CHAPTER

15

Fungal biofactories as potential inulinase sources for production of fructooligosaccharides

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15.1 Introduction

In recent years, urbanization and changing lifestyles have caused an increase in different health disorders like obesity, cancer, diabetes, and various neurodegenerative diseases (Das et al., 2012). However, medical advancements have not completely addressed the treatment of these diseases. Considering these issues, there is a switchover in the perspectives of individuals who are looking for natural, healthy, and low-calorific foods. In response to these increased demands, a number of functional and probiotic foods are currently available in the market. Among the available functional foods, fructooligosaccharides (FOS) represent an important source of components that favor the growth of probiotic microorganisms.

Fructan polysaccharides like inulin are present in nature in plentiful amounts and serve as storage carbohydrates in plants like Asparagaceae (*Agave americana*, *Asparagus racemosus*), Amaryllidaceae (*Allium sativum*, *Allium cepa*, *Allium ampeloprasum*), Campanulaceae (*Campanula rapnculus*), Poaceae (*Saccharum officinarum*), Liliaceