Fungal Inulinolytic Enzymes: A Current Appraisal

15

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Abstract

Inulinolytic enzymes produced by molds and yeasts have many applications. Inulin is being looked upon as an abundant and renewable source of fructose, a low-calorie sweetener, and a readily fermentable substrate. Inulin can be exploited at industrial scale for generation of high-fructose syrup (HFS) using fungal exoinulinases and may also be selectively hydrolyzed using endoinulinase for generation of prebiotic inulooligosaccharides (IOS). Some members of Aspergilli, Penicillia, and a yeast, *Kluyveromyces marxianus*, are known as potential producers of inulin-hydrolyzing enzymes; however, recently, it has been characterized from extremophilic and marine-derived microorganisms as well. Inulinases find applications in nutraceutical, feed, pharmaceutical, and biofuel industries. This chapter discusses production, molecular aspects, and biotechnological applications of inulinases.

Keywords

Exoinulinase · Endoinulinase · Fungi · Yeast · Inulin · Fructose

Introduction

Inulin is non-structural polysaccharide, used as energy-rich compounds, and also has role in plant metabolism and energy storage. After starch, inulin is one such storage polysaccharide found widely dispersed in many plants. Inulin is made of β-(2 → 1) linked linear poly-fructose units (2–60) terminated by a sucrose residue

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(Fig. 15.1) (Kango and Jain 2011; Rawat et al. 2015a). Plants that store and synthesize inulin usually do not store other materials as energy reserve. Several temperate and tropical plants, such as dandelion (*Taraxacum officinale*), chicory (*Cichorium intybus*), Jerusalem artichoke (*Helianthus tuberosus*), dahlia (*Dahlia pinnata*), and asparagus (*Asparagus officinale*) (Table 15.1), reserve this polymer (Kango 2008; Chi et al. 2011; Rawat et al. 2016).

Commercial production of fructose by inulin hydrolysis is more effective than other conventional method which required starch hydrolysis by the action of group of enzymes (α-amylase, amyloglucosidase, and glucose isomerase) liberating less fructose (∼45%) in the end product. Inulin hydrolysis using microbial inulinase yields 90–95% fructose solution. Fructose syrup production from inulin-rich material is a major area of inulinase application (Kango 2008; Vijayaraghavan et al. 2009; Liu et al. 2013).

Inulin is utilized by a variety of fungi, bacteria, and yeasts which degrade and modify inulin by enzymes such as endoinulinase, exoinulinase, and invertase. Exoinulinase (EC 3.8.1.80; β-D-fructohydrolase) hydrolyzes the terminal unit of fructose which is linked by β-fructofuranosidic bonds and liberates fructose.

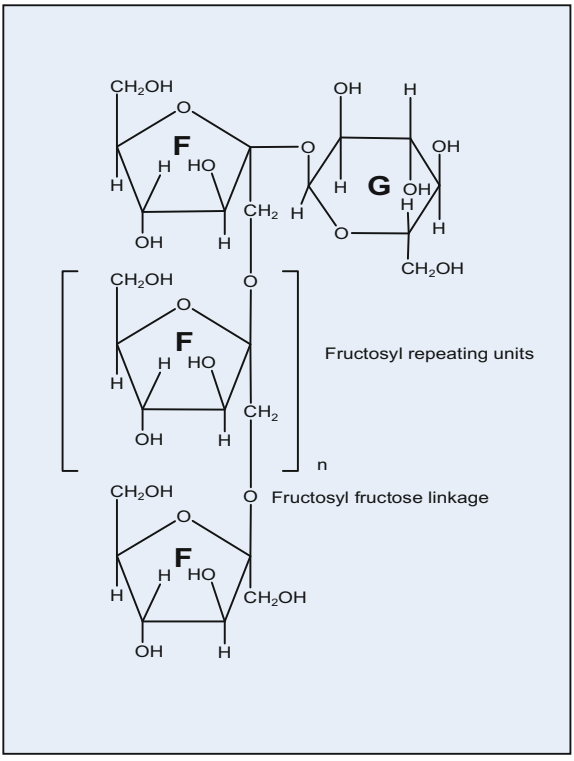

Fig. 15.1 Structure of inulin (F—fructose, G—glucose)

