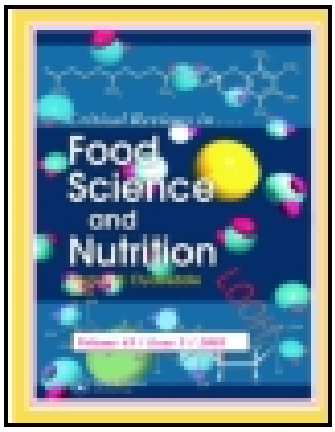

Critical Reviews in Food Science and Nutrition
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/bfsn20

Inulin Potential for Enzymatic Obtaining of Prebiotic Oligosaccharides

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Accepted author version posted online: 06 Mar 2015.

Inulin Potential for Enzymatic Obtaining of Prebiotic Oligosaccharides

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ABSTRACT

Oligosaccharides have been marketed since the 1980s as low calories agents and recently have gained interest in the pharmaceutical and food industry as functional sweeteners and prebiotic enriching population of Bifidobacteria. Currently, they have an approximated value of $ 200 per kg and recently, inulin has been proposed as a feedstock for production of oligosaccharides through selective hydrolysis by action of endoinulinase. High optimum temperature (60 °C) and thermostability are two important criteria which determine suitability of this enzyme for industrial applications as well as enzyme cost, a major limiting factor. Significant reduction in cost can be achieved by employing low-value and abundant inulin rich plants as Jerusalem artichoke, dahlia, yacon, garlic and onion, among others. In general, the early-harvested tubers of these plants contain a greater amount of highly polymerized sugar fractions, which offer more industrial value than late-harvested tubers or those after storage. Also, development of recombinant microorganisms could be useful to reduce the cost of enzyme technology for large

scale production of oligosaccharides. In the case of fungal inulinases, several studies of cloning and modification have been made to achieve greater efficiency. The present article reviews inulin from vegetable sources as feedstock for oligosaccharides production through the action of inulinases, the impact of polymerization degree of inulin and its availability and some strategies to increase oligosaccharides production.

Keywords: endoinulinases, inulin, polymerization degree, prebiotics, thermostability.

Introduction

Both, modern lifestyle and high levels of stress have forced consumers to change their eating habits. An increasing healthy awareness has triggered the demand of dietary carbohydrates because their beneficial effects are well documented (Rocha *et al*., 2006; Chi *et al*., 2009). These carbohydrates include dietary fibers, resistant starch and non-digestible oligosaccharides (Nacos *et al*., 2006; Nabarlatz *et al*., 2007). Oligosaccharides have been marketed since the 1980s as low calorie agents and have recently gained interest in the pharmaceutical and food industry. In 1991, some oligosaccharides were classified as "food for specified health uses" (FOSHU) in Japan and, since 2000, oligosaccharides were designated as GRAS by the U.S. FDA (Food and Drug Administration) in accordance with the agency´s proposed regulation. Currently, they have an approximated value of $ 200 per kg (Godshall, 2007). These low molecular weight carbohydrates are non-digestible, contain sugar residues with a polymerization degree from 3 to 10, and are intermediate between simple sugars and polysaccharides (Weijers *et al*., 2008).

In non-digestible oligosaccharides (NDOs), the anomeric carbon (C1 or C2) of the monosaccharide units possess a ɓ-type configuration, which makes their links, non susceptible to hydrolytic activity of human digestive enzymes, so they can reach the large intestine with their structure intact (Roberfroid & Slavin, 2000). Because of that, they can act as a competitive ligand that protects from pathogen and also as dietary fiber having beneficial effect on the intestinal bacterial populations, which ferment them to produce short-chain fatty acids (propionic, butyric, acetic and lactic acid), lowering the pH (de Genaro *et al*., 2000; Quigley,

2010). Through the re-absorption of these acids, an energy supply of 1-1.5 kcal/g cab be rescued, which represents 40-50% of those digestible carbohydrates such as sucrose (Mussatto & Manchilha, 2007).

As result of their high biological activity, the food industry has driven research on such type of compounds aiming at prospective practical applications; this research has increased in the last years (Crittenden & Playne, 1996; Rivero-Urgell & Santamarina-Orleans, 2001). Also, their low sweetness has promoted many food industrial applications which involve inulin-type fructans as diabetic food components (Blize *et al*., 1994; Rivero-Urgell & Santamarina-Orleans, 2001).

