.2.4. Acid hydrolysis pre-treatment of *Gelidium amansii*

In order to obtain galactose from *Gelidium amansii*, dilute acid hydrolysis was selected as pre-treatment. The biomass was washed with distilled water to remove salinity and impurities, followed by drying at 50 °C in an incubator overnight. In the final volume of solution to be added, algae moisture measured by dry weight was considered. In a reactor (Series 4520 Bench Top Reactor; Paar) of stainless steel, 4%, 8% and 12% (w/w) of dry biomass was packed with 400 mL of the desired sulphuric acid concentration (0.5 and 1.5% (w/w)).

Approximately 5 min of preheating was necessary to reach the desired temperature (130, 140 and 150 ^{0}C). Reaction temperature was controlled using temperature controller (Series 4848 Reactor Controller; Paar). Mixing was done at approximately 200 rpm. Once the reaction was complete, the reactor was cooled down to room temperature. Solid fraction and liquid fraction were separated by filtration to be analysed. Solid phase was analysed following the procedure described in section 3.2.2. Liquid phase was analysed directly by HPLC for determination of monosaccharides.

3.2.5. Acid hydrolysis pre-treatment of κ-carrageenan

Optimization of operational conditions to hydrolyse κ-carrageenan were carried out using a factorial experimental design. The following variables were evaluated: treatment time (15, 30 and 45 min) and sulphuric acid concentration (0.5, 2.25 and 4.0%, (w/w)). The temperature (126°C) and the solid concentration (2% (w/w)) were kept constant. **Table 5** shows the assays performed for a final volume of 25 mL using a stock solution of 96% sulphuric acid. After the hydrolysis using an autoclave, hydrolysates were frozen in order to precipitate the non-hydrolysed fraction (that remains as insoluble polysaccharide).

Table 5. Factorial experimental design of acid hydrolysis pre-treatment of κ-carragenan. The temperature (126°C) and the solid concentration (2% (w/w)) were kept constant.

Run	Treatment time (min)	Acid concentration (%)
1	15	0.50
2	30	0.50
3	45	0.50
4	15	2.25
5	30	2.25
6	30	2.25
7	30	2.25
8	45	2.25
9	15	4.00
10	30	4.00
11	45	4.00

To calculate the yield, the molar concentration of κ-carrageenan and the stoichiometry of reactions was considered. The theoretical maximum concentration of galactose was estimated (9.33 g/L for 2% (w/v) of κ-carrageenan) [89].

Response surface methodology (RSM) is a useful tool to evaluate the independent variables. For that, experimental data obtained from acid hydrolysis of κ-carrageenan (galactose concentration and galactose yield) were correlated to independent variables (treatment time and percentage of sulphuric acid) following the second-order polynomial equation:

$$y_i = \beta_{0i} + \beta_{1i}x_1 + \beta_{2i}x_2 + \beta_{11i}{x_1}^2 + \beta_{22i}{x_2}^2 + \beta_{12i}x_1x_2 \quad \textbf{Equation (1)}$$

where, y_i (i= 1 to 2) are the dependent variables (corresponding to galactose concentration or galactose yield); x_1 and x_2 value of independent variables; β_{0i}, β_{1i} and β_{2i} are regression coefficients calculated from experimental data by multiple regression using the least-squares method. The experimental data were fitted to the proposed model using commercial software (Statgraphics). The goodness of model fitting was evaluated by the coefficient determination R^2 and the statistical significance by the Fisher´s F-test for analysis of variable with a 95 % confidence level.

After that, percentage of κ-carrageenan was increased, in order to obtain a higher concentration of galactose in the hydrolysate. For that, hydrolysis with 6%, 8% and 12% (w/w) of solid concentration were also performed using a sulphuric acid concentration of 2.25% (w/w).

3.2.6. Enzymatic hydrolysis treatment of cheese whey powder

In order to obtain a galactose concentration within 20 g/L as the result obtained for *Gelidium amansii*, 8% (w/w) of cheese whey was taken and dissolved in sterile water for lactose hydrolysis. The mixture was sterilized by pasteurization incubated at 60 ^{0}C for 60 minutes. For hydrolysis was used a β-galactosidase (Saphera 2600L, Novozymes) (147.1 U/mL) from *Bifidobacterium bifidum*. The hydrolysis was carried out using an enzyme loading of 5 UI/g of lactose incubating at 65 ^{0}C overnight [94].

3.3. Isomerization of D-galactose to produce D-tagatose

3.3.1. Preparation of D-galactose-rich liquors for isomerization assays

The liquors obtained from *Gelidium amansii* and κ-carrageenan hydrolysis were neutralized with $CaCO_3$ (BIOCHEM Chemopharma), achieving a final pH of 7, followed by the separation of the precipitated $CaSO_4$ from the supernatant by centrifugation (10000 g, 10 min) and filtration (Sterile Syringe Filter 0.45 μm).

Activated charcoal (Sigma-Aldrich) was used to remove HMF from *Gelidium amansii* hydrolysate obtained by dilute acid hydrolysis treatment. 1 g of activated charcoal per 10 g of liquor was added. The

mixture was left to stir at room temperature for 1 hour. The supernatant was recovered by centrifugation (10000 g, 10 min) and filtration (Sterile Syringe Filter 0.45 μm). HMF and galactose concentrations were determined using HPLC.

3.3.2. Isomerization assays conditions

After purification of BSAI protein, different elute volumes with a final protein concentration of 7 mg/mL and 3 mg/L were used for the different isomerization assays included in the scheme of **Figure 7**.

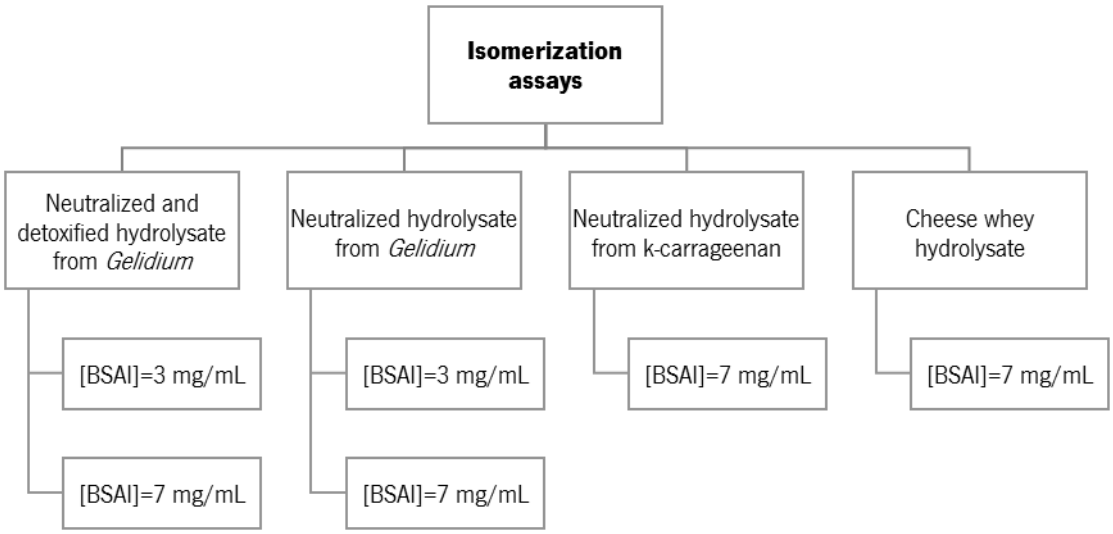

Figure 7. Isomerization assays. The assays were performed at 42 ^{0}C and 200 rpm.