Table 15.1 Inulin content of some plants

Source of inulin	Scientific name	Storage organ	Inulin content[a]
Asparagus	*Asparagus racemosus*	Root	10–15
Banana	*Musa cavendishii*	Fruit	0.3–0.7
Camas	*Camassia quamash*	Bulb	12–22
Chicory	*Cichorium intybus*	Root	15–20
Dandelion	*Taraxacum officinale*	Root/leaves	12–15
Dahlia	*Dahlia pinnata*	Tuber	9–13
Garlic	*Allium sativum*	Bulb	9–16
Jarusalem artichoke	*Helianthus tuberosus*	Tuber	14–19
Onion	*Allium cepa*	Bulb	2–6
Yacon	*Smallanthus sonchifolius*	Root	3–10

[a]% of fresh weight (Modified from Kango and Jain 2011)

Endoinulinase (EC 3.2.1.7; β-D-fructan fructanohydrolase) breaks the internal glycosidic bonds of inulin and generates inulobiose (F2), inulotriose (F3), inulotetraose (F4), and inulopentaose (F5) as important end products (Fig. 15.2). β-D-fructofuranoside fructohydrolase (EC 3.2.1.26; invertase) hydrolyzes β-2, 1-fructosidic bond of sucrose to release fructose and glucose. Inulin is also degraded by yeast non-specific β-fructosidases which release fructose units from its reducing end. Relative activities toward sucrose and inulin are represented as inulinase/sucrase (*I/S*) ratio to differentiate between inulinase and invertase. High *I/S* ratio indicates predominant inulinase activity (Chi et al. 2009; Kango and Jain 2011; Rawat et al. 2015b; Singh et al. 2016).

Properties and Molecular Biology of Fungal Inulinases

Protein sequence of fungal inulinases has revealed several conserved motifs. Six highly conserved motifs of inulinases are WMND(E)PNGL, EC(V)P, SVEVF, FS(T), RDP, and Q that played an important role in substrate binding and inulin catalysis (Fig. 15.3). SVEVFV and Q are segments common in fungal exo- and endoinulinases, while SVEVFV amino acid segment was not noticed in yeast exoinulinases (Liu et al. 2013; Rawat et al. 2016). Fungal inulinases have been expressed in *Yarrowia lipolytica* (Liu et al. 2010), *S. cerevisiae* (Yuan et al. 2013), *P. pastoris* (Cao et al. 2013; Ma et al. 2015), *Kluyveromyces lactis* (Yu et al. 2010), and *E. coli* (Zhou et al. 2015). Such yeasts hydrolyze inulin and ferment fructose into ethanol simultaneously paving the way for consolidated bioprocessing (CBP) (Yuan et al. 2012).

Inulin rich substrates (Dahlia, Chicory, Dandelion)

Extraction

Inulin

Reducing end

β,2-1 fructosyl linkage

Exoinulinase

Endoinulinase

Fructose

(Low calorie sweetener, Feed stock)

Inulooligosaccharides

(Prebiotics, Nutraceutical)

Fig. 15.2 Schematic showing generation of fructose syrup and inulooligosaccharides from inulin-rich plant extract

Among fungal strains, *Aspergillus* spp. (Kango 2008; Rawat et al. 2015a) and *Penicillium* spp. (Rawat et al. 2015b), are common sources of exo- and endoinulinase (Table 15.2). Characteristics of some fungal inulinases are described in Table 15.3. Incidence of both exo- and endo-acting inulinolytic enzymes has been reported in fungi. For instance, exoinulinase gene from *A. niger* (*inuE*) encoded