Oligosaccharides can be extracted from natural sources or synthesized by physical, chemical or enzymatic methods (Courtois, 2009). Actually, they are mainly produced at industrial scale from disaccharide sucrose by microbial enzymes having transfructosylating activity (β-fructofuranosidase-FFase-EC 3.2.1.26). However, the oligosaccharide production yields by this process are normally low (55-60 %), since the enzymes involved in this reaction have, besides transfructosylation activity, hydrolytic activity giving glucose and fructose as reaction by-products (Nishizawa, *et al.,* 2001), which could act as enzyme inhibitor. Inulin has been proposed as a feedstock for production of inulo-oligosaccharides (IOS) such as inulotriose and inulotetraose through selective hydrolysis by action of endoinulinase (Yun *et al.,* 2000), improving production yields.

IOS have very similar structure and functionalities to fructo-oligosaccharides (FOS) whose beneficial effects on humans and animals have been well characterized as functional sweeteners. Thus, IOS can be used as soluble dietary fiber, a functional sweetener or as prebiotic for enriching population of Bifidobacteria (Hidaka *et al*., 1991; Roberfroid, 1993). In this paper, we review some vegetable sources of inulin for obtaining IOS and its different characteristics, microbial endoinulinase producers, factors affecting their production and strategies for increasing oligosaccharides production.

Inulin as feedstock

Sucrose and starch are the primary vegetative storage carbohydrates in tropical and subtropical grasses, while temperate and cool zone grasses mainly accumulate fructose polymers called fructans (French & Waterhouse, 1993). It has been reported that from 12 to 15% of angiosperms contain fructans (Hendry, 1987) and within them, inulin can be found (Hendry & Wallace, 1993). Inulin is a natural linear biopolymer build by 20 to 30 D-fructose units connected through β-(2,1) glycosidic linkages. As terminal monosaccharide residue, it presents α-D-glucose bonded (1→2) to fructose from inulin chain (Ronkart *et al*., 2007; Dan *et al*., 2009). Glucose unit occurs in the inulin macromolecule in pyranose form ($^4C_1$ conformation), and fructose is present in furanose form. Inulin can be found in some monocot family members like Liliaceae, Agavaceae, Amaryllidaceae and Iridaceae, as well as in some dicotyledonous members like Compositae, Boraginaceae, Malpighiaceae, Primulaceae, Violaceae and Styracaceae (Carpita *et al*., 1996).

Inulin was also designated as GRAS since 2002 for use in foods, including meat and poultry products, as a bulking agent and for use in baby foods, excluding infant formula, at levels up to 1 gram per serving. Some other inulin applications include: synthesis of biodegradable plastics, production of high fructose syrups and crystalline fructose (Nakamura *et al*., 1995; Wenling *et al*., 1999), production of low molecular weight FOS and pet food (Kim *et al*., 1997). It can also be used as a fat substitute (Kocsis *et al*., 2007) and due to its health promoting properties, inulin can be considered as functional food (Milner, 1999; Roberfroid, 2007), nutritional compound (Coussement, 1999) and medicine in food and pharmaceutical industries (Monti *et al*., 2005; Valluru & Van den Ende, 2008).

Inulin polymerization degree

Polymerization degree of inulin (DP) in plants varies from 2 to 60 and depends on factors such as plant source, climate, growing conditions, maturity at harvest and storage time and conditions after harvest (Prosky & Hoebregs, 1999). For example, inulin from chicory (*Cichorium intybus*) has a considerably lower degree of polymerization than inulin found in artichokes (about 10) (*Cynara scolymus*), globe thistle (*Echinops ritro*) and *Viguiera discolor* (Raccuia & Melilli, 2010). The variation in length of inulin chain in Asteraceae species could be the result of different enzymatic characteristics present in these plants. The inulin stored in *C. intybus* and *H. tuberosus* has a mean DP of about 10 as estimated by acid-hydrolysis. Higher-DP inulins are found, for example, in *E. ritro* (Vergauwen *et al*., 2003; mean DP 30) and in the blossom disk of *C. scolymus* (Hellwege *et al*., 2000; mean DP 65).