The assays were performed at 42 ^{0}C and 200 rpm in an orbital incubator. To avoid contamination ampicillin was added with a final concentration of 100 μg/mL. Samples were withdrawn at desired times and analysed by HPLC to determine the bioconversion efficiency.

3.4. Analytical Methods

The samples collected from the hydrolysates and from hydrolysates treatment, as well as the quantifications of components of *Gelidium amansii*, were analysed for glucose, galactose and HMF concentration by HPLC utilizing an Agilent BioRad 87H column, operating at 60 ^{0}C, with a mobile phase

0.005 M H_2SO_4 and flow rate of 0.6 mL/min. The peaks were detected using a JASCO 830-IR intelligent refractive index detector.

For the samples from isomerization assays were analysed for galactose and tagatose concentration by HPLC utilizing a Rezex™ RCM-Monosaccharide Ca^{2+} (8%) column, operating at 85 ^{0}C, with sterile water as mobile phase and flow rate of 0.6 mL/min. The peaks were detected using a JASCO RI-4030 refractive index detector. Tagatose and galactose standards were prepared together.

3.5. Statistical analysis

Experiments with liquors obtained from *Gelidium amansii* (detoxified and non-detoxified) were performed in duplicate and the values obtained are presented with the means and respective standard deviations. Two-way ANOVA tests were performed using GraphPad Prism 6.0 software (GraphPad Software, California, USA) to estimate significant differences ($p<0.05$) among samples in graphics of columns with a confidence interval of 95%.

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1. Recombinant L-arabinose isomerase expression and purification

As previously described, the BSAI was overexpressed in *E. coli* BL21 (DE3). In order to confirm its correct expression, the supernatants of induced cultures were analysed by SDS-PAGE.

As can be observed in **Figure 8** (black box) BSAI appears with a molecular weight close to the to the previously reported of 56 kDa [21]. Moreover, BSAI enzyme exhibited a strong protein band, which indicated its overexpression.

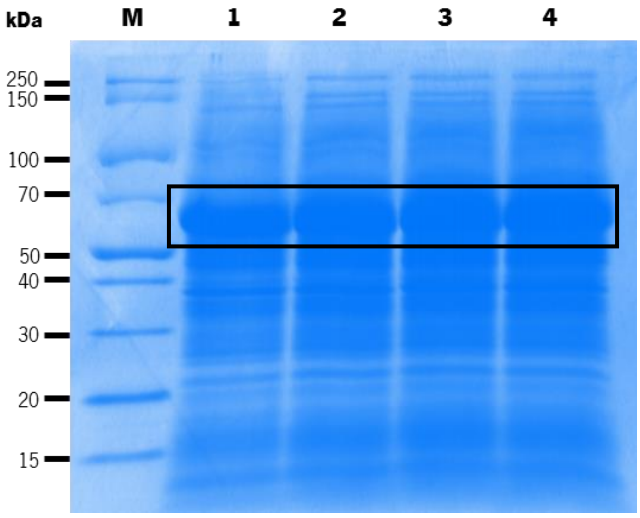

Figure 8. SDS-PAGE analysis of recombinant BSAI expression. M - protein markers; 1, 2, 3 and 4 - total cell. Bands that correspond to the BSAI enzyme are in black box.

After the confirmation of the correct expression of BSAI, the protein was purified by IMAC and the samples were analysed by SDS-PAGE in order to confirm the level of purity (**Figure 9**).

Purification efficiency, estimated by the ratio between the protein amount in eluted samples and the wet cell weight, was approximately of 34 mg/g cells.

The analysis of the protein extract after cell disruption (SF) showed the presence of BSAI, exhibited a strong protein band in the expected position (**Figure 9**, black box), indicating that sonication method used for cell lysis was efficient. The flow through (FT), collected after applying SF into the column, showed all proteins that were not attached to the column and indicated that most BSAI was attached to the column, as expected.

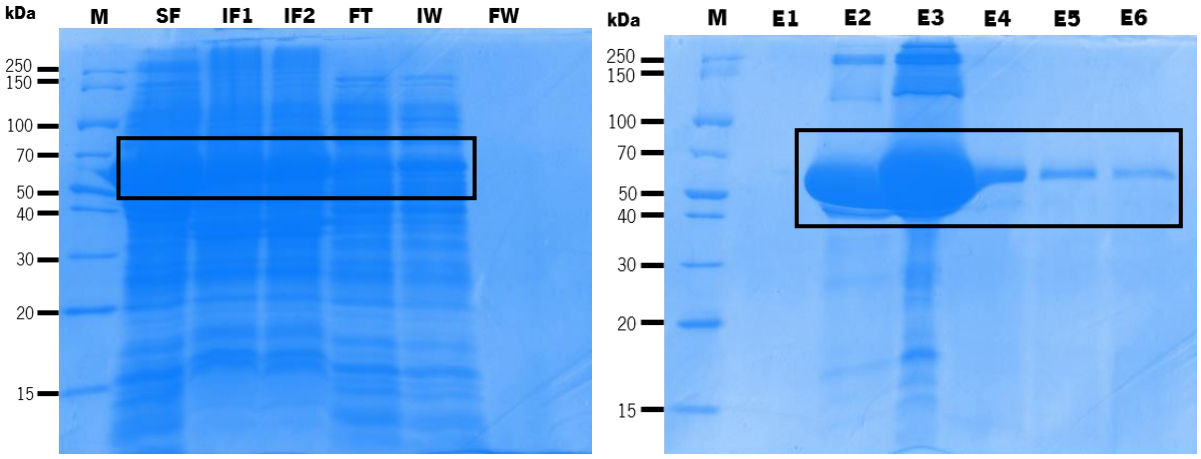

Figure 9. SDS-PAGE analysis of recombinant BSAI purification. M – protein markers; SF – Soluble Fraction; IF1 – Insoluble Fraction 1; IF2 –Insoluble Fraction 2; FT – Flow Through; IW – Initial Wash; FW – Final Wash; E1, E2, E3, E4, E5 and E6 – Eluted Fraction 1, 2, 3, 4, 5 and 6, respectively. Bands that correspond to the BSAI enzyme are in black box.

The analysis of the initial wash (IW) showed that some target protein was lost in the washing step, probably due to an excess of protein loaded that can exceed the column binding capacity. In the final wash (FW) there were no bands, indicating the efficiency of the washing step. The elution fractions (E2, E3, E4, E5 and E6) presented a predominant protein corresponding to BSAI showing a high level of purity.

4.2. Hydrolysis of several raw materials to produce liquors rich in D-galactose

4.2.1. Chemical characterization of *Gelidium amansii*

Representative sample of *Gelidium amansii* was used for the determination of galactan content in seaweed following the procedures described in section 3.2.2. Chemical characterization of main polysaccharides present in the seaweed was as follow (expressed in g/100 g of dry weight): 11.89 ± 0.28 of glucan and 19.51 ± 0.30 of galactan. These results were slight lower than reported in literature. Malihan *et al.* reported a content of 22.87% of glucan and 29.97% of galactan [95]. Carbohydrates contents of 77.2% [78] and 71.43% [77] were also reported. The content in carbohydrates in red seaweed depends of season and area of production [96]. Moreover, *Gelidium amansii* can be also composed by protein (10-18 %), ashes (2.5-8 %) and 0.2-7.4 % of lipids [77], [78], [95].