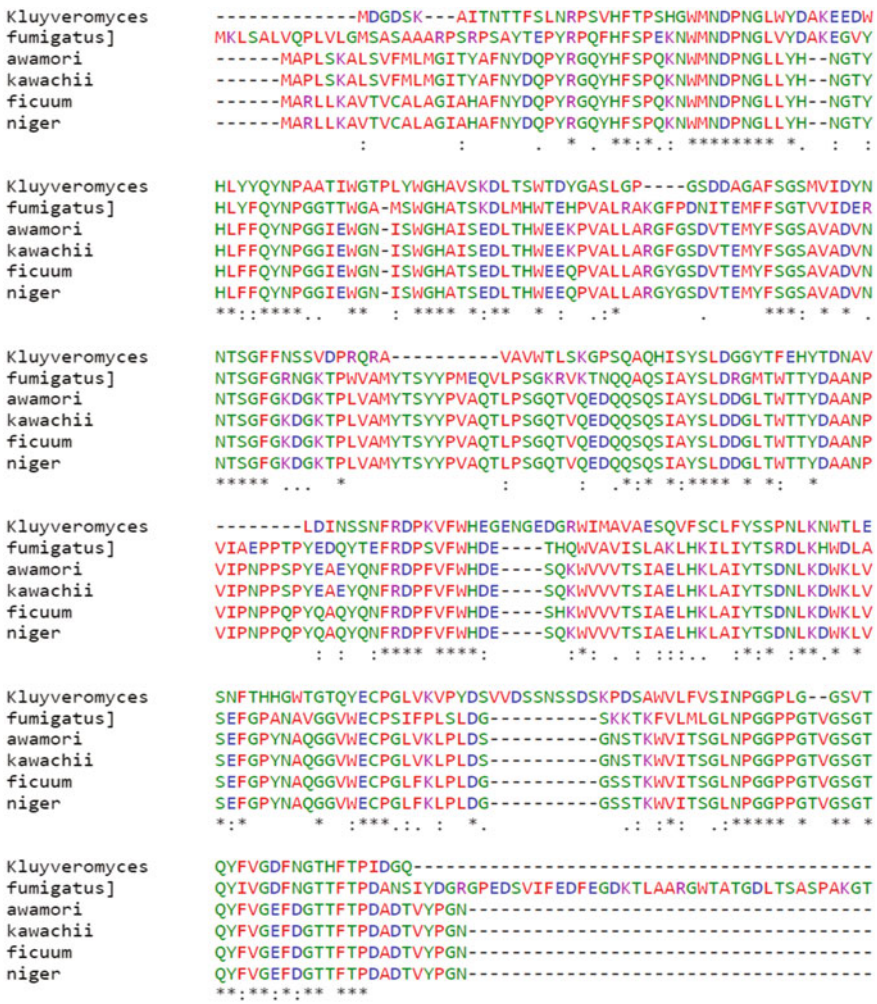

Fig. 15.3 Amino acid sequence alignment of various fungal exoinulinase using the Clustal Omega software

protein of 57 kDa (Goosen et al. 2008), while endoinulinase gene from the same strain encoded 54 kDa (Ohta et al. 1998). Another inulinase gene, *Inu*2 from *A. ficuum* ATCC 1688L encoded 55.1 kDa protein, while *endo1* gene from *A. ficuum* JNSP5-06 consisted of 1482 bp and was 98% identical to *A. niger* CBS513.88 and 60% identical to *A. fumigatus* AF293 inulinase (Chen et al. 2012). Exoinulinase genes, (*inu*D) and (*inu*A1), from *Penicillium* sp. (TN-88) and *P. janthinellum* (B01) have been isolated and cloned (Moriyama et al. 2002; Wang et al. 2011). Details of various inulinase genes cloned from molds and yeasts are summarized in Table 15.4.