Respecting to maturity at harvest, the early-harvested tubers contain a greater amount of highly polymerized sugar fractions, which offer more industrial value than late-harvested tubers or those after storage (Schorr-Galindo & Guiraud, 1997). Degradation of inulin to sucrose and fructo-oligosaccharides is higher after cold shock. For example, Saengthongpinit & Saijaanantakul (2005) reported that storage temperature and time affected quality and DP distribution proýle of inulin. Increased sucrose and DP 3ï 10, with decreased DP > 10 fractions were observed in the 2 and 5 °C cold storage tubers after 4ï 6-weeks storage, whereas inulin composition remained unchanged with frozen storage (-18 °C) for 3 months, whereas oligosaccharides as inulo-triose (GF3) and inulotetraose (GF4) where predominant throughout the 12-week study period. This decreasing on inulin content and an average polymerization degree is due to de-polymerization of high molecular weight carbohydrate (Leroy *et al*., 2010). This process often precedes growth, and is already initiated during dormancy. A role for inulin on expansion of þowers has also been suggested. Thus, the oligosaccharides liberated during breakdown of inulin probably contribute to the osmotic driving force that is involved on expansion of þowers. In general, inulin content increased with increase in dry matter of the underground organs (Raccuia & Melilli, 2010).

The degree of polymerization can inþuence some properties of inulin such as digestibility, prebiotic activity, caloric value, sweetening power, water binding capacity, etc. (López-Molina *et al*., 2005; Van de Wiele *et al.,* 2007). For example, Roberfroid *et al.* (1998) reported 50% lower degradation rate for inulin with DP > 10 compared with oligofructose (DP < 10) over a 4-hour fermentation. Decreased pH was seen in fermenters with oligofructose and inulin, but the decrease was less pronounced for inulin, indicating a slightly lower metabolic rate. Also, Stewart

et al. (2008) observed that rate and proportions of produced SCFAs are influenced by chain length (DP), with FOS (DP < 10) being rapidly fermented and producing higher proportions of butyrate than that value obtained with inulin (DP > 10). Thus, rate of fermentation may be of clinical interest for those desiring SCFA absorption in the proximal colon.

The solubility of inulin also depends on chain length (as well as molecular weight) of the polymer and further advantages can be taken of the solubility of different molecular weight of inulins in water. Shorter oligomers are much soluble than long chain polymers (Blecker *et al.*, 2001; Blecker *et al.,* 2002; Roberfroid, 2005). This means that different applications can be utilized either inulinôs solubility or its insolubility, depending on which polymer length is utilized. For example, in the food industry is generally required that inulin need to be dissolved in processing to make the gels that are important for texture and bulk (Blecker *et al.,* 2001). In contrast, crystalline forms of inulin with low solubility in water activate the complement system, relevant to its use as a vaccine adjuvant (Cooper *et al.,* 1991; Silva *et al.,* 2004) or for cancer treatment (Korbelik & Cooper, 2007).

Vegetal sources of inulin

In species of the Liliaceae, Amaryllidacea and Compositae families, inulin is usually stored in organs such as bulbs, tubers and tuberous roots so it can be easily extracted and purified in absence of interfering compounds. The two species usually employed in industry to produce inulin belong to the Compositae family, the tubers of Jerusalem artichoke (*Helianthus tuberosus*)

and chicory roots (*Chicorium intibus*), but also inulin from Dahlia tubers (*Dahlia variabilis*) is in the market (Kaur & Gupta, 2002; Ritsema & Smeekens, 2003; Kango, 2008; Gao *et al*., 2009; Leroy *et al*., 2010).

Jerusalem artichoke has some agronomical advantages such as cold and drought tolerance, salt and pest resistance. Recently, it was found that Jerusalem artichoke is one of the most promising candidates as raw material for industrial production of fructose from inulin. This plant accumulates around 50-70 g/kg of fresh weight as inulin-type fructans and has a crop yield of 5.4 ton/ha (Li & Chan-Halbrendt, 2009).

Although plants of the Compositae family are preferred, other species have been tested for inulin degradation and oligosaccharides production. Yacon (*Smallanthusson chifolius*) which belongs to the Asteraceae family stores 3 to 10% of inulin into its tubers. Dandelion (*Taraxacum officinale* syn) is a flowering plant also from Asteraceae family, with a large amount of inulin (12-15%) and oligofructans in the main root (Kango, 2008).

Inulin is also found in *Sphenoclea* sp. (Sphenocleceae), which belongs to the complex of Asterales-Campanulaceae (Porembski & Koch, 1999). Garlic (*Allium sativum* L.) belongs to the Liliaceae family and stores carbohydrates corresponding to 75% of its dry matter (Baumgartner *et al*., 2000). On the other hand, asparagus is a perennial plant with roots which contain up to 15% inulin. In addition, the annual world production of this plant is greater than chicory, Jerusalem artichoke and dahlia (Singh & Bhermi, 2008).