4.2.2. Hydrothermal pre-treatment and enzymatic saccharification of *Gelidium amansii*

Hydrothermal pre-treatment *of Gelidium amansii* was performed in order to hydrolyse the agar into oligosaccharides. An assay was performed with a treatment temperature of 140 °C and a treatment time of 20 minutes for 4% (w/w) of dry biomass. These treatment conditions resulted in a liquid phase composed of agar. Cho *et al.* [83] reported that agar cannot be hydrolysed by enzymatic hydrolysis. However, another assay was performed with a treatment temperature of 170 °C and a treatment time of 40 minutes for 4% (w/w) of dry biomass. Increased temperature and pre-treatment time resulted in a liquid phase composed of oligosaccharides.

After hydrothermal pre-treatment (40 minutes at 170 °C for 4% (w/w) of dry biomass) *of Gelidium amansii,* the liquid phase (composed by oligosaccharides) was submitted to enzymatic saccharification in order to hydrolyse the oligosaccharides into monomeric sugars. The enzymatic saccharification was performed by adding 20 FPU/g substrate of Cellic CTec2 (Novozyme) or mixed enzymes by adding the same load of Cellic CTec2 and Viscozyme L (Novozyme) in proportion 1:2. **Table 6** shows the concentration of galactose obtained after hydrothermal pre-treatment (time 0 hours of enzymatic saccharification) and at the end of enzymatic saccharification.

A final galactose concentration of 1.01 g/L was obtained in the assay using only the Cellic CTec2 enzyme. Considering that the concentration of galactose in the liquor before enzymatic saccharification (0.56 g/L), only 0.45 g/L of galactose was obtained with the enzyme action. These results show that it is not feasible to use the Cellic CTec2 enzyme for the hydrolysis of oligosaccharides into monosaccharides.

Table 6. Hydrothermal pre-treatment and enzymatic saccharification of *Gelidium amansii*. Concentration of D-galactose (g/L) obtained after hydrothermal pre-treatment (0 hours) and during enzymatic saccharification (4, 24 and 48 hours) by Cellic CTec2 and mixed enzymes (Cellic CTec2 and Viscozyme L). For 4% (w/w) of dry biomass.

Enzymes	Time (hours)	D-galactose concentration (g/L)
Cellic ctec2 Viscozyme L	0	0.56
	4	4.03
	24	4.41
	48	4.46
Cellic ctec2	0	0.56
	4	0.67
	24	0.90
	48	1.01

However, a final galactose concentration of 4.46 g/L was obtained in the assay using mixed enzymes (Cellic CTec2 and Viscozyme L). Considering that the concentration of galactose in the liquor before enzymatic saccharification (0.56 g/L), 3.90 g/L of galactose was obtained with mixed enzymes treatment. Cho *et al.* reported that the mixed enzyme treatment (Celluclast 1.5 L and Viscozyme L) showed a synergistic effect and the maximum efficiency for saccharification of fiber to glucose. Despite the different enzymatic mixture or the type of residue used, this may indicate that the enzymes used in this study also have a synergistic effect. Still, these enzymes are used for cellulose hydrolysis and do not have agarase activity.

One of the main objectives of this study was to obtain galactose rich liquors for further isomerization assays. Galactose concentrations obtained in these assays were not adequate for a proper valorization of this biomass. So, acid hydrolysis was performed in order to obtain higher concentrations of galactose.

4.2.3. Acid hydrolysis pre-treatment of *Gelidium amansii*

Gelidium amansii was submitted to acid hydrolysis treatment in order to hydrolyse the polysaccharides into monomeric sugars. Different concentrations of biomass were evaluated in the pre-treatment to evaluate the extraction efficiency. Therefore, to obtain high concentrations of galactose for the isomerization assays, the use of several dry biomass (4, 8 and 12 % (w/w)) with 0.5 g of sulphuric acid per 100 g of dissolution was evaluated (**Table 7**). Maximum dry biomass was chosen according to literature. C. Ra *et al.* [85] reported that increasing the biomass content over 12 % (w/v) during hyper thermal acid hydrolysis resulted in a decrease of efficiency of the pre-treatment. High biomass content leads to high viscosity, which causes difficulty in handling and in mixing, leading to insufficient action of the acid catalyst, reducing its efficacy [97]. Operational conditions were chosen trying to use the lowest acid concentration possible. The different treatments were performed at 140 °C and 20 minutes. **Table 7** shows the maximal galactose concentration obtained in the acid hydrolysates.

The results show that using the same sulphuric acid concentration (0.5% (w/w)) for the different solid loadings, the galactose concentration obtained is higher, 5.94 g/L, when 4% (w/w) of dry biomass is used. When dry biomass is increased to 8 and 12% (w/w) the galactose concentration decreases to 3.72 g/L and 1.10 g/L, respectively. Considering the galactan content in the raw material, the galactan conversion to galactose was of 68.49, 21.45 and 4.23 % for 4, 8 and 12 % of solid loading, respectively.

Table 7. Acid hydrolysis with 0.5% (w/w) sulphuric acid concentration of 4, 8 and 12% (w/w) of dry biomass. Treatment time of 20 min at 140 ^{0}C. D-galactose concentration (g/L) obtained in each test.

Dry biomass %(w/w)	D-galactose concentration (g/L)
4	5.94
8	3.72
12	1.10

Increased dry biomass percentage in the pre-treatment decreased the extraction efficiency, probably due to poor mass transference. Moreover, the percentage of sulphuric acid was insufficient for the hydrolysis of 8 and 12% of seaweed biomass. To obtain higher concentrations of galactose by increasing biomass it was necessary increase sulphuric acid concentration as well. For this, the acid/biomass ratio of the first assay (4 % of solid loading) was calculated, obtaining a ratio of 0.125 g of sulphuric acid/g of *Gelidium amansii*. An additional experiment was carried out using 12% (w/w) of dry biomass and sulphuric acid concentration of 1.5% (w/w). Moreover, the effect of temperature was also evaluated.

For 12% (w/w) of dry biomass hydrolysis with a sulphuric acid concentration of 1.5% (w/w) different temperatures (130, 140 and 150 ºC) were tested to determine the influence of the temperature on hydrolysis (**Table 8**). Reaction temperatures and treatment time were chosen according to literature. C. Ra *et al.* [85] reported an increase in galactose concentration with increasing reaction temperatures from 110 to 150 ºC. Furthermore, it was also reported that galactose concentration and the efficiency of pre-treatment decreased when thermal hydrolysis time increased beyond 10 min. This suggests that extended hydrolysis time may have a negative effect on galactose concentration due to monosaccharide degradation and consequently increased formation of inhibitor compounds [85]. It was also reported by Sukwong *et al.* [82] that the efficiency of hyper thermal acid hydrolysis decreases with increasing hydrolysis time at temperatures higher than 140 °C. So, a treatment time of 10 minutes was used for these tests. **Table 8** shows the maximal galactose concentration obtained in the acid hydrolysates and the HMF concentration.

The results show that galactose concentration increases with increasing temperature, however the formation of degradation compounds (such as HMF) are also present in the hydrolysates and the HMF concentration also increases with the temperature.

Table 8. Acid hydrolysis of 12% (w/w) of dry biomass with a sulphuric acid concentration of 1.5% (w/w) at different temperatures (130, 140 and 150 ^{0}C). Treatment time of 10 minutes. D-galactose and HMF concentration (g/L) obtained in each test.