Table 15.2 Fungal sources of inulinases

Source	Microorganism	Type[a]	References
Molds	*Aspergillus niger* NK 126	Exo, and endo	Kango (2008)
	Aspergillus ficuum JNSP5-06	Exo and endo	Chen et al. (2011)
	Aspergillus tubingensis CR-16	Exo	Trivedi et al. (2012)
	Penicillium subrubescens FBCC 1632	Exo	Mansouri et al. (2013)
	A. niger ATCC 26011	Exo and endo	Dinarvand et al. (2013)
	A. niger AUMC 9375	Exo, and endo	Housseiny (2014)
	A. niger CICIM F0620	Endo	He et al. (2014)
	Aspergillus fumigatus Cl1	Endo	Chen et al. (2015)
	A. niger 20 OSM	Exo, and endo	Trytek et al. (2015)
	Aspergillus awamori MTCC 2879	Exo	Rawat et al. (2015a)
	Penicillium citrinum MTCC 1256	Endo	Rawat et al. (2015a)
	Penicillium sp. NFCCI 2768	Exo, and endo	Rawat et al. (2015b)
Yeasts	*Pichia guilliermondii* M-30	Exo	Yu et al. (2009)
	Kluyveromyces marxianus MTCC 188	Exo	Dilipkumar et al. (2013)
	K. marxianus NRRLY-7571	Exo	Treichel et al. (2009)
	P. guilliermondii	Exo	Zhang et al. (2009)
	K. marxianus NRRL Y-7571	Exo	Mazutti et al. (2010)
	Candida guilliermondii TISTR 5844	Exo	Songpim et al. (2011)
	K. marxianus MTCC 3995	Exo	Jain et al. (2012)

[a]Exo–Exoinulinase; Endo–Endoinulinase

Heterologous Expression of Inulinase Genes of Fungi

Several exo- and endoinulinases encoding genes of filamentous fungi have been cloned in yeasts and characterized (Chi et al. 2011; Liu et al. 2013). The inulinase gene from *K. marxianus* CBS6556 was expressed in *Y. lipolytica* ACA-DC50109, and inulinase activity up to 41 U/ml was obtained (Zhao et al. 2010b). Recombinant yeast containing inulinase gene was used in inulin hydrolysis, with production of citric acid and SCP. The *K. marxianus* (*INU* 1) gene was expressed in *S. cerevisiae,* and the recombinant enzyme showed improved thermostability due to hyperglycosylation (Kim et al. 1997).

The endoinulinase gene (*inu* B) of *A. ficuum* was expressed in the mutant (Suc Z) *S. cerevisiae*. The recombinant inulinase was free from sucrase and exoinulinase activity, and the endoinulinase yield was up to 83 U/ml (Park et al. 2001). Yu et al. (2011) isolated inulinase gene from *Kluyveromyces cicerisporus* and expressed in a hexokinase muted *S. cerevisiae* strain. The yield of inulinase reached up to 31 U/ml, and recombinant yeast accumulated glucose-free fructose in fermentation broth containing Jesrusalem artichoke tubers. Workers have also expressed inulinase genes in high ethanol producing yeast for direct processing of inulin into ethanol.

