

Research Article

Fungal Inulinases as Potential Enzymes for Application in the Food Industry

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Abstract: Inulinase is a versatile enzyme used in many fields, especially in food industry, to produce high fructose syrups and Fructo-Oligosaccharides (FOS). In this review study, fungal inulinases were investigated with a particular emphasis on their production, properties and their potential applications in the food industry. The production of inulinases has been reported from various fungal and yeast strains such as *Penicillium*, *Kluyveromyces* and *Aspergillus* sp. Microorganisms are the best sources for inulinases production, as are easy to be cultivated and produce high enzymes yields.

Keywords: Food industry, fungal inulinases, microorganisms, properties, substrates

INTRODUCTION

Inulinases are enzymes capable of degrading inulin a widespread naturally occurring poly-fructan in plants (Carpita *et al.*, 1989; Hendry, 1987; Marchetti, 1993), consisting of linear chains of β (2,1)-linked fructose units attached to a terminal sucrose molecule (Vandamme and Derycke, 1983). Fructose is a GRAS (Generally Recognized as Safe under U.S. FDA regulations) sweetener that enhances flavor, color and product stability (Hanover and White, 1993) and is considered as a safe alternative sweetener to sucrose because it has beneficial effects in diabetic patients, increases iron absorption in children and has a higher sweetening capacity (Pawan, 1973; Roberfroid and Delzenne, 1998) and is used as dietary fibers because of its fat like texture. Sucrose, on the other hand, is known to cause problems related to corpulence, carcinogenicity and arteriosclerosis (Vandamme and Derycke, 1983). In addition, fructose is more soluble than sucrose, less viscous and in low levels it can be metabolized without a need of insulin (Fleming and Groot Wassink, 1979).

Fructo-oligosaccharides are emerging fast as important ingredients in the food and pharmaceutical industry. Fructo-oligosaccharides have good functional and nutritional properties such as low calorie diet, Bifidus stimulating factor and source of dietary fiber in food preparations (Roberfroid and Delzenne, 1998; Elmer, 1986; Kim and Kim, 1992). These oligosaccharides are therefore now widely used to replace sugars in many food formulations such as

confectionery and dairy products (Vandamme and Derycke, 1983; Ohta *et al.*, 1993).

Both fructose and fructooligosaccharides can be produced from inulin by chemical hydrolysis (pH 1-2 at 80-100°C), but the low pH results in fructose degradation and the process also gives rise to the formation of Di-Fructose Anhydride (DFA), which is a colored byproduct with almost no sweetening capacity (Barthomeuf *et al.*, 1991). Microbial inulinases play an important role in the hydrolysis of inulin for the production of fructose syrups (Zittan, 1981) and FOS (Sangeetha *et al.*, 2005; Skowronek and Fiedurek, 2006).

The present review will focus mainly on the production, purification and characterization of fungal inulinases as well as their application in the food industry.

CLASSIFICATION AND MODE OF ACTION

Inulinases can be divided into exo- and endo-inulinases according to their mode of action on inulin (Nakamura *et al.*, 1988). Exo-inulinases (β -D-fructan fructohydrolase, EC 3.2.1.80) split off terminal fructose units successively from the non-reducing end of the inulin molecule. The exo-acting inulinases hydrolyze sucrose and the fructose portion of raffinose in addition to inulin. However, fructan exohydrolases obtained from a protein preparation of Jerusalem artichoke tubers have been reported to be specific for β -(2 \rightarrow 1) linkages while being inactive against sucrose (Edelman and Jefford, 1964). In contrast, endo-inulinases (2, 1- β -D-fructan fructanohydrolase, EC 3.2.1.7) are specific

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Table 1: Microorganisms employed for production of inulinases

Microorganism	Nature of enzyme and maximal activities ^a	Reference
Moulds		
<i>A. niger</i> NK-126	E, 55	Kango (2008)
<i>Aspergillus</i> sp.	E, 75	Viswanathan and Kulkarni (1996)
<i>A. aureus</i> MTCC 151	E, 1-1.2	Gupta <i>et al.</i> (1994)
<i>A. ficuum</i>	E, 3000, ^b 3366 ^b	Carniti <i>et al.</i> (1991), Kim and Rhee (1989)
<i>A. fischeri</i> MTCC 150	E, 1-1.2	Gupta <i>et al.</i> (1994)
<i>A. flavus</i> MTCC 277	E, 1-1.2	Gupta <i>et al.</i> (1994)
<i>A. nidulans</i> MTCC 344	E, 1-1.2	Gupta <i>et al.</i> (1994)
<i>A. niger</i> A42	E, 4600, ^b 43.7	Ongen-Baysal <i>et al.</i> (1994), Azhari <i>et al.</i> (1989)
<i>A. niger</i> AUP19	E, 176 ^c	Kumar <i>et al.</i> (2005)
<i>A. niger</i> MTCC 281	E, 1-1.2	Gupta <i>et al.</i> (1994)
<i>A. niger</i> mutant 817	E, 160	Nakamura <i>et al.</i> (1995)
<i>Cladosporium</i> sp.		Viswanathan and Kulkarni (1996)
<i>Chrysosporium pannorum</i> AHU 9700	E, 115	Xiao <i>et al.</i> (1988,1989)
<i>F. oxysporum</i> NCIM 1072	E, I, 80, ^c 21, ^d 63.3 ^d	Gupta <i>et al.</i> (1988); Gupta <i>et al.</i> (1990); Gupta <i>et al.</i> (1992)
<i>Penicillium</i> sp. 91-4	E, 50	Wenling <i>et al.</i> (1994)
<i>Penicillium</i> sp. TN 88	E, 9.9	Nakamura <i>et al.</i> (1997)
<i>P. rugulosum</i>	E, 54	Barthomeuf <i>et al.</i> (1991)
Yeast		
<i>C. kefir</i> DSM70106		Pessoa <i>et al.</i> (1996)
<i>Kluyveromyces</i> sp. Y-85	E, 68.9 ^a	Wenling <i>et al.</i> (1999)
<i>K. fragilis</i> ATCC 12424	E, I, 355, 1000, ^b 19 ^c	Pandey <i>et al.</i> (1999) Thonart <i>et al.</i> (1988)
<i>K. marxianus</i> ATCC 36907	E, 2.60 ^a	Passador <i>et al.</i> (1996)
<i>K. marxianus</i> ATCC 52466	E, 122.88 ^b	Selvakumar and Pandey (1999)
<i>K. marxianus</i> CDBB-L-278	E, 82	Cruz-Guerrero <i>et al.</i> (1995)
<i>K. marxianus</i> var. <i>marxianus</i> CBS 6556	E, 3000 ^c	Hensing <i>et al.</i> (1994)
<i>K. marxianus</i> var. <i>Bulgaricus</i> ATCC 16045	E, 8.6-11.6	Silva-Santisteban <i>et al.</i> (2006)
<i>K. marxianus</i> CBS 6556	E, I, 58 ^c	Rouwenhorst <i>et al.</i> (1988)
<i>K. marxianus</i> YS-1	E, 47,3 ^c , 40.2	Singh <i>et al.</i> (2006); Singh <i>et al.</i> (2007)
<i>Pichia</i> sp.		Zhang <i>et al.</i> (2005)