Temperature (^{0}C)	D-galactose concentration (g/L)	HMF concentration (g/L)
130	8.25	1.52
140	12.01	2.07
150	22.28	6.56

The galactan extraction as galactose was 31.7, 46.16 and 85.63 % for treatment temperatures at 130, 140 and 150 °C, respectively. These results revealed that temperatures lower than 150°C were not suitable for the complete extraction and degradation of galactan into galactose. C. Ra *et al.* [85] reported that the most effective hyper thermal acid hydrolysis conditions were 12% (w/v) biomass content and sulphuric concentration of 144 mM at 150 ºC for 10 min. Under these conditions, the concentration of galactose obtained by them was approximately 27.5 g/L and the concentration of HMF was 3.7 g/L [85]. Sukwong *et al.* [82] reported that the most effective hyper thermal acid hydrolysis conditions were sulphuric concentration of 358.3 mM at 142.6 ºC and 11 min for 12% (w/v) biomass content, resulted in hydrolysate containing 20.3 g/L galactose and 3.8 g/L HMF. The difference in results may be due to carbohydrate content in red algae depending on season and area of production [96].

Nevertheless, under the most severe conditions of pre-treatment (150 °C), 11.40 % of hexoses (galactose and glucose) were dehydrated to HMF. HMF is considered an inhibitor compound of biological processes. Considering the results obtained, treatment at 150 °C was selected to produce tagatose. The effect of inhibitor compounds (as HMF) on BSAI activity was also evaluated. For that, hydrolysate obtained at 150 °C was submitted to a detoxification step with activate charcoal to compare detoxified and non-detoxified hydrolysates.

4.2.4. Experimental design: evaluation of acid hydrolysis of κ-carrageenan

In order to determine the best conditions for the hydrolysis of κ-carrageenan a factorial experimental design was carried out. The following variables were evaluated: treatment time (15, 30 and 45 min) and sulphuric acid concentration (0.5%, 2.25% and 4.0%, (w/w)). The temperature (126°C) and the solid

concentration (2% (w/w)) were fixed variables. The theoretical maximum concentration of galactose was estimated as described in section 3.2.4, to calculate the yield of galactose (**Table 9**).

Table 9. Factorial experimental design for the hydrolysis of κ-carrageenan. Factors: treatment time (15, 30 and 45 min) and sulphuric acid concentration (0.5%, 2.25% and 4.0%, (w/w)). Constants: temperature (126°C) and solid concentration (2% (w/v)).

Run	Treatment time (min)	Acid concentration (%)	D-galactose concentration (g/L)	Yield (%)
1	15	0.50	0.87	9.3
2	30	0.50	2.14	22.9
3	45	0.50	2.39	25.7
4	15	2.25	3.66	39.2
5	30	2.25	4.57	49.0
6	30	2.25	4.35	46.6
7	30	2.25	4.52	48.4
8	45	2.25	4.66	49.9
9	15	4.00	4.68	50.2
10	30	4.00	4.65	49.8
11	45	4.00	4.62	49.5

The results show that galactose concentration increases with the increase of sulphuric acid concentration. Meinita *et al.* [98] tested the effect of sulphuric acid concentration on the hydrolysis of κ-carrageenan waste. They reported that the highest production of galactose was obtained with 0.2 M of acid sulphuric under standard conditions of hydrolysis at 120 °C for 15 min. Furthermore, reported that at lower and higher concentrations, galactose concentration decreased. The differences might be due to the use of a different κ-carrageenan (a commercial one was used in this work).

At different treatment times for the same sulphuric acid concentration of 0.5% there are significant differences in galactose concentration (run 1-3). However, at higher sulphuric acid concentrations (2.25% and 4.0%) there appears to be no significant differences in galactose concentration with increasing treatment time. Meinita *el al.* reported [98] that increasing the hydrolysis time to longer than 15 min resulted in decreased production of galactose. However, as mentioned above, the polysaccharide used was not the same.

Experimental data obtained from acid hydrolysis of κ-carrageenan (galactose concentration and galactose yield) were correlated to independent variables (treatment time and percentage of sulphuric acid) following the **equation 1**.

The regression coefficients and the statistical significance (base on the Student´s test), and the statistical significance of the model (based on Fischer´s F parameter) are listed in **Table 10**. The parameters revealed the good fitting of the evaluated variables by empirical models (R^2 > 0.99).

Table 10. Regression coefficients and statistical parameters measuring the correlation and significance of models. (a) Coefficients significant at the 90% confidence level; (b) Coefficients significant at the 95% confidence level.

Coefficient	Galactose concentration (g/L)	Galactose Yield (%)
$\mathbf{b_{0j}}$	4.47	47.84
$\mathbf{b_{1j}}$	1.43 (a)	15.27 (a)
$\mathbf{b_{2j}}$	0.41 (a)	4.40 (a)
$\mathbf{b_{11j}}$	-1.05 (a)	-11.24 (a)
$\mathbf{b_{22j}}$	-0.29 (b)	-3.04 (b)
$\mathbf{b_{12j}}$	-0.40 (a)	-4.28 (a)
Statistical Parameters		
$\mathbf{R^2}$	0.99	0.99
Fexp	124.67	124.62
significance level	> 99	> 99

Therefore, the effect of these independent variables (treatment time and percentage of sulphuric acid) were evaluated by RSM. **Figure 10** shows the effect of treatment time and percentage of sulphuric acid on galactose concentration and yield, with a fixed treatment temperature (126°C) and solid concentration (2% (w/w)).