A large amount of fructans have been also found in the "head" or "pineapple" of Agavaceae family plants as the tequila agave (*Agave tequilana* Weber) (Mancilla & Lopez, 2002; Lopez *et al*., 2003) and mezcal potosino agave (*Agave salmiana* L.), among others (Peña *et al*., 2004). These sources of inulin have gained interest as they represent a renewable, cheap and abundant raw material for production of high fructose syrups, ethanol, citric acid, inulo-oligosaccharides (IOS) and other chemicals (Chi *et al*., 2009; Zhao *et al*., 2010). Table 1 shows various vegetable sources of inulin and their polymerization degree.

In general, to obtain inulin from these vegetables sources, a simple process is followed. First, tubers, roots or leaves or the whole plant are washed, peeled and cut into small pieces. Material can be dried and then, milled and sieved to obtain a fine powder that can be used directly as carbon source without any further treatment (Park & Yun, 2001). On the other hand, to obtain a more pure inulin, an extract of this material can be obtained with boiling water (at pH 9.5, adjusted with NaOH) and then filtered and neutralized at 65 °C. The filtrate can be concentrated with 2-propanol and then washed with acetone and dried. For additional purification, the obtained powder can be dissolved in hot water and cooled down at 4 °C. The precipitate is recovered by filtration and washed several times with acetone and then dried to obtain inulin fine powder. Panchev *et al.* (2011) extracted inulin from four Jerusalem artichoke (*Helianthus tuberosus* L.) cultivars, following this procedure, with a maximum yield of 16.5 % (Energina cultivar) and 91 % of purity and a DP of 33. In Table 2 some properties of inulin extracted from these different Jerusalem artichoke cultivars are compared.

Enzymatic hydrolysis of inulin

De-polymerization of inulin involves action of the enzymes called inulinases. These enzymes hydrolyze the ɓ-(2,1) bonds in the inulin chain to yield fructose and glucose units. They can be designated as 2,1-ɓ-D-fructan- fructanohydrolases (EC 3.2.1.7) and are divided into endo- and exoinulinases according to the mode of action on inulin.

1. Endoinulinase (2,1-ɓ-D-fructan fructano-hydrolase; EC 3.2.1.7) is specific for inulin and hydrolyze the internal ɓ-(2,1) fructofuranosidic linkages of the inulin molecule to yield a mixture of inulo-oligosaccharides with different polymerization degrees as the main products, e.g. inulotriose, inulotetraose and inulopentaose. This enzyme is not capable of breaking the Ŭ-(1,2) linkage which connects glucose to the main chain of inulin (Nakamura *et al*., 1995; Kang *et al*., 1998).
2. Exoinulinase (ɓ-D- fructan fructohydrolase EC 3.2.1.8) successively split off terminal fructose units from the non-reducing end of inulin, and also hydrolyze sucrose and raffinose. It produces a mixture of free glucose and fructose, since this enzyme hydrolyses the glycosidic bond Ŭ- (1,2) which connects glucose to the main inulin chain (Nakamura *et al*., 1978).

Production of FOS, fructose and glucose has become a big business with the use of enzymes (Montes & Magana, 2002). In the industrial processes for obtaining high fructose syrups and crystalline fructose, exoinulinases are used alone or in combination with endoinulinases for total hydrolysis of inulin. On the other hand, endoinulinases are responsible for inulo-oligosaccharides

(IOS) production. IOS have wide applications in food industry: confectionary, milk desserts, yoghurt and cheese production, bakery, chocolate, ice-cream and sauces (Chi *et al.*, 2011). It was found that the major IOS obtained after inulin hydrolysis with endo-inulinases have a degree of polymerization of 3 and 4, like 1-kestose (GF_2), nistose (GF_3) and 1-fructosyl-nistose (GF_4).