^aUnits/mL, unless otherwise specified; E, Extracellular; I, Intracellular; ^bUnits/g; ^cUnits/L; ^dSpecific activity; ^eUnits/milligram; ^fTotal activity from ground cell and medium in wild type; ^gTotal activity from ground cell and medium in mutant; ^hUnits enzyme/gds. U/gds

for inulin and hydrolyze the internal β -(2 \rightarrow 1)-fructofuranosidic linkages to yield inulotriose, inulotetrose and inulopentose as the main products. Both exo- and endo-inulinases belong to family 32 of the glycosyl-hydrolases (Henrissat, 1991; Henrissat and Bairoch, 1993; Pons *et al.*, 1998).

MOULDS AND YEASTS PRODUCING INULINASE

Several strains of moulds and yeasts have been used for the production of inulinases (Table 1). Among them, mould strains belonging to *Aspergillus* sp. and yeast strains belonging to *Kluyveromyces* sp. are apparently the most common and preferred choice. Viswanathan and Kulkarni (1996) isolated several fungal strains including *Aspergillus*, *Penicillium*, *Sporotrichum* and *Cladosporium* from dahlia rhizosphere and reported *A. niger* as the highest inulinase producer (75 U/mL). When the culture was grown in a medium containing inulin and corn steep liquor, the enzyme yields were 1.5-fold higher than those obtained with synthetic medium (Poorna and Kulkarni, 1995). Following ultraviolet radiation, the *A. niger* strain yielded 3.1 times more enzyme in comparison to the parent strain (Viswanathan and Kulkarni, 1995a). Nakamura *et al.* (1995) also reported higher enzyme titers from a mutant strain of *A. niger* 817. Gupta *et al.* (1994) reported that 5 strains of *Aspergillus* sp., namely, *A. fischeri*, *A. aureus*, *A. flavus*, *A. niger* and *A. nidulans*, showed

maximum production of 1000-1200 U/L of extracellular inulinase after 9 days of growth. Ongen *et al.* (1994) achieved very high yields of inulinases (4600 U/g) from a culture of *A. niger* A42. Carniti *et al.* (1991) stated that Novo Industries (Denmark) has developed a commercial preparation of inulinase from *A. ficuum* (Novozym 230). Kim and Rhee (1989) have also used inulinase from *A. ficuum* to produce fructose syrup, whereas, Gill *et al.* (2006) succeeded to hydrolyze inulin using *Aspergillus fumigatus*. Gouda (2002) tested inulinase and invertase production using a local isolate identified as *Aspergillus fumigatus*. Kumar *et al.* (2005) isolated from different soil samples 5 strains showing a high inulinase activity and one of them was identified as *Aspergillus niger* AUP19 with the maximum productivity of inulinase being 176 U/mL after 72 h. Nakamura *et al.* (1978a, 1978b) reported on the characterization of an extra-cellular inulinase from *Aspergillus* sp., whereas Peters and Kerkhooft (1983) obtained enzyme preparations from *Aspergillus phoenicis* with a high inulinase activity. Skowronek and Fiedurek (2004) optimized inulinase production by *Aspergillus niger*, while Norman and Hojer-Pedersen (1989) used *Aspergillus ficuum* strain to produce fructo-oligosaccharides from inulin (Table 1). Several species of *Penicillium* have also been used for the production of inulinase which was recovered from the culture broth, i.e., extracellular in nature (Barthomeuf *et al.*, 1991; Moriyama *et al.*, 2002; Pessoni *et al.*, 1999). Wenling *et al.* (1994) have isolated from soil a high-

inulinase producing strain *Penicillium* sp. designated as 91-4. Inulinase preparation from *P. rugulosum* proved to be a better choice for the production of high fructose syrup than *A. niger* (Barthomeuf *et al.*, 1991). Gupta *et al.* (1990); Gupta *et al.* (1992); Kaur *et al.* (1992), prepared inulinase from *Fusarium oxysporum* and found that the nature of enzyme, extra- or intracellular, varied with the substrate used for cultivation. Xiao *et al.* (1988); Xiao *et al.* (1989), isolated from soil a mold, *Chrysosporium pannorum* AHU 9700, was found to produce a very active inulin-hydrolyzing enzyme.