Table 15.3 Characteristics of fungal inulinases

Microorganism	M (r) kDa	Optimum		K_m	V_{max}	Action	References
		pH	T (°C)				
Molds							
Aspergillus fumigatus CL1	58	6.0	55	2.18 mM	1590 μmol min^{-1} mg^{-1}	Endo	Chen et al. (2015)
Aspergillus ficuum JNSP5-06	63	4.0	60	7.1 mM	1000 μmol min^{-1} mg^{-1}	Exo	Chen et al. (2013)
A. ficuum JNSP5-06	70	4.5	45	43.1 mg ml^{-1}	32.7 mg min^{-1} ml^{-1}	Exo	Chen et al. (2009)
A. ficuum	63	5.4	50	4.75 mM	833.3 μmol min^{-1} ml^{-1}	Exo	Mutanda et al. (2009)
Aspergillus niger	68.1	6.0	50	3.53 mM	666.7 μmol min^{-1} ml^{-1}	Endo	Mutanda et al. (2008)
Penicillium janczewskii	80	4.0–5.5	60	6.3×10^{-2} M	2.09×10^{-2} μmol min^{-1} ml^{-1}	Exo	Pessoni et al. (2007)
A. fumigatus	62	6.0	60	1.25 mM	526 μmol min^{-1} mg^{-1}	Exo	Gill et al. (2006)
Chaetomium sp. C34	66	6.0	55	0.199 mM	115 μmol min^{-1} mg^{-1}	Endo	Zhang et al. (2004)
Aspergillus awamori var. 2250	69	4.5	–	0.003 mM	–	Exo	Arand et al. (2002)
Yeasts							
Kluyveromyces cicerisporus	90	4.5	55	0.32 mM	4317 μmol min^{-1} ml^{-1}	Exo	Ma et al. (2015)
Cryptococcus aureus G7a	60	5.0	50	20.06 mg ml^{-1}	0.0085 mg min^{-1}	Exo	Sheng et al. (2008)
Kluyveromyces marxianus Y1	–	5.5	55	–	–	Exo	Yuan and Bai (2008)
Pichia guilliermondii	–	6.0	60	–	–	Exo	Gong et al. (2007)

Table 15.4 Characteristics of some inulinase genes from molds and yeasts

Molds/yeast	Gene type	Size (bp)	Accession no.	References
Kluyeromyces cicerisporus	Exo *kcINU1*	1665	AF178979	Ma et al. (2015)
K. marxianus	Exo *rKmINU*	3223	X68479	Zhang et al. (2012)
Meyerozyma guilliermondii	Exo *INU1*	1732	EU195799	Liu et al. (2014)
Aspergillus ficuum	*Endo 1* gene	1482	FJ 984582	Chen et al. (2012)
A. fumigatus CL1	*Endo*	1561	EAL86248.1	Chen et al. (2015)
A. niger CICIM FO620	*En Inu*	1614	XM_001395842	He et al. (2014)
A. ficuum JNSP5-06	*Exo I* gene	1600	HM587130	Chen et al. (2012)
Penicillium janthinellum B01	*Exo inu A1*	2115	JF961344	Wang et al. (2011)
P. citrinum ESS	*Exo*	1608	KM364035	Flores-Gallegos et al. (2015)

For instance, inulinase (*INU* 1) gene was isolated from marine *Pichia guilliermondii* strain 1 and expressed in *Saccharomyces* sp. W0 (Zhang et al. 2010). Wang et al. (2011) noticed that *INU* 1 gene integration into rDNA in *Saccharomyces* sp. W0 leads to production of more inulinase and ethanol from inulin in less time as compared to *Saccharomyces* sp. W0 strain carrying *INU* 1 gene in plasmid. Codon-optimized inulinase gene (*INU1Y*) from yeast *Meyerozyma guilliermondii* was expressed in *Saccharomyces* sp. W0, and recombinant strain (W0 Y13) produced 43 U/ml inulinase which was higher than native gene *INU1* containing recombinant yeast (Liu et al. 2014).

Moriyama et al. (2002) have expressed *inu* E gene from *A. niger* strain 12 in *P. pastoris* yielding 16 U/ml inulinase having larger molecular mass (86 kDa) than inulinase produced by wild-type *A. niger* strain 12. Similarly, Wang et al. (2004) have expressed endoinulinase gene from *A. niger* 9891 (CGMCC 0991) in *P. pastoris* and obtained 291 U/ml yield in inulin-containing medium. Wang et al. (2011) obtained 11-fold high exoinulinase production (272 U/ml) when *inu* A gene from *P. janthinellum* strain B01 was expressed in *P. pastoris* X-33. Recombinant *P. pastoris* containing gene *KmInu* of *K. marxianus* produced 6667 U/ml inulinase (Zhang et al. 2012). The recombinant inulinase showed good stability up to 50 °C and 5.0 pH as compared to native enzyme. Zhou et al. (2014) disrupted *MIG1* gene in *K. marxianus* and developed a derepressed mutant producing high inulinase (133 U/ml).