However, cost of enzyme is one major limiting factor for applications at industrial scale. Significant reduction in cost can be achieved by employing low-value and abundant inulin rich plant parts (as the ones mentioned in previous section) for inulinase production and thus efforts are underway to develop a cheaper process. Use of low value natural complex substrates of plant origin as carbon source has been shown to enhance enzyme production, particularly, in case of inducible glycosidases (Ongen-Baysal *et al.,* 1994; Kango *et al.,* 2003). Tubercles of yacon (*Polymnia sanchifolia*), also a member of Asteraceae, have been reported as an inexpensive substrate for inulinase production from *Kluyveromyces marxianus* (Cazetta *et al.*, 2005). Recently, garlic bulbs (*Allium sativum*) have been used for inulinase production from *Streptomyces* sp. (Sharma *et al.,* 2006).

Microbial inulinases production

In the last decades, a large number of fungal, yeast and bacterial strains has been used for inulinase production. As example, it can be found *Yarrowia* lipolitica, *Cryptococcus aureus* (Gao *et al.,* 2007), *Arthrobacter* sp. (Kang *et al*., 1998), *Pseudomonas* sp. (Chi *et al*., 2009), *Paenibacillus* sp. (Gern *et al*., 2001*) Kluyveromyces* sp. Y-85, and *Aspergillus ficuum* JNSP5-06 (Chen *et al*., 2009). However, among the various microbial strains, *Kluyveromyces marxianus* and *Aspergillus niger* are reported as the most common and preferred microorganisms for

inulinase production (Pandey *et al.*, 1999; Singh & Gill, 2006; Chi *et al.*, 2009). Furthermore, it is reported that all *Kluyveromyces marxianus* and *Aspergillus niger* strains produce the enzyme in an extracellular manner, except for *Aspergillus niger* strain12 (Nakamura *et al*., 2001) and *Kluyveromyces*sp-Y-85 (Wei *et al*., 1997).

Bacterial production

Data on inulinase biosynthesis using bacterial strains are scarce and mainly about endoinulinases (Neagu & Bahrim, 2011). Some *Bacillus* species are active producers of extracellular inulinase (Zherebstov *et al*., 2002) as well as *Pseudomonas* sp. (Kim *et al*., 1997) and *Arthrobacter* sp. (Kang *et al*., 1998). In a study carried out by Ayyachamy *et al.* (2007), inulinase production of 101 and 117 U g/ds were obtained from *Xanthomonas campestris pv phaseoli* using garlic and onion as substrate, respectively, and in a submerged culture at an optimum pH of 7.0, temperature of 37 ºC and agitation of 150 rpm, an inulinase production of 17.42 U/mL . On the other hand, Dilipkumar *et al*. (2011) had a maximum inulinase activity of 89 U/gds using *Streptomyces* sp. and pressmud as carbon source.

Fungal production

Gern *et al*., (2001) tested sixteen fungal strains reported in literature as endo-inulinase producers and three bacterial strains, isolated from dahlia rizosphere. Among bacterial strains, *Paenibacillus* sp. CDB 003 was the most suitable for endo-inulinase production, as this enzyme produced inulobiose as the principal substrate as well as inulo-oligosaccharides with polymerization degrees of 3-5. Regarding to fungal strains, based on results obtained by TLC, *Aspergillus niger* DSM 2466 was selected as the best endo-inulinase producer. Ge and Zhang

(2005) also used an *Aspergillus niger* strain and obtained a maximum inulinase activity of 100 U/mL in the presence of S-770 sucrose ester as nutritive substrate added into the fermentative medium. Kumar *et al*. (2005) obtained a maximum inulinase activity of 176 U/mL at a 5% (w/v) inulin concentration in the medium, using a soil isolated fungal strain and identified as *A. niger*. In a study using an infusion prepared from dandelion tap roots, Kango (2008) obtained 52.5 U/mL inulinase activity after 96 h of cultivation with an *Aspergillus niger* strain while Naveen (2008) obtained 52.3 U/mL using the same substrate and *A. niger* NK-126. However, the results obtained using extract of chicory where lower (12.3 U/mL). Besides, other species of *Aspergillus* have been reported in literature for inulinase production such as *A. parasiticus* (Ertan *et al.,* 2003), *A. awamori* (Nagem *et al*., 2004), *A. fumigatus* (Gill *et al*., 2006) and *A. ochraceus* (Guimaraes *et al.,* 2007).