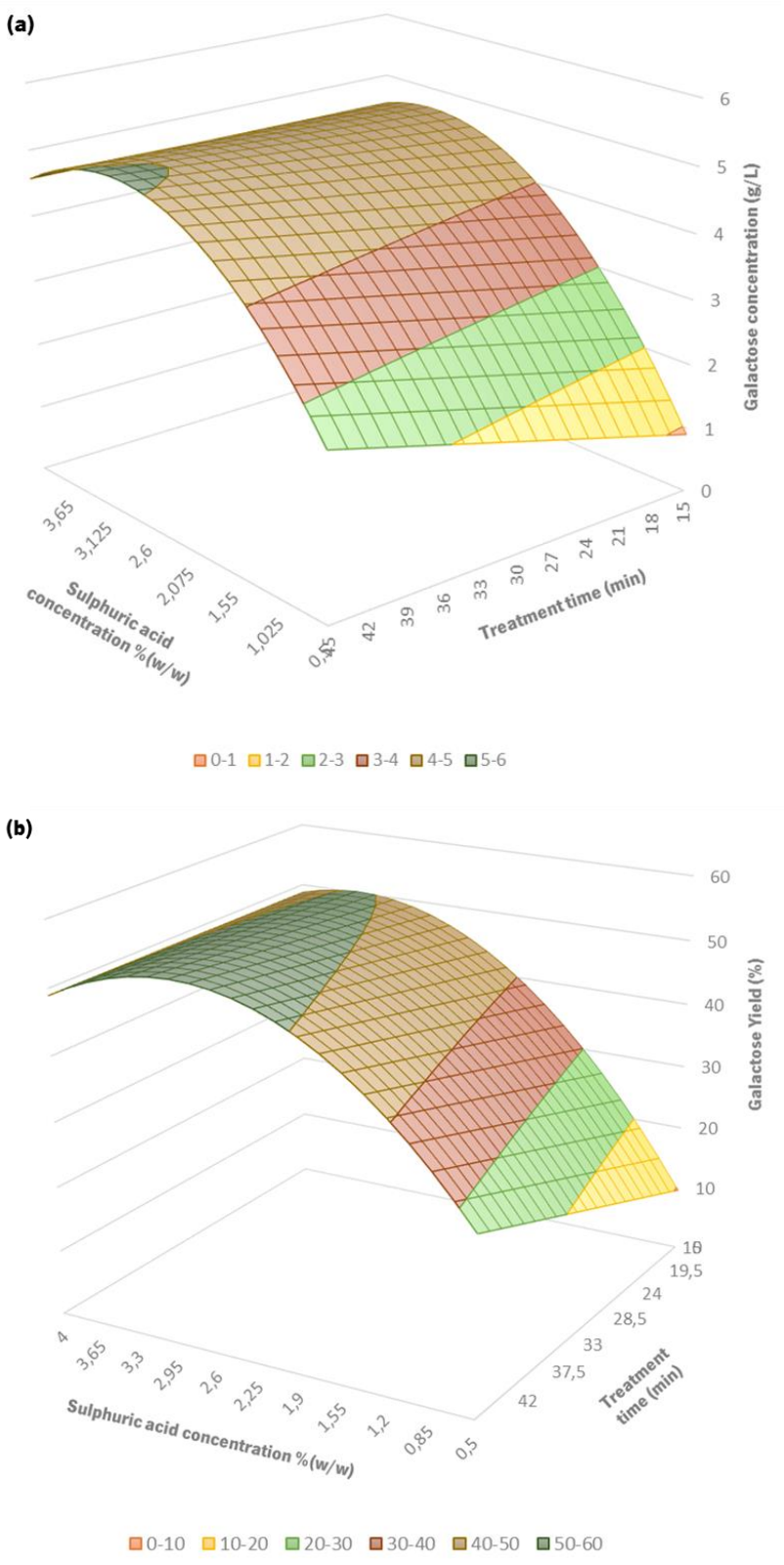

Figure 10. Effect of treatment time and percentage of sulphuric acid on galactose concentration (a) and yield (b). Fixed treatment temperature (126°C) and solid concentration (2% (w/w)). Evaluated by response surface methodology assessment (RSM).

As seen in **Figure 10**, galactose concentration higher than 4 g/L was obtained when the percentage of sulphuric acid was > 2.25% and treatment time > 15 minutes. On the other hand, galactose yield higher than 40% was obtained when percentage of sulphuric acid was > 2.25%, independently of treatment time. Percentage of sulphuric acid had a remarkable positive effect on galactose concentration, while the treatment time effect was lower.

Considering the results obtained, run 8 (treatment time of 45 min and sulphuric acid concentration of 2.25% (w/w)) was chosen to perform more tests in order to increase the concentration of galactose for isomerization tests. Considering the low concentration of galactose obtained in the hydrolysates, concentration of κ-carrageenan was increased. The maximum concentration was chosen according to the literature. Meinita *et al.* [98] tested carrageenan waste concentration between 0-12% (w/w). Thus, hydrolysis with 6%, 8% and 12% (w/w) of solid concentration was also performed using a sulphuric acid concentration of 2.25% (w/w) and a treatment time of 45 minutes (**Table 11**).

These assays were intended to obtain a galactose concentration liquor within 20 g/L as the result obtained for *Gelidium amansii* hydrolysis. **Table 11** shows the maximal galactose concentration obtained in the acid hydrolysates.

Table 11. Acid hydrolysis of 6, 8 and 12% (w/w) of κ-carrageenan concentration. Assays performed using a sulphuric acid concentration of 2.25% (w/w) and a treatment time of 45 minutes. Galactose concentration and yield obtained in each assay.

Solid concentration %(w/w)	Galactose concentration (g/L)	Galactose Yield (%)
6	15.30	54.7
8	19.95	53.6
12	30.34	54.2

Galactose concentrations of 15.30, 19.95 and 30.34 g/L were obtained with 6, 8 and 12% (w/w) of solid concentration, respectively. Galactose yield of 54.7, 53.6 and 54.2% were obtained with 6, 8 and 12% (w/w) of solid concentration, respectively. The target was a liquor with approximately 20 g/L. So, the liquor obtained with 19.95 g/L of galactose was used for the isomerization assays.

4.2.5. Hydrolysis of lactose from cheese whey

In order to obtain a galactose concentration within 20 g/L as the result obtained for *Gelidium amansii*, 8% (w/w) of cheese whey was used.

The hydrolysis of 8% (w/w) was carried out using an enzyme loading of 5 UI/g of lactose incubating at 65 ^{0}C overnight [94]. A galactose concentration of 26.67 g/L was obtained and used for the isomerization assays.

4.3. D-tagatose production – Isomerization assays

The liquors obtained from *Gelidium amansii* and κ-carrageenan hydrolysis were neutralized with $CaCO_3$. Furthermore, activated charcoal was used to remove HMF from *Gelidium amansii* hydrolysate following acid hydrolysis. After detoxification process the hydrolysate was analysed by HPLC utilizing an Agilent BioRad 87H column for HMF concentration. HMF was not detected in the detoxified liquors.

The samples from isomerization assays were analysed for galactose and tagatose concentration by HPLC utilizing a Rezex™ RCM-Monosaccharide Ca^{2+} (8%) column. Tagatose and galactose standards were prepared together. The chromatogram of the standards is shown in **Figure 11.**

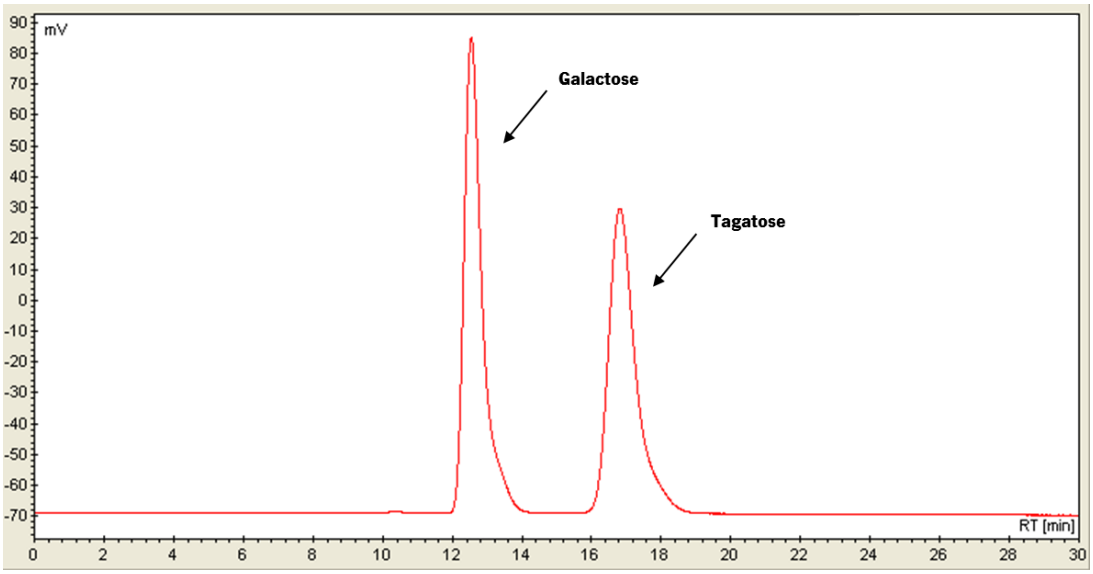

Figure 11. Chromatogram of tagatose and galactose standards. HPLC with a Rezex™ RCM-Monosaccharide Ca^{2+} (8%) column, operating at 85 ^{0}C, with sterile water as mobile phase and flow rate of 0.6 mL/min. The peaks were detected using a JASCO RI-4030 refractive index detector.

The detoxified and non-detoxified liquors obtained from *Gelidium amansii* hydrolysis were used for isomerization assays at 42 °C. In both, the conversion of galactose into tagatose by the enzyme BSAI was verified. **Figure 12** shows chromatograms obtained from detoxified and non-detoxified liquor assays. There is a peak corresponding to tagatose according to the standard shown in **Figure 11**, for both assays.

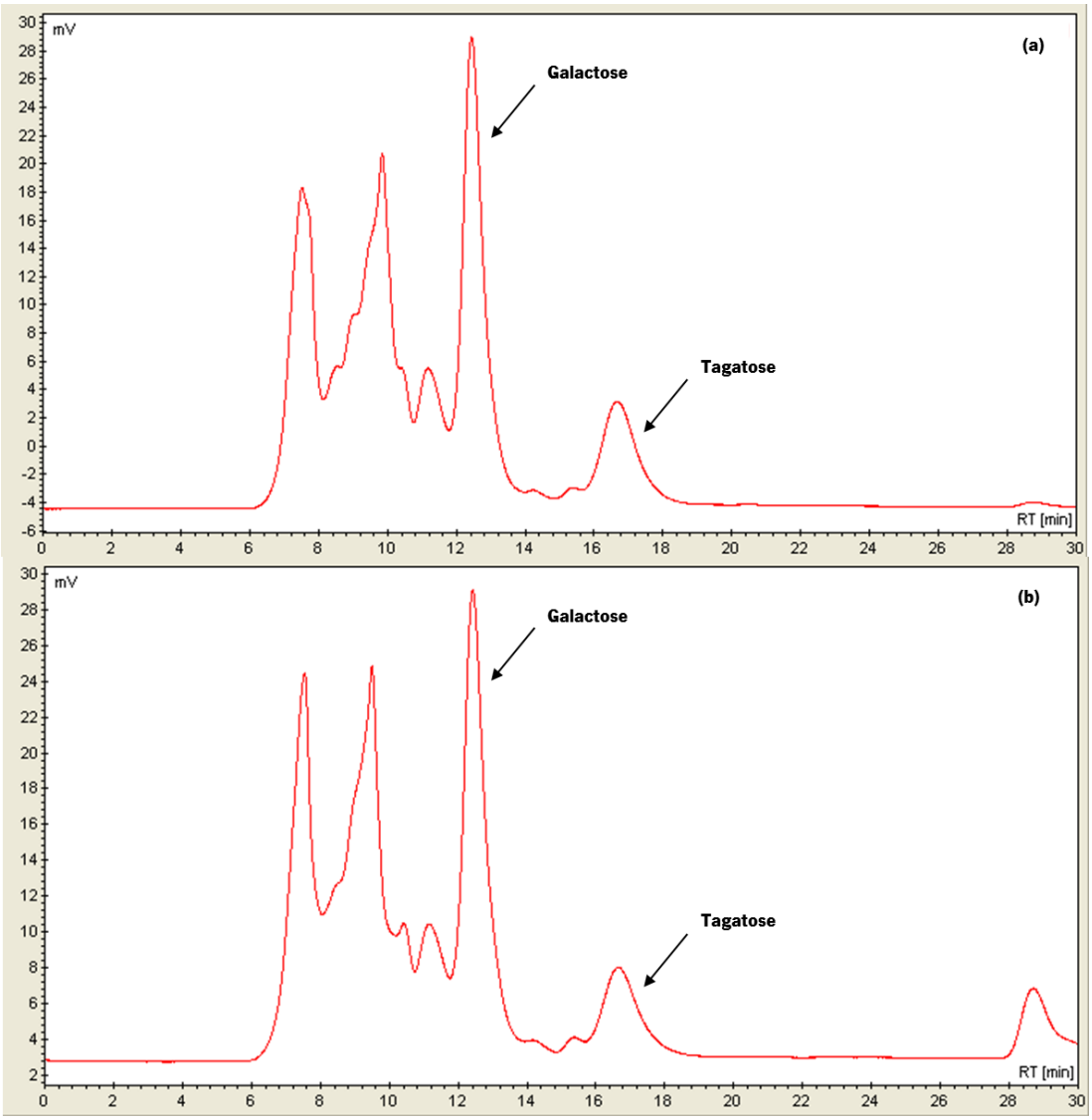

Figure 12. Chromatograms obtained from detoxified (a) and non-detoxified (b) liquor assays. HPLC with a Rezex™ RCM-Monosaccharide Ca^{2+} (8%) column, operating at 85 ^{0}C, with sterile water as mobile phase and flow rate of 0.6 mL/min. The peaks were detected using a JASCO RI-4030 refractive index detector.

Both liquors (detoxified and non-detoxified) were used for assays with BSAI concentration of 7 and 3 mg/mL (**Figure 13**). **Table 12** shows the final tagatose concentration obtained and the initial galactose concentration in one of the tests performed for each liquor.

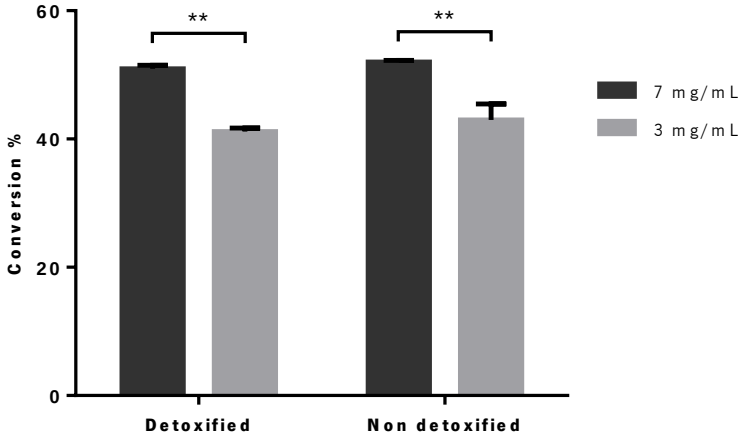

Figure 13. Conversion obtained in isomerization assays with detoxified and non-detoxified liquors obtained from *Gelidium amansii* hydrolysis. BSAI concentration of 7 and 3 mg/mL. Isomerization performed at 42 ^{0}C. Values are the mean ± SD (n=2). Statistical analysis was performed for detoxified and non-detoxified liquors with the same BSAI concentration and for different BSAI concentration with the same liquor by two-way ANOVA. **p<0.01.

An average conversion of 50.9% was obtained in the detoxified liquor assays with a BSAI concentration of 7 mg/mL. In the non-detoxified liquor assays and the same BSAI concentration (7 mg / mL) an average conversion of 52.0% was obtained. The results show that there are no significant differences in the conversion of galactose to tagatose between the detoxified and non-detoxified liquors. So, this means that HMF, in this concentration range, did not inhibited the action of the BSAI enzyme.

On the other hand, using the same liquor with different BSAI concentrations lead to significant variation of the galactose-to-tagatose conversion. For the detoxified liquor assays average conversions of 50.9 and 40.7% are obtained for BSAI concentrations of 7 and 3 mg/mL, respectively. For the non-detoxified liquor assays average conversions of 52.0 and 43.0% are obtained for BSAI concentrations of 7 and 3 mg/mL, respectively. An approximately 10% difference in conversion occurs with different concentrations of BSAI enzyme. Thus, the concentration of BSAI used influences the conversion of galactose to tagatose in both detoxified and non-detoxified liquors.