Several inulinase genes have been expressed in *Kluyvermyces lactis* and *E. coli* (Liu et al. 2013; Rawat et al. 2016). Yu et al. (2010) have isolated inulinase gene (*Kcinu*) from *K. cicerisporus* and expressed in mutant *K. lactis*. They have noticed

twofold increase in inulinase activity (391 U/ml) than wild-type strain. Kwon et al. (2000) expressed inulinase gene (*inuZ*) of *Pichia mucidolen* in *E. coli,* and recombinant inulinase was a monomeric protein with MW of 55 kDa. Endoinulinase of *A. ficuum* was expressed in *E. coli* expression system (Chen et al. 2012). This endoinulinase was used in IOS production from inulin. Chen et al. (2013) have expressed exoinulinase gene from *A. ficuum* in *E. coli* and characterized recombinant enzyme.

Application of Fungal Inulinases

Hydrolysis of inulin can be selectively directed using microbial inulinases (exoinulinases and endoinulinases) for production of fructose-rich syrup and fructooligosaccharides (FOS), and this preparation can also be used as feedstock for production of single-cell protein (SCP), citric acid, ethanol, and other useful products (Chi et al. 2011; Kango and Jain 2011). Hydrolysis of inulin for the fructose bioconversion to ethanol by utilizing fructose from engineered yeast which has a prominent ability of consolidated bioprocessing (CBP) of inulin (Yuan et al. 2012).

Inulin is a renewable and commonly occurring polysaccharide that can be used as fructose feedstock generation. *S. cerevisiae* can easily utilize and convert fructose into ethanol (Chi et al. 2009; Nevoigt 2008). The same yeast strain was used for SCO production using Jerusalem artichoke (JA) tuber extract. This yeast strain accumulated 48.8% (w/w) and 52.2% (w/w) oil while growing on hydrolyzates of inulin (Zhao et al. 2010a). *Y. lipolytica*, an oleaginous yeast, accumulated 0.44-0.54 g lipid/g of biomass and produced 9-12 g/l dry biomass (Papanikolaou et al. 2002). An inulin utilizing mutant of this strain, *Y. lipolytica* ACA-DC 50109 (uracil mutant) containing inulinase gene of *K. marxianus* CBS 6556, was developed (Papanikolaou and Aggelis 2003).

Inulinase enzyme secreted by this engineered yeast was used to hydrolyze JA juice followed by SCO production from inulin (Zhao et al. 2010b) (Table 15.5).

Inulinase preparations, therefore, can be used in feed, pharmaceutical, biofuel, and nutraceutical industries. Endoinulinase of *Aspergillus niger* was immobilized on chitosan and prepared for continuous generation of inulooligosaccharides (IOS) syrup from artichoke juice. This syrup contained IOS with DP 3–7 (Nguyen et al. 2011). Inulinase of *A. niger* was immobilized on Concanavalin-A (lectins) for the generation of syrup of fructose (Altunbas et al. 2013). IOS are also applicable in animal nutrition for significant change in colonic bacterial populations (Kelly, 2009). IOS also have various applications in food industries like chocolate, ice cream, milk desserts, confectionary, and sauces (Kuntz et al. 2013). Inulin and IOS contribute in improvement of the mineral balance of Ca, Mg, and Fe and show anti-carcinogenic effect by enhancing the bifidogenic flora which improves immunity of the system (Kango and Jain 2011). Endoinulinase *inuA* gene of *A. niger* was cloned and expressed in *S. cerevisiae.* The resultant recombinant enzyme

Table 15.5 Potential uses of inulinase in generation of industrially and nutritionally important end products using fungal inulinases