Yeast production

Yeasts have been used in enzyme production, as they are easier to grow and handle in comparison with bacteria. Among the reported inulinase-producing yeasts, *Kluyveromyces* sp., *Pichia* sp. and *Candida* sp. have high potential. *Kluyveromyces* species are well known for their ability to grow on fructans such as inulin. The inulinase production by *Kluyveromyces marxianus* YS-1 was investigated using as substrate roots of *Asparagus racemosus* obtaining 47.1 U/mL (Singh *et al.,* 2006) and 50.2 U/mL (Singh & Bhermi, 2008), showing that inulinase yield is six times higher when produced in a bioreactor in comparison with shake flask trials. However, these values were lower than those obtained by Kalil *et al.* (2001) using a strain of *Kluyveromyces marxianus* and an optimized medium (127 U/mL). In other optimization studies,

Mazutti *et al.* (2007) obtained a maximum inulinase activity of 250 U/gds using solid-state fermentation and 47.2 U/mL in submerged fermentation (Mazutti *et al.,* 2010). On the other hand, Treichel *et al.* (2009) obtained 1317 U/mL using agro-industrial residues as substrate.

Thermostability

Bacterial strains are used for inulinase production, mainly because of their thermostability. It is an important factor to consider because inulin is insoluble in cold water and only slightly (5 %) soluble even in water at 55 °C. Thus, a thermostable and inulinolytic enzyme would be expected to play an important role in food and chemical industries. However, most of the reported inulinases lose their activity after few hours at this temperature. It has been reported that the thermophile *Geobacillus stearothermophilus* KP1289 strain grew between 41 °C and 69 °C and produced an inulinase which is active at 30-75 °C with an optimum at 60 °C (Tsujimoto *et al.,* 2003). *Clostridium thermoautotrophicum* isolated from dahlia tubers showed that cell-bound and cell-free inulinase functioned optimally at 60 °C under neutral pH conditions (Wim & Jan, 1991). In yeast, *Pseudozyma* sp. CCMB 300 showed the smallest reduction in its inulinase activity, only 1.72% after 50 min, whereas inulinase from *K. marxianus* CCMB 322 retained only 55.28 % of its original activity after 50 min at 60 °C. At higher temperatures, although the inulinase produced by *K. marxianus* CCMB 322 lost 100 % of its activity after 10 min of heat treatment at 70 °C those inulinases produced by *Pseudozyma* sp. CCMB 300 showed a non-linear lost, decreasing as time and temperature increased during pre-incubation (Lima *et al.,* 2009). Among the different fungal strains, the extracellular extract of *Aspergillus fumigatus*

exhibited thermostable inulinase activity. At an optimum temperature of 60 °C, the ammonium sulphate fraction retained= 70% of its inulinase activity at the end of 72 hours incubation in the absence of inulin, and in the presence of inulin the isoform II retained 54 % of its activity (Prabhjot *et al.,* 2006). A comparison between optimal temperature and thermostability of inulinases from different sources is showed in Table 3.

Factors affecting inulinase production

Concerning to inulinase production by fermentation, different components and mediums have been used. For example, in the case of nitrogen source, it has been observed that complex nitrogen sources such as yeast extract, peptone, corn steep liquor and beef extract are better than inorganic sources ($NaNO_3$, $NH_4H_2PO_4$ or NH_4Cl) (Gill *et al*., 2003; Zhang *et al*., 2005). Yeast extract has been reported as the best nitrogen source for inulinase production in the case of *Aspergillus niger, Streptomyces* sp. and *Cryptococcus aureus* (Gill *et al.,* 2003; Skowronek & Fiedurek, 2006; Sheng *et al., 2*007; Kango, 2008). It could be due to the presence of vitamins and trace elements that enhance inulinase production. However, concentrations above 0.5 % (w/V) of yeast extract in the fermentation medium repressed inulinase activity of marine yeast, *Cryptococcus aureus* (Shen *et al.,* 2007). Finally, a positive influence of inorganic salts such as Mn^{2+}, Ca^{2+} (Singh & Gill, 2006; Singh & Bhermi, 2008), Mg^{2+}, Fe^{2+} and K^{+} (Derycke & Vandame, 1984) for inulinase synthesis has been reported.

Characterization of purified endoinulinases

Most reports on purification of extracellular microbial endoinulinases include various conventional methods like centrifugation, precipitation with salts or organic solvents or ultra-filtration followed by ion exchange chromatography and gel filtration (Wei *et al*., 1997; Uhm *et al*., 1999; Nakamura *et al.*¸ 2001; Chen *et al.*, 2009). In some cases, fast protein liquid chromatography (FPLC) (Ettalibi & Baratti, 1987), hydrophobic interaction chromatography (Kang *et al*., 1998; Cho & Yun, 2002; Skowronek & Fiedurek, 2006) and preparative electrophoresis (Uhm *et al*., 1999; Chen *et al.*, 2009) have been used. Although most endoinulinases purified from fungi and bacteria are extracellular, when they are produced in an intracellular way, purification needs a preliminary step of cell disruption.