Many species of yeast are generally preferred for inulinase production because their enzymes are able to hydrolyze both inulin and sucrose. There are reports on inulinase production using strains of *Candida* sp., *Sporotrichum* sp., *Pichia* sp. and *Kluyveromyces* sp. Two species of *Kluyveromyces*, *fragilis* and *marxianus*, have high potential for producing commercially acceptable yields of inulinase.

Passador *et al.* (1996) used a micro-titer reader system for screening yeast belonging to the genera of *Kluyveromyces*, *Candida*, *Debaryomyces* and *Schizosaccharomyces*. Four strains belonging to *K. marxianus* (CBS 6397, DSM 70792, ATCC 36907 and IZ 619) were found suitable, with the strain ATCC 36907 being the most suitable for inulinase production. Similarly, a strain of *K. marxianus* CDBB-L-278 (hyper-producing strain) gave a 3.3-fold increase in enzyme yield in comparison with the control strain, *K. marxianus* NCYC-1429 (Cruz-Guerrero *et al.*, 1995; Cruz *et al.*, 2006). The inulinase produced by most of the strains was extracellular in nature but, Rouwenhorst *et al.* (1991) reported only about 50% enzymes in the culture liquid. Parekh and Margaritis (1986) also reported only 25% total inulinase activity of extracellular nature with the remaining 75% being cell associated. Hensing *et al.* (1994) employed a strain of *K. marxianus* var. *marxianus*, CBS 6556, for the production of extracellular inulinase in high cell density fed-batch culture and yields as high as 3,000,000 U/L were achieved. However, when this strain was grown in a continuous culture in a chemostat under various experimental conditions, a substantial portion of the enzyme was associated with the cell wall and this could be released from the cell wall by a simple chemical treatment (Rouwenhorst *et al.*, 1988). Gupta *et al.* (1994) tested *K. fragilis* NCIM 3217, *K. marxianus* NCIM 3231, *Hansenula polymorpha* NCIM 3377, *Pichia fermentan* NCIM 3408, *P. polymorpha* NCIM 3419 and *D. castellii* NCIM 3446 for inulinase production and found that only *K. fragilis* was suitable.

Guiraud *et al.* (1987) isolated respiratory-deficient mutant strains of *K. fragilis* LM92 by treatment with ethidium bromide at 2.8 mg/L and one of the mutants, S23, was characterized with improved inulinase activity apart from other improved characteristics. Treatment with 0.5% ethylmethanesulfonate of another strain, *K. fragilis* LG, also resulted in mutants with enhanced activity of inulinase synthesis; one of them, D9, produced 1.5 times the amount of enzyme that the

parent strain produced and secreted 3 times that amount into the medium (Bourgi *et al.*, 1986). Thonart *et al.* (1988) obtained 50 mutants of the strain of *K. fragilis* (ATCC 12424) using nitrosoguanidine and found that the most important improvement was intracellular inulinase activity which was notably increased with the KF28 mutant strain. In a study on recovery of extracellular inulinase from *C. kefir* DSM 70106 which showed good capacity to synthesize the enzyme, it was found that the enzyme could be recovered directly from the culture broth and the presence of yeast cells did not cause any problem (Pessoa *et al.*, 1996). Singh *et al.* (2006); Singh *et al.* (2007) used a root tubers of *Asparagus officinalis* as a source of raw inulin for the production of exoinulinase (EC 3.2.1.7) from *Kluyveromyces marxianus* YS-1 a newly isolated strain. Root extract prepared at 10 kg/cm² pressure for 10 min showed maximum inulinase production. Medium components and process parameters were standardized to improve the enzyme production. After optimization, the enzyme production was 4.8 times more than the basal medium. Silva-Santisteban *et al.* (2006) cultivated a strain *Kluyveromyces marxianus* ATCC 16045 in a batch on minimal medium to overproduce inulinase and optimized the production of inulinase. Zhang *et al.* (2005) studied the INU1 gene encoding an exoinulinase from *Kluyveromyces marxianus* KW02 expressed in *Pichia pastoris*. Laloux *et al.* (1991) also studied the INU1 gene encoding an exoinulinase from *Kluyveromyces marxianus* Var. *Marxianus* ATCC 12424 and come across that it was successfully expressed in *S. cerevisiae*. The expressing product of the recombinant was functional. However, it cannot be put into industry application, because of its low expression level.

SUBSTRATES USED FOR INULINASE PRODUCTION

Inulin is the most commonly used substrate for the production of inulinase. Inulin is a carbohydrate reserve found in the roots and tubers of several plants such as Jerusalem artichoke (*Helianthus tuberosus* L.) (Edelman and Jefford, 1964; Wenling *et al.*, 1999; Bhatia *et al.*, 1974), Chicory (*Cichorium intybus* L.) (Flood *et al.*, 1967; Bhatia *et al.*, 1974; Gupta *et al.*, 1985; Gupta *et al.*, 1986; Gupta *et al.*, 1989; Van Waes *et al.*, 1998), Dahlia (*Dahlia variabilis* L.) (Barthomeuf *et al.*, 1991; Drent and Gottschal, 1991; Yun *et al.*, 1997), Yacon (*Polymnia sonchifolia*), (Asami *et al.*, 1989; Wei *et al.*, 1991; Vilhena *et al.*, 2003; Cazetta *et al.*, 2005) and also in burdock (*Arctium lappa*) (Ishimaru *et al.*, 2004), Agave (*Agave Americana*) (Bhatia and Nandra, 1979; Nandra and Bhatia, 1980) and Dandelion (*Taraxacum officinale*) (Trojanova *et al.*, 2004; Schutz *et al.*, 2006; Kango, 2008). Although a variety of substrates (carbon or energy source) has been used for inulinase production, using different fungi (Table 2). They include pure substances,