Hydrolysates obtained from κ-carrageenan and cheese whey were also used for isomerization assays at 42 °C. Both liquors were used for assays with BSAI concentration of 7 mg/mL, since this was the concentration that allowed for better results previously. **Figure 14** shows chromatograms obtained from κ-carrageenan hydrolysate and cheese whey hydrolysate assays. There is a peak corresponding to tagatose according to the standard shown in **Figure 11**, for both assays.

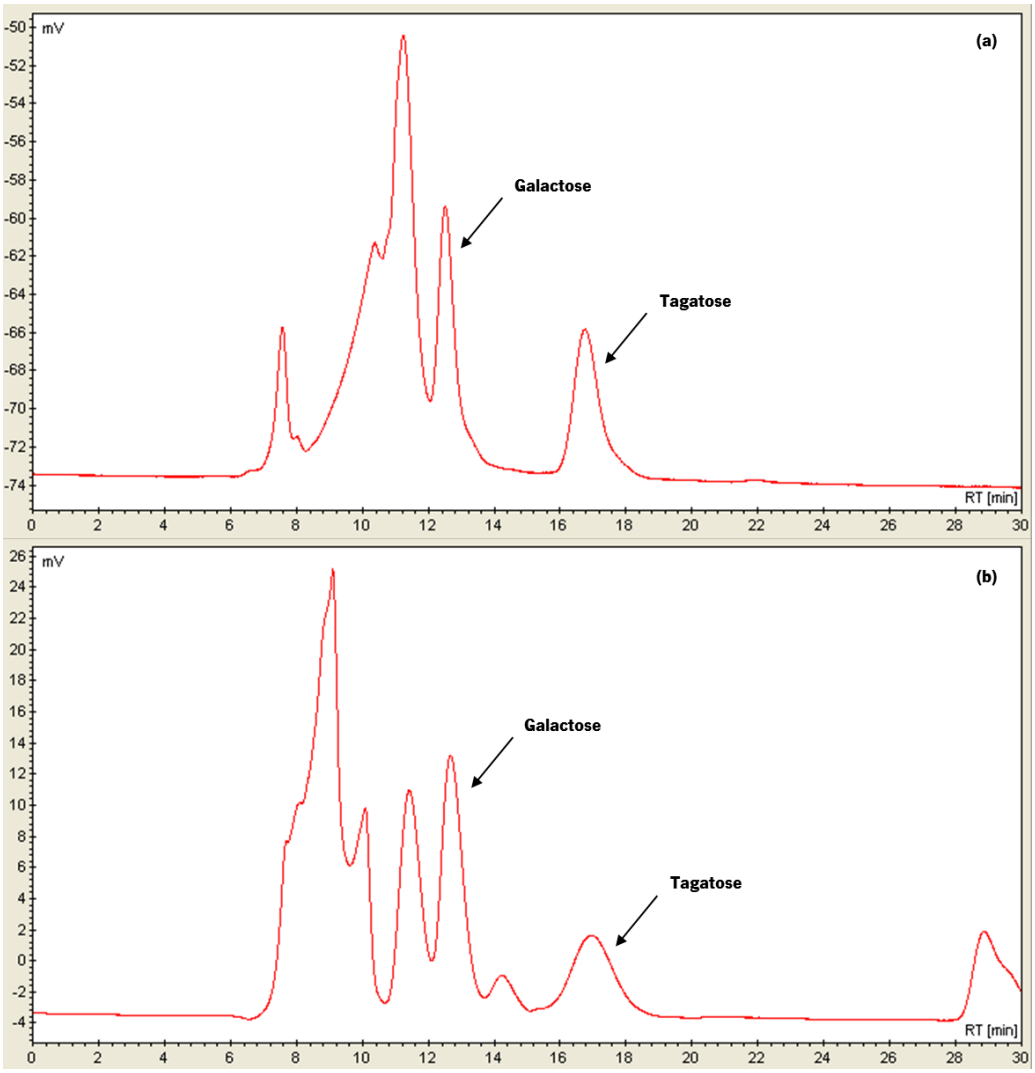

Figure 14. Chromatograms obtained from cheese whey (a) and κ-carrageenan (b) liquors assays. HPLC with a Rezex™ RCM-Monosaccharide Ca^{2+} (8%) column, operating at 85 ^{0}C, with sterile water as mobile phase and flow rate of 0.6 mL/min. The peaks were detected using a JASCO RI-4030 refractive index detector.

Figure 15 shows the conversions of galactose to tagatose obtained with the different liquors rich in galactose. In all these assays an enzyme concentration of 7 mg/mL was used.

Conversions of 50.9, 52.0, 55.6 and 27.8% were obtained in the isomerization assays with *Gelidium amansii* detoxified and non-detoxified liquor, cheese whey hydrolysate and κ-carrageenan hydrolysate, respectively. The highest conversion of galactose to tagatose obtained was using cheese whey (55.6%). This may be due to the fact that cheese whey is a less complex raw material. The results obtained are very similar to those obtained in the group previously with pure galactose solutions. This suggests that the use of liquors instead of pure solutions does not affect BSAI enzyme activity.

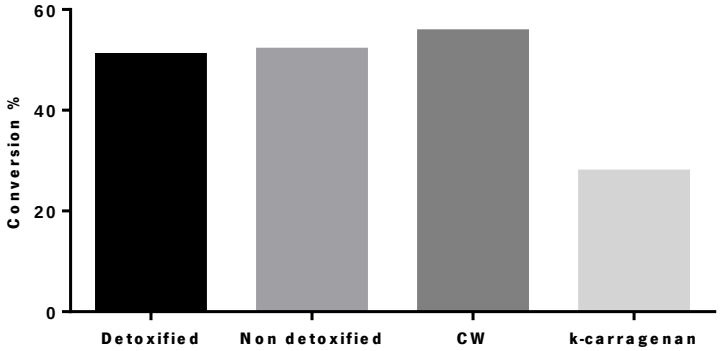

Figure 15. Conversion obtained in isomerization assays with the different liquors rich in D-galactose. BSAI concentration of 7 mg/mL. Isomerization performed at 42 ^{0}C. CW, Cheese Whey.

Table 12 shows the final tagatose concentration obtained and the initial galactose concentration in one of the tests performed for each liquor.

Table 12. Conversion (%) obtained in isomerization assays, final concentration of D-tagatose (g/L) and initial concentration of D-galactose (g/L). BSAI concentration (3 and 7 mg/mL) used in each assay. *NA, not available.

	7 mg/mL			3 mg/mL		
	Initial concentration of D-galactose (g/L)	**Final concentration of D-tagatose (g/L)**	**Conversion (%)**	**Initial concentration of D-galactose (g/L)**	**Final concentration of D-tagatose (g/L)**	**Conversion (%)**
Detoxified hydrolysate from *Gelidium amansii*	4.20	2.12	50.9	7.28	2.97	40.7
Non-detoxified hydrolysate from *Gelidium amansii*	4.69	2.43	52.0	7.12	2.93	43.0
k-carrageenan hidrolysate	8.99	2.50	27.8	NA*	NA	NA
Cheese whey Hydrolysate	5.52	3.07	55.6	NA	NA	NA

Maximum conversion in all assays were attained within 4 hours of enzymatic isomerization. Temperature selected to perform these assays was based on previous results obtained in this research group, in which 42°C was the optimal temperature for enzyme activity. Still, enzymatic activity could

decrease with increasing temperatures, which could explain the reason why the conversion was not increased after 4 h of enzymatic assay.

The results obtained in here show that the enzyme BSAI has activity for galactose substrate, contrary to the previously reported [21]. Supporting our results, Roh *et al.* [69] obtained a final concentration of tagatose of 0.56 g/L from colonies expressing the same enzyme used in this study. The conversion medium used contained 10 g of D-galactose in 3 mL of medium and the cells resuspended were maintained at 37°C for 72 h. The concentration of tagatose obtained is low and better results were obtained in this study. However, they used cells expressing the enzyme BSAI whereas in this study the pure enzyme was used.

In this study, BSAI was shown to be able to convert galactose to tagatose not only using pure solutions as well as using liquors obtained from several raw materials, suggesting a possible valorization route for these bioresources.

Considering the L-AI enzymes previously reported for the conversion of galactose to tagatose, only three showed slightly higher conversion than the enzyme used in this study. Namely, *Anoxybacillus flavithermus* [54], *Thermotoga maritima* [57] and *Thermotoga neapolitana* [58], with maximum conversions of 60, 56 and 68%, respectively. The optimum temperature of activity of these enzymes is between 85-95 ºC. Studies of thermophilic L-AI have shown that they have better conversion because the interconversion equilibrium between galactose and tagatose shifts toward tagatose at higher temperatures [19]. However, L-AI which are active at low temperatures have been of great interest [28]. Furthermore, the maximum conversion reported is with pure galactose solutions.

To produce tagatose cheese whey and onion juice residue has already been used as renewable sources of galactose. However, the use of liquors obtained from *Gelidium amansii* or κ-carrageenan has not been reported.

Jayamuthunagai *et al.* [28] produced tagatose by direct addition of alginate immobilized *Lactobacillus plantarum* cells expressing L-AI to lactose hydrolysed whey permeate. They obtained the maximum conversion of 38% with successive lactose hydrolysis by β-galactosidase from *Escherichia coli* and galactose isomerization using L-AI from *Lactobacillus plantarum*. The conversion obtained was lower than that obtained in this study, suggesting that the BSAI enzyme has higher affinity for galactose than the enzyme reported by Jayamuthunagai *et al.* However, using cell immobilization expressing BSAI enzyme may improve the conversion obtained in this study, since immobilization improves the biochemical

properties of recombinant cells and L-AI enzymes, such as the optimal temperature and thermostability [42].

Zheng *et al*. [76] performed a dual production of valuable tagatose and bioethanol from lactose and cheese whey. A total of 23.5 g/L of tagatose from cheese whey containing 100 g/L lactose was obtained. Substrate concentration used as well as tagatose concentration obtained are not comparable with this study.

Onion juice residue was used for the production of tagatose and bioethanol achieving a tagatose conversion yield of 41.7% [63]. The conversion obtained was lower than that obtained in this study, suggesting that the BSAI enzyme has higher affinity for galactose than the enzyme reported by Kim *et al*. The differences may also be due to the type of raw material, initial galactose concentration or enzyme concentration used.

A dual production of tagatose and bioethanol reported by Zheng *et al*. [76] and Kim *et al*. [63] is interesting since it is possible to obtain two valuable products from the same raw material.

CONCLUSIONS AND FUTURE PERSPECTIVES

5. CONCLUSIONS AND FUTURE PERSPECTIVES

The main objective of this thesis was the production of tagatose from several renewable resources by enzymatic isomerization of galactose using a BSAI enzyme. For that, production and purification of BSAI enzyme to be used in the bioconversion of galactose into tagatose and hydrolysis of *Gelidium amansii*, κ-carrageenan and cheese whey to obtain enriched liquor in galactose was performed.

The analysis of the protein extract after cell disruption (SF) showed the presence of BSAI, exhibiting a strong protein band in the expected position. This indicates that the sonication method used for cell lysis was efficient. Furthermore, purification efficiency, estimated by the ratio between the protein amount in eluted samples and the wet cell weight, was approximately of 34 mg/g cells.

Hydrothermal treatment using water as reaction media without catalysts was carried out to hydrolyse the agar present in *Gelidium amansii* into oligosaccharides. After pre-treatment at 170 °C for 40 minutes using 4% (w/w) of dry biomass it was obtained a liquid phase composed by oligosaccharides and no agar was recovered. Enzymatic saccharification with commercial mixed enzymes (Cellic CTec2 and Viscozyme L) was the most appropriated combination to hydrolyse the oligosaccharides into monomeric sugars, achieving 4.46 g/L of galactose in comparison with results obtained with the enzymes, individuality.

On the other hand, acid hydrolysis of *Gelidium amansii* was performed in order to obtain higher concentrations of galactose. Sulphuric acid concentration of 1.5% (w/w) was required to hydrolysed 12% of *Gelidium amansii.* In addition, the galactose concentration increases with increasing pre-treatment temperature (up to a temperature of 150 ºC), however HMF concentration also increases. A pre-treatment at 150 ºC and a sulphuric acid concentration of 1.5% (w/w) for 10 minutes using 12% (w/w) of dry biomass was suitable for the extraction of galactan as galactose achieving a concentration of 22.28 g/L.

After evaluating the best conditions for the hydrolysis of κ-carrageenan (sulphuric acid concentration of 2.25% (w/w) and a treatment time of 45 minutes at 126 ºC), the solid concentration was increased to 8% (w/w) obtaining a galactose concentration of 19.95 g/L.

Moreover, lactose present in cheese whey powder was successfully hydrolysed resulting in a galactose rich liquor of the desired concentration (26.67 g/L).

Tagatose production by enzymatic method using BSAI enzyme was tested using different enriched liquor in galactose. Production of tagatose by BSAI enzyme was verified in all assays with different liquors, existing a peak corresponding to tagatose detected by HPLC. In addition, it was also verified that the

concentration of BSAI used influences the conversion of galactose to tagatose. For the same BSAI concentration (7 mg/mL) conversions of 50.9, 52.0, 55.6 and 27.8% were obtained in the isomerization assays with *Gelidium amansii* detoxified and non-detoxified liquor, cheese whey hydrolysate and κ-carrageenan hydrolysate, respectively. Furthermore, HMF did not inhibited the action of the BSAI enzyme when used non-detoxified liquor. Therefore, tagatose was successfully produced from several renewable resources of galactose and the results obtained are very similar to those obtained with pure galactose solutions.

Tagatose production by enzymatic method using BSAI enzyme can be improved. Strategies for improving BSAI conversion, including immobilization (cells expressing BSAI or BSAI immobilization) and operational modes (like fed batch) should be evaluated. In addition, the temperature at which the BSAI enzyme is most stable should be determined, since for the temperature used in this study the enzymatic activity decreased after 4h.

The valorization of these renewable sources may also consider the simultaneous production of other products. To produce tagatose only the galactose present in these raw materials is used, however, high glucose concentrations are also present in these sources. A dual production of tagatose and other products such as bioethanol, for example, can be performed for maximum valorization of these raw materials. Other renewable sources should also be evaluated, for example, cheese whey permeate considering the added value of cheese proteins as food and pharmaceutical ingredient, in order to integral valorization of cheese whey residue. In addition, can also be evaluated different residues combination.

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6. REFERENCES

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