In 1978, Nakamura *et al.* purified for the first time the extracellular P-III endoinulinase from *Aspergillus niger* strain 13 by ethanol precipitation, anion exchange and gel permeation chromatography, obtaining its crystalline form by precipitation with ammonium sulfate. The enzyme contained 6.7 % of carbohydrates, being the major components mannose and galactose. Subsequently, five exoinulinases (Exo I, II, III, IV and V) and three endoinulinases (Endo I, II and III) as well as an invertase (Inv) were purified from a commercial preparation of inulinase of *A. ficuum* ATCC 16882 (Novozyme 230, Novo A/S, Bagsvaerd, Denmark) by conventional techniques. All exoinulinases showed the same molecular mass (74 kDa), whereas the endoinulinases showed 64 kDa (Ettalibi & Baratti, 1987). Additionally, two endoinulinase isoforms (P-1A and P-1B) have been purified from *Aspergillus niger* mutant 817 (Nakamura *et al*., 1994). Their specific activities were 3.5 higher than the endoinulinase III of the wild strain, with a lower Km.

Recently, three exoinulinases (Exo-I, Exo-II and Exo-III) and two endoinulinases (Endo-I and Endo-II) have been purified from *A. ficuum* JNSP5-06 culture broth (Chen *et al*., 2009). All endoinulinases were stable at 50 ºC with an optimum activity at 45 ºC. Their optimal pH was 4.5 and 5.0 for exoinulinase and endoinulinase, respectively. Inulinases from *Penicillium purpurogenum*, *Chrysosporium pannorum* and *Penicillium* sp. strain TN-88 have also been characterized (Onodera & Shiomi, 1988; Xiao *et al.*, 1988; Nakamura *et al.,* 1997). In general, these enzymes are glycoproteins with molecular mass between 58 and 70 kDa with and optimum pH value from 4.5 to 7.0 and are stable in a temperature range from 45 to 55 ºC.

Strategies to increase production of functional oligosaccharides

Generally, enzymatic processes are expensive because the high production, purification and stabilization cost. For large scale production of oligosaccharides, it is necessary to use cheaper enzyme technology and develop recombinant microorganisms. Two major areas of biotechnology have solved this, biocatalysis and metabolic engineering, through genetic engineering and molecular biology techniques have been obtained many modified enzymes with enhanced properties compared to their natural counterparts (Poppe & Novak, 1992; Kim *et al*., 1999).

In the case of fungal inulinases, several studies of cloning and modification have been made to achieve greater efficiency. Nakamura *et al*. (1997) obtained 70% of inulotriose as the main product when using the endoinulinase P-II from *Penicillium* sp. TN-88. Also, *A. niger* 12 (a

strain that produces constitutively exo and endoinulinases) was used to create the mutant 817, which proved to have four times higher inulinase activity than the wild strain under submerged culture.

Furthermore, the gene encoding for the *Aspergillus ficuum* endoinulinase (INU2) has been cloned and sequenced. Alignment of amino acidic sequences revealed a 73.9 % similarity between endoinulinases from *A. ficuum* and *Penicillium purpurogenum*. Endoinulinase gene INU2 has been successfully expressed in *Saccharomyces cerevisiae* and the enzyme produced by the recombinant yeast was used to hydrolyze inulin and produce fructooligosaccharides. It was found that the main product was 1-kestose (Kim *et al.*, 1999). Also, an increase in the production of *A. ficuum* endoinulinase was achieved in the heterologous expression system by the SUC2 gene deletion (Park *et al.*, 2001).

Future perspectives

Currently, costumers have a high standard for what they are consuming. The demand for good-tasting foods, and reduced fat or calories has increased. The oligosaccharides are a source of fiber which satisfies such considerations. Market for these compounds is substantial and continues to expand. Development of functional foods is a great opportunity to contribute in improving quality of consumer health and welfare. IOSôs are currently recognized for their bifidogenic effect, beneficial effect on health and represent one of the oligosaccharides mostly produced and used in the food industry. Inulin containing plants represent an inexpensive,

abundant and renewable raw material for large scale production of prebiotic oligosaccharides. To make this process industrially suitable, further efforts are needed to develop cheaper enzyme technologies through characterization of new endoinulinase producers, thermostability and process optimization studies, genetic engineering and efficient purification systems. Finally, the study of IOS metabolism and function as prebiotic in human should be performed. With this knowledge in combination with enzyme engineering, new potential prebiotic compounds could be developed to further ensure the quality of life of consumers.

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Table 1. Inulin content and chain length in some vegetal sources.

Vegetal source	Part	% fructans on fresh	DP	Reference
Onion (*Allium cepa*)	Bulb	1.1-7.5 (average 3.6)	1-12	van Loo *et al*., 1995
Jerusalem artichoke (*Helianthus tuberosus*)	Tubers	17-20.25	2-19=74% 19-40=20%, >4=6%	van Loo *et al*., 1995; Gupta & Kaur, 1997; Partida *et al.,* 1998
Artichoke (*Cynara scolymus*)	Leaves/heart	1.8% (fresh), 1.7% (cooked)	>5=95%	van Loo *et al.,* 1995
Chicory (*Chicorium intybus*)	Roots	15.2-20.5 (average 16.2)	2-19=55% 19-40=28%, >40=17%	van Loo *et al*., 1995; Gupta & Kaur, 1997; Partida *et al.,*

				1998
Asparagus (*Asparagus officinalis*)	Root tubers	10-15		Gupta & Kaur, 1997
Leek (*Allium ampeloprasum* var. *porrum*)	Bulb	3-10	12	van Loo *et al*., 1995
Garlic (*Allium sativum*)	Bulb	12.98	>5=75%, average=15	van Loo *et al*., 1995
Banana (*Musa cavendishii*)	Fruit	0.7	<5=100%	van Loo *et al*., 1995
Wheat (*Triticum aestivum*)		1.17 (white flour)	7-8	Collins & Rastall, 2008
Rye (*Secale cereale*)	Grains	0.6%		van Loo *et al*., 1995
Barley (*Hordeum vulgare*)	Grains	22.1 (young kernals), 1.1 (mature kernals)		van Loo *et al*., 1995

Dandelion (*Taraxacum officinale*)	Leaves	12.8 (leaves)	van Loo *et al.,* 1995
Dahlia (*Dahlia* sp.)	Root tubers	15-20	Gupta & Kaur, 1997
Shatavari (*Asparagus racemosus*)	Root tubers	10-15	Gupta & Kaur, 1997
Agave (*Agave americana*)	Lobes	7-10	Partida *et al.,* 1998

Table 2. Characteristics of inulins extracted from different Jerusalem artichoke cultivars.

	Jerusalem artichoke		
	Energina	**Topstar**	**Spindel**
Yield (%)	16.5 ± 0.9	14.0 ± 6	15.7 ± 0.8
Molecular weight (kDa)	5.6 ± 0.5	4.8 ± .4	5.2 ± .4
DP	33	28	30
Purity (%)	91	89	88

Table 3. Properties of some microbial inulinases

Microorganism	Optimum temperature (° C)	Temperature stability (° C)	Maximal activity	References
K. marxianus	55	40	18743 U/mL	Kushi *et al.*, 2000
			1317 U/mL	Treichel *et al*., 2009
			47.2 U/mL	Mazutti *et al*., 2010
Yarrowia lipolitica	50	50	22.5 U/mg	Liu *et al,* 2010
			62.85 U/mL	Gao *et al*., 2007
Cryptococcus aureus	50	65	85.0 U/mL	Sheng *et al*., 2008
			52.37 U/mL	Gao *et al*., 2007
Pichia guilliermondii	60	60	60.1 U/mL	Gong *et al*., 2008
			130.38 U/mL	Yu *et al*., 2009
Streptomyces sp.	60	60-70	524 IU/L	Sharma *et al*., 2006
			89 U/gds	Dilipkumar *et al*., 2011
Bacillus sp.	--	25-40	42.36 U/mL	Zherebtsov *et al*.,

				2002
A.ochraceus	60	60	108 Total U	Guimaraes *et al*., 2007
A. niger	55	65		
A. ficuum	60	60-70	193.6 U/gds	Chen *et al*., 2011
Geotrichum candidum	--	--	45.65 IU/mL	Mughal *et al*., 2009