Table 2: Substrates Used for Production of Inulinases

Substrate and concentration	Microorganism	Reference
Pure substrates		
Glucose (1–2%)	<i>K. Marxianus</i>	Espinoza <i>et al.</i> (1992), Schwan <i>et al.</i> (1997)
Fructose (1%)	<i>K. Marxianus</i>	Nakamura <i>et al.</i> (1995)
Fructose (1%)	<i>K. fragilis</i>	Tsang and Groot-Wassink (1988)
Sucrose (0.2–5%)	<i>A. Niger</i>	Nakamura <i>et al.</i> (1994)
	<i>K. marxianus</i> , and several other yeasts strains	Rouwenhorst <i>et al.</i> (1988), Hensing <i>et al.</i> (1995)
Lactose (1%)	<i>K. Fragilis</i>	Tsang and Groot-Wassink (1988)
Fructan (3%)	<i>F. Oxysporum</i>	Gupta <i>et al.</i> (1990)
Fructosan (3%)	<i>F. oxysporum</i>	Gupta <i>et al.</i> (1988)
Inulin (0.5–2%)	<i>A. niger</i>	Poorna and Kulkarni, (1995)
	<i>K. marxianus</i>	Parekh and Margaritis (1986)
	<i>C. kefyri</i> and other soil isolates	Pessoa <i>et al.</i> (1996)
Caproyl-inulin (0.5%)	<i>K. marxianus</i>	Fontana <i>et al.</i> (1994)
Cholesteryl-inulin (0.5%)	<i>K. marxianus</i>	Fontana <i>et al.</i> (1994)
Natural substrates		
Dahlia rhizosphere (1, 3%)	Yeasts and fungal strains	Barthomeuf <i>et al.</i> (1991)
Jerusalem artichoke (0.2–8%)	<i>A. niger</i>	Ongen <i>et al.</i> (1994); Ongen and Sukan (1996)
	<i>P. purpurogenum</i>	
Chicory roots (0.2–8%)	<i>P. purpurogenum</i>	Gupta <i>et al.</i> (1989)
	<i>F. oxysporum</i>	Gupta <i>et al.</i> (1990)
Kuth roots (1%)	<i>A. niger</i>	Gupta <i>et al.</i> (1988)
Wheat bran (40%) ^a	<i>K. marxianus</i> (123 U/gds) ^b	Selvakumar and Pandey <i>et al.</i> (1999)
Mixed substrates		
Inulin (0.25–1.0%) +2-deoxyglucose(0.01–0.1%)	<i>K. marxianus</i>	Cruz-Guerrero <i>et al.</i> (1995)
Glycerol (0.25–1.0%) +2-deoxyglucose(0.01–0.1%)	<i>K. marxianus</i>	Cruz-Guerrero <i>et al.</i> (1995)
Chicory roots + fructans (1%)	<i>K. fragilis</i>	Gupta <i>et al.</i> (1994)
Inulin +wheat (1%)	<i>A. fumigatus</i>	Gouda (2002)

^aSolid-state fermentation; ^bU/gds, units per gram of dry substrate

naturally occurring inulin-rich materials and mixed substrates.

Among the pure substrates, which are mostly sugars of mono-, di-, or polysaccharide nature, inulin and sucrose have been employed preferably as carbon sources. In general, if the fungal strain showed only inulinase activity, inulin served as the best source of carbon (Poorna and Kulkarni, 1995; Pessoa *et al.*, 1996, Pandey *et al.*, 1998). However, if the fungus exhibited inulinase activity coupled with invertase activity, sucrose served as a better carbon source for enzyme production. There are many reports describing the use of sucrose as carbon source primarily for inulinase production (Rouwenhorst *et al.*, 1988; Hensing *et al.*, 1994; Nakamura *et al.*, 1994; Hensing *et al.*, 1995). Poorna and Kulkarni (1995) carried out a study on the use of various carbon sources singly or in combination and found that inulinase production by fungal culture was probably inducible and subject to catabolic repression (Table 2).

In their study on microbial inulinase secretion, Fontana *et al.* (1994) used chemically modified inulin for enzyme production. Caproyl and cholesteryl derivatives of native dahlia inulin were prepared from the respective chloride donors and the light derivatization was monitored by ¹³C-Nuclear Magnetic Resonance (NMR) and Fourier Transform Infrared spectroscopy (FTIR). These inulin derivatives were employed as carbon sources and as inulinase inducers using different strains of the inulinolytic yeast, *K. marxianus*. Compared to the cholesteryl derivative, caproylated inulin was superior as an inulinase inducer, which gave a 6.8- or 4.9- fold increase in the inulinase

titers in the presence or absence of ammonium phosphate, respectively. Fontana *et al.* (1994) concluded that inulinase induction or the secretion process is affected by the presence of ammonium phosphate. In a sucrose-limited fed-batch fermentation using a yeast culture of *K. marxianus* (Hensing, 1995) when the temperature was 40°C (in comparison with 30°C), the formation of organic acids, particularly acetic acid, was pronounced. A non structured Monod-type equation, describing the relationship between specific growth rate and specific extracellular inulinase production rate, was incorporated in a model for the production of biomass and extracellular inulinase in a high cell density fed-batch culture of yeast strain (grown on sucrose).