SN	Fungal strain used	End product[a]	Enzyme type	Fermentable substrate	References
1.	*Aspergillus niger* NK 126	F, IOS	Exo, endo	Chicory inulin	Kango (2008)
2.	*Saccharomyces* sp. W0	Ethanol	Inu	JA	Zhang et al. (2010)
3.	*Yarrowia lipolytica*	Citric acid	Inu	Inulin	Liu et al. (2010)
4.	*Cryptococcus aureus* G7a	SCP	Inu	Yacon	Zhao et al. (2010a, b)
5.	*Yarrowia lipolytica*	SCP	Inu	Inulin	Cui et al. (2011)
6.	*A. niger* (Megazyme)	Oligofructose syrup	Endo	JA	Nguyen et al. (2011)
7.	*Rhodotorula mucilaginosa* TJY15a	SCO	Inu	JA	Zhao et al. (2011)
8.	*A. niger* (Fructozyme L)	Tequila	Exo, endo	*Agave tequilana*	Waleckx et al. (2011)
9.	*Kluyveromyces marxianus*		Inu	JA	Yuan et al. (2012)
10.	*Aspergillus niger* (Fructozyme)	FOS	Inu	Inulin, Sucrose	Kuhn et al. (2012)
11.	*Kluyveromyces marxianus*, *S. cerevisiae*	Ethanol	Inu	JA	Hu et al. (2012)
12.	*Pichia guillermondii* Pcla22	SCO	Inu	Inulin	Wang et al. (2012)
13.	*Saccharomyces* W0	Ethanol	Inv	Inulin	Li et al. (2013)
14.	*Saccharomyces cerevisiae*	Ethanol	Inu	JA	Yuan et al. (2013)
15.	*Pichia pastoris*	IOS	Endo	Inulin	He et al. (2014)
16.	*Meyerozyma guillirmondii, Saccharomyces* W0	Ethanol	Exo	Inulin	Liu et al. (2014)
17.	*Rhodosporidium toruloides* 2F5	SCO	Inu	Inulin	Wang et al. (2014)
18.	*Kluyveromyces marxianus*	Ethanol	Inu	JA	Gao et al. (2015)
19.	*Aspergillus* spp., *Penicillium* spp., *Fusarium oxysporum*, *Kluyveromyces*	F, IOS, FOS	Exo, endo	Chicory inulin, Sucrose	Rawat et al. (2015a)
20.	*Penicillium* sp. NFCCI 2768	F, IOS	Exo, endo	Dahlia inulin	Rawat et al. (2015b)

[a] *F* fructose; *IOS* inulooligosaccharides; *FOS* fructooligosaccharides; *SCP* single cell protein; *LA* lactic acid; *SCO* single cell oil; *CA* citric acid; *Exo* exoinulinase; *Endo* endoinulinase; *Inu* inulinase; *Inv* invertase; *JA* Jerusalem artichoke

showed maximum activity 3.1 U/ml and ethanol concentrations 55.3 g/L (Yuan et al. 2013).

Conclusions and Future Perspectives

Inulin hydrolysis for generation of fructose and fructooligosaccharides has recently received considerable interest. Some yeasts and fungi have been noticed to produce exoinulinases, while endoinulinase production is limited to few fungal and bacterial strains. Unconventional raw materials with high inulin content are being explored for optimized inulinase production. Cloning and expression of novel inulinases in suitable hosts, mostly yeasts such as *Pichia*, *Saccharomyces*, has been useful in consolidated bioprocessing of inulin to bioethanol. Thermostable inulinases from *Bacillus smithii* T7 and *Sphingomonas* sp. JB13 indicate possibility of finding robust inulinases among extremophiles. Inulin can be obtained from horticultural crops such as Jerusalem artichoke, chicory and utilized for generation of high-fructose syrup or oligosaccharides. Search for novel inulinase producers, parametric optimization for production and application, enzyme immobilization, and cloning of inulinase gene in suitable hosts are some of the challenges in this area. Efforts for the development of cost-effective bioprocesses suiting to food and nutraceutical industries using inulooligosachrides and fructose in commercial preparations would be helpful in realizing the applications.

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