In another sucrose-limited chemostat study (Rouwenhorst *et al.*, 1988) found that the production of enzyme was negatively controlled by the residual substrate (sugar) concentration. High enzyme activities were seen during growth on non sugar substrates, indicating that enzyme synthesis was a result of a depression/repression mechanism. A mutant fungal culture of *A. niger* produced high levels of inulinase, irrespective of the carbon sources. When sucrose was used as substrate, the addition of sucrose fatty acid ester in the fermentation medium as surfactant resulted in enhanced yields of the enzyme (Fontana *et al.*, 1994). Although inulinase synthesis is regulated by both induction and repression, reasonable amounts of enzyme were produced in the absence of inducer with cultures grown on glucose as the sole carbon source (Schwan *et al.*, 1997). Espinoza *et al.* (1992) evaluated yeast culture of *K. marxianus* for the simultaneous

production of two enzymes (one being inulinase) using glucose as carbon source. Notably higher activities of inulinase were produced in two-enzyme fermentation.

Tsang and GrootWassink (1988) used fructose and lactose for inulinase production by yeast culture *K. fragilis* and found that inulinase yields were twice as high as the peak yields on lactose. Gupta *et al.* (1988); Gupta *et al.* (1990) succeeded the cultivation of *F. oxysporum* on the aqueous extract of chicory roots in fructan-and fructosan containing media for production of extracellular inulinase.

Among the various sugars tested, sucrose showed the greatest inhibitory effect on inulinase synthesis and led to a significant increase in the formation β -D-fructo-furanosidase (EC 3.2.1.26).

Roots and tubers of several Compositae and Gramineae appeared to be good sources for either direct fermentation for inulinase production or the use of compounds such as fructan and inulin isolated from them. Dahlia (*Dahlia pinnata*) rhizosphere (Barthomeuf *et al.*, 1991), Jerusalem artichoke (*Helianthus tuberosus*) (Ongen *et al.*, 1994; Ongen and Sukan, 1996), Chicory (*Cichorium intybus*) roots (Gupta *et al.*, 1985) and Kuth (*Saussurea lappa*) roots Viswanathan and Kulkarni (1995b) have widely been used for this purpose. Cazetta *et al.* (2005) investigated the inulinase production by yeast *K. marxianus var. bulgaricus* growing in yacon extract. The microorganism showed good development in yacon, higher enzymatic activities were achieved at 30% and 40% (v/v) of extract. Pessoa and Vitolo (1999) cultivated *K. marxianus* DSM 70106 for inulinase production.

When a strain of *K. marxianus* was cultivated in a medium containing inulin as a unique carbon source in the presence of 2-deoxyglucose, the enzyme yields were 3.3 times higher than the medium with only inulin. When glycerol was used as the sole carbon source, the inulinase yields were 3.6-fold higher. Although the strain was able to produce enzyme in the presence of 2-deoxyglucose, it was shown that the strain was not depressed, since enzyme production was reduced when the concentration of glucose or fructose was increased in the medium. Since inulinase was produced in a glycerol medium lacking an inducer, it was thought that enzyme production was partially constitutive (Rouwenhorst *et al.*, 1991). Gupta *et al.* (1994) using *K. fragilis*, found that an aqueous solution of chicory roots with 1% fructan was a better carbon source for inulinase production than inulin. Ongen and Sukan (1996) investigated inulinase activity produced by a mixed culture of *Aspergillus niger* and *Kluyveromyces marxianus* growing on Jerusalem artichoke powder. *Kluyveromyces marxianus* cells with inulinase (2, 1- β -D-fructan fructanohydrolase, EC 3.2.1.7) activity have been immobilized in open pore gelatin pellets with retention of >90% of the original activity (Bajpai and Margartis, 1985). Inulase production by *Kluyveromyces fragilis* on various fermentable and non-fermentable carbon sources was examined in carbon-limited continuous culture

(Grootwassink and Hewitt, 1983). Fructose and sucrose supported superior inulase yields [above 24 μ mol sucrose hydrolysed min⁻¹ (mg cell dry wt)⁻¹ at pH 5.0, 50°C], while some other carbon sources, including lactose, galactose, ethanol and lactate, did not stimulate inulase formation beyond basal levels. Thus fructose was identified as the primary physiological inducer.

Gouda (2002) found that about 91% inulinase was extracellular enzyme in a culture medium containing inulin and wheat bran as sole carbon source (1% for each).

FERMENTATION TECHNIQUES

SmF (Submerged Fermentation) is the preferred fermentation method for the production of most microbial products, unless there is a particular reason why SSF (Solid State Fermentation) should be chosen. In general, SmF is less problematic than SSF because heat transfer and media homogeneity is facilitated (Pandey *et al.*, 2001). In SSF the main problem is related to the heat removal, while in SmF the major consideration is the supply of oxygen to the bioreactor (Mitchell *et al.*, 2000). Reasons for evaluating SSF processes include the use of low-cost substrates, simplified downstream process, reduced energy requirement and simplicity of bioreactor design (Xiong *et al.*, 2007).

Although the entire commercial production as well as the reported literature on microbial inulinases involved submerged fermentation (batch, fed-batch, or continuous mode) as the technique of fermentation, it has been reported that SSF offers several advantages in comparison to liquid fermentation (Pandey, 1992; Nigam and Singh, 1994; Pandey *et al.*, 1994).

PURIFICATION AND PROPERTIES OF INULINASES

Most of the reports on purification of extra-cellular inulinases produced by microorganisms have been dealing with the conventional method of centrifugation and/or ultra-filtration, salt or solvent precipitation, followed by column chromatography (Barthomeuf *et al.*, 1991; Yun *et al.*, 1997; Azhari *et al.*, 1989). Intra-cellular inulinases required the usual step of cell wall destruction and then followed the similar procedures. Table 3 (a) and (b), summarizes the optimal conditions and properties of some fungal inulinases obtained after purification. Inulinases of fungal origin have mostly been extracellular and have generally been exo-acting. However, Gupta *et al.* (1988) found only 14% total inulinase activity in a strain of *F. oxysporum* as extracellular whereas the remaining activity was mycelial bound. Both inulinases (extra- and intra-cellular) were purified by column chromatography on Sephadex G-100 and yields between 65 and 75% were obtained. Both inulinases also hydrolyzed sucrose, raffinose and stachyose and were primarily exo-acting. The intracellular and the extracellular enzymes from

Table 3a: Optimal pH and temperature of inulinases

Source	Forms	Mol wt	pH range	pH optima	Temperature range (°C)	Temperature optima (°C)	Reference
<i>Penicillium sp.</i>			5.0-7.0	5.6	Below 45	45	Wenling <i>et al.</i> (1994)
<i>K. fragilis</i>	Exo	250,000		4.75		55	Gupta <i>et al.</i> (1994)
<i>F. oxysporum</i>				5.5-6.5 ^b		37, 45 ^b	Gupta <i>et al.</i> (1992)
<i>F. oxysporum</i>	Extra intra	300,000 300,000		6.2, 5.8		37, 30	Gupta <i>et al.</i> (1988)
<i>P. rugulosum</i>				5.5-5.6		55	Barthomeuf <i>et al.</i> (1991)
<i>F. oxysporum</i>				6.0, 6.2b		37, 45 ^b	Gupta <i>et al.</i> (1990)
<i>C. pannorum</i>		58,000	4.5-8.5 ^a	6.0-7.0		50	Xiao <i>et al.</i> (1989)
<i>Aspergillus sp.</i>	Endo Exo	53,000 81,000					Azhari <i>et al.</i> (1989)
<i>A. niger</i>	P-IA P-IB	70,000 68,000		5, 5		40, 40	Nakamura <i>et al.</i> (1994)
<i>A. ficuum</i> JNSPS	Exo I	70,000	4.0-8.0	4.5	Below 50	45	Chen <i>et al.</i> (2009)
	Exo II	40,000	—	4.5	—	—	
	Exo III	46,000	—	4.5	—	—	
	Endo I	34,000	—	5.0	—	—	
	EndoII	31,000	—	5.0	—	—	
<i>Penicillium sp.</i> strain TN-88	Endo II	68,000		5.2		50	Nakamura <i>et al.</i> (1997)
<i>Penicillium janczewskii</i>	Extra	80,000		4-5.5		60	Pessoni <i>et al.</i> (2007)
	—	—		—		—	
	—	—		—		—	
<i>C. aureus</i>	Exo	60,000		5		50	Sheng <i>et al.</i> (2008)

Kluyveromyces fragilis were precipitated with tannic acid and further purified on a DEAE-Sephadex A-50 column (Negoro, 1978).

Kaur *et al.* (1992) purified four different forms of invertase and inulinase named as invertase, I, II, III, IV and inulinase I, II, III, IV from a culture filtrate of *Fusarium oxysporum* grown on an inulin containing medium and found that none of the invertases showed activity with inulin.

Barthomeuf *et al.* (1991) purified *P. rugulosum* inulinase from the supernatant by centrifugation, ultra-filtration and acetone precipitation with a yield of 32%. The enzyme was stable for 2 h at 50°C, lost 12% of its activity after 2 h at 55°C and was totally inactivated after 30 min at 60°C. A crude inulinase preparation from *Aspergillus sp.* was dialyzed for 48 h and the lyophilized enzyme powder was loaded onto a CM-Sephadex column for ion-exchange chromatography. This step was selective for separating two forms of the enzyme. After high-performance gel permeation chromatography, endo- and exo-inulinases were obtained in 5 and 13.9% yields, respectively. Both forms exhibited invertase activity as well (Azhari *et al.*, 1989). Two forms of inulinase were also obtained from the culture broth of *A. niger* by chromatography on a DEAE cellulofine A-500 column and then further separated on a Q-Sephadex HP column with yields of 18.2 and 18.9% for P-IA and P-IB, respectively (Nakamura *et al.*, 1994). Xiao *et al.* (1989) purified and characterized an endo-inulinase from *C. pannorum*. The enzyme was a glycoprotein and had a pH isoelectric at 3.8. The enzyme was active on inulin but not on levan or sucrose and catalyzed the production of inulotriose, inulotetraose and inulopentaose.

Pessoa *et al.* (1996) studied the purification of an extra-cellular inulinase produced by yeast cells by adsorption of culture broth on ion-exchange adsorbents in the absence and presence of cells. The favorable adsorption pH was 6.5-7.0 using the weak anion-exchanger DEAE and pH 4.0 with the strong cation-exchanger (Streamline SP). Inulinase could be purified 93% directly from the culture broth without cell removal, the enrichment and concentration factors being 5.8 and 2.8, respectively.

The extra-cellular inulinase of *K. marxianus* was fractionated from the fermentation broth using acetone, yielding more than 81% enzyme activity (Ku and Hang, 1994). An enzyme preparation from *K. fragilis* was purified from the fermented broth by ethanol precipitation and chromatography on Sephadex G-200, DEAE-cellulose and CM-cellulose. The enzyme was primarily exo-acting.

Purification of an endo-inulinase (obtained from a commercial preparation) was carried out at 4°C by CM-Sephadex CL-6B and DEAE-cellulose column chromatography, followed by dialysis in 1% glycine. After isoelectric focusing and gel filtration on a high-performance liquid chromatography Bio-Sil SEC-250 column, high-purity enzyme was achieved (Zhang *et al.*, 2004). About 70% the total activity was extra-cellular and all but 9% of the remainder was easily recoverable from cell-soluble fractions. Ettalibi and Baratti (1987) isolated from a commercial inulinase preparation derived from *Aspergillus ficuum* 1 invertase, 5 exo-inulinases (Exo I, II, III, IV, V) and 3 endo-inulinases (Endo I, II, III) and reported the strain to be the first organism containing the 3 activities: invertase, exo and endo-inulinase.

Kim and Rhee (1989) characterized the immobilized inulinase from *A. ficuum*. The immobilized enzyme exhibited 23% initial enzyme activity and was best active at pH 4.5. Wenling *et al.* (1999) purified intracellular inulinase from *Kluyveromyces sp.* Y-85 was immobilized covalently by adsorb-crosslinking onto macroporous ionic polystyrene beads. Using a 4.5% (w/v) fructan solution from Jerusalem artichoke tuber as substrate, in a continuous bed column reactor packed with 70 mL of the immobilized inulinase beads, the maximum volumetric productivity of 234.9 g reducing sugars l⁻¹ h⁻¹ was obtained with inulin hydrolysis of 75%. The hydrolytic product was a mixture of 85% D-fructose and 15% D-glucose.

Gupta *et al.* (1994) immobilized *K. fragilis* inulinase using the metallink chelation method on cellulose. Only 40% inulinase, which could be immobilized, showed a half-life of 5 days at 25°C. In

Table 3b: Properties of inulinases produced by some microorganisms

Source	Km (mmol/L)	Specific activity(U/mg)	Purification fold	Surfactants	Enzyme activators	Enzyme inhibitors	Reference
<i>Penicillium</i> sp.				PCMB (P-chloromercuribenzoic acid)	Mn ²⁺ c		Wenling <i>et al.</i> (1994)
<i>K. fragilis</i>			26				Gupta <i>et al.</i> (1994)
<i>F. oxysporum</i>	0.25- 0.44 ^{cd}						Gupta <i>et al.</i> (1992)
<i>F. oxysporum</i>		63.3 21					Gupta <i>et al.</i> (1988)
<i>P. rugulosum</i>							Barthomeuf <i>et al.</i> (1991)
<i>F. oxysporum</i>							Gupta <i>et al.</i> (1990)
<i>C. pannorum</i>							Xiao <i>et al.</i> (1989)
<i>Aspergillus</i> sp.	570 ^d , 60 ^d	150, 258	18.5, 31.9			Fe ³⁺ , Mn ²⁺ , Mg ²⁺	Azhari <i>et al.</i> (1989)
<i>A. niger</i>	0.48 ^d , 0.50 ^d	350, 340	177, 177				Nakamura <i>et al.</i> (1994)
<i>A. ficuum</i> JNSPS	43.1 ^d g 31.5 ^d g 25.3 ^d g 14.8 ^d g 25.6 ^d g			SDS — — — —	— — — —	Mg ²⁺ , Ag ⁺ , Fe ³⁺ , Al ³⁺	Chen <i>et al.</i> (2009)
<i>Penicillium</i> strain TN-88	0.20	105		p-Cl-mercuribenzoate, carbodiimide, N-bromosuccinimide		Ag ⁺ , Hg ²⁺	Nakamura <i>et al.</i> (1997)
<i>Penicillium janczewskii</i>	8.11 10 ⁻⁴ 2.62 10 ⁻³ 3.7 10 ⁻⁴	231.7 364.5 78	15 24 26				Pessoni <i>et al.</i> (2007)
<i>C. aureus</i>	20.06 ^g			PMSF, iodoacetic acid, EDTA and 1,10-phe nanthroline	Ca ²⁺ , K ⁺ , Na ⁺ , Fe ²⁺ , Cu ²⁺	Mg ²⁺ , Hg ²⁺ , Ag ⁺	Sheng <i>et al.</i> (2008)

a: For 24 h; b: Immobilized enzyme; c: K_m for sucrose; d: K_m for inulin; e: Enzyme activation; f: μmol/dm; g: mg/mL

general, not much difference in several properties such as pH and temperature optima of inulinases obtained from different fungi. The optimal pH varied between 4.5 and 7.0 for moulds, whereas for yeasts it ranged from 4.4 to 6.5. The optimal temperature was in the mesophilic as well as thermophilic range for moulds, while being relatively high for yeasts (Table 3a).

Chen *et al.* (2009) purified 3 exo-inulinases (Exo-I, Exo-II and Exo-III) and 2 endo-inulinases (Endo-I and Endo-II) from the culture broth of *Aspergillus ficuum* JNSP5-06 by ammonium sulphate precipitation, DEAE cellulose column chromatography, Sepharose CL-6B column chromatography and preparative electrophoresis.

These 5 inulinases were stable below 50°C with optimum activity at 45°C and were stable at a pH range of 4-8 with an optimum activity at pH 4.5 and 5.0 for exo-inulinase and endo-inulinase, respectively. The inulinase activity was completely inhibited by Ag⁺ and strongly inhibited by Fe²⁺ and Al³⁺, whereas K⁺, Ca²⁺, Li²⁺, EDTA and urea had no significant influence on the inulinase activity. Pessoni *et al.* (2007) isolated *Penicillium janczewskii* from the rhizosphere of *Vernonia herbacea*. Three different extra-cellular β-fructo-furanosidases (2 inulinases and 1 invertase) were purified from fungal cultures grown on sucrose or inulin, through precipitation with ammonium sulfate and anion-exchange, hydrophobic interaction and gel filtration chromatographies. The 3 enzymes exhibited an optimum temperature of approximately 60°C, an optimum pH of 4-5.5 and an apparent molecular mass of 80 kDa. The Km values determined for invertase were 3.7 10⁻⁴ and 6.3 10⁻² M with sucrose and inulin, respectively. The values of Km obtained for the 2 inulinases (8.11 10⁻⁴ and 2.62 10⁻³ M) were lower for

inulin when compared to those obtained for sucrose. The inulinases did not produce oligofructans from inulin, indicating that they were primarily exo-inulinases (Table 3b).

An extra-cellular inulinase present in the supernatant of cell culture of the marine yeast *Cryptococcus aureus* G7a was purified to homogeneity with a 7.2-fold increase in specific inulinase activity by ultra-filtration, concentration, gel filtration chromatography (Sephadex G-75) and anion-exchange chromatography (DEAE sepharose fast flow anion-exchange). The molecular mass of the purified enzyme was estimated to be 60.0 kDa. The optimal pH and temperature of the purified enzyme were 5.0 and 50°C, respectively. The enzyme was activated by Ca²⁺, K⁺, Na⁺, Fe²⁺ and Zn²⁺. However, Mg²⁺, Hg²⁺ and Ag⁺ acted as inhibitors in decreasing the activity of the purified inulinase. The enzyme was strongly inhibited by phenylmethanesulphonyl fluoride (PMSF), iodoacetic acid, EDTA and 1, 10-phenanthroline. The Km and Vmax values of the purified enzyme for inulin were 20.06 mg/mL and 0.0085 mg/min, respectively. A large amount of mono-saccharides were detected after the hydrolysis of inulin with the purified inulinase, indicating that the purified inulinase had a high exo-inulinase activity (Sheng *et al.*, 2008).

APPLICATIONS OF INULINASES IN THE FOOD INDUSTRY

Fungal inulinases are an important class of industrial enzymes, which are usually extra-cellular and inducible. Inulinase (β-D-fructan-fructanohydrolase,

EC 3.2.1.7) is a versatile enzyme which is used for the production of various carbohydrate-based products.

Inulinase offers interesting perspectives in view of the growing need for the production of pure fructose syrups and may present an alternative way to produce the so-called Ultra High Fructose Syrups from inulin. In contrast, the chemical approach for fructose production is currently associated with some drawbacks (Gill *et al.*, 2006; Pandey *et al.*, 1999). Acid hydrolysis has always resulted in an undesirable coloring of the inulin hydrolysate, whereas changes in taste and aroma are minimal on enzymatic hydrolysis. Acid hydrolysis also results in the formation of Di-Fructose Anhydride (DFA), which has practically no sweetening properties and cannot be further hydrolyzed enzymatically into fructose.

The best procedure involves the use of microbial inulinases, which can hydrolyze inulin to yield 95% pure fructose in a single-step enzymatic reaction. Thus, the production of fructose syrup from inulin or inulin-rich materials (Kim and Rhee, 1989; Nakamura *et al.*, 1995; Partida *et al.*, 1997) is the major area of application for inulinases. An exo-inulinase preparation from *K. marxianus* YS-1 was successfully used to hydrolyze pure and raw inulins from *Asparagus racemosus* for the preparation of high-fructose syrup. In a batch system, the exo-inulinase hydrolyzed the pure and raw inulins at 84.8 and 86.7%, respectively.

Fructo-Oligosaccharides (FOS) constitute one of the most popular functional food components because of their bifidogenic and health-promoting properties. Inulin can be selectively hydrolyzed by the action of endo-inulinase into Inulo-Oligosaccharides (IOS) such as inulotriose and inulotetraose (Yun *et al.*, 1997). When endo-inulinase is used for the hydrolysis of inulin, Fructo-Oligosaccharides (FOS) can be produced in one step to yield more than 80%. An endo-inulinase from the recombinant yeasts was used to hydrolyze inulin and produced fructo-oligosaccharides which were represented mainly by 1-kestose (Kim *et al.*, 1999). The endo-inulinase P-II from *Penicillium* sp. TN-88 hydrolyzed inulin to the extent of 70% and liberated inulotriose as the main product (Nakamura *et al.*, 1997).

The sugar industry has recently faced intense competition from high-fructose syrup, which is used as a low-cost alternative sweetener. Partida *et al.* (1997) described a method for the production of fructose syrup from agave plant pulp, which involved the hydrolysis of poly-fructose extract of the pulp with inulinase. The syrup was claimed to be of high purity with desirable color, stable over time and suitable for human consumption in a wide variety of foods and beverages.

Nakamura *et al.* (1995) developed a process for the continuous production of fructose syrup from inulin using immobilized inulinase from *A. niger*. The production was carried out in a packed-bed column reactor and the volumetric productivity was 410 g/(L.h) reducing sugars. Kim and Rhee (1989) also used an immobilization technique for the production of fructose

from Jerusalem artichoke. They evaluated the batch and continuous processes using *A. ficuum* inulinase immobilized on chitin.

CONCLUSION

Microbial inulinases play an important role in the hydrolysis of inulin for the production of fructose syrups and FOS. After the inulin hydrolysis by exo- and endoinulinases the raw materials can be used for many practical applications in food industry. Inulin and inulin containing plants represent a renewable, inexpensive and abundant raw material for industry. Obviously many advances in fungal inulinases production, purification and characterization as well as their application in the food industry have been made in the last years, yet can be supplementary significantly improved.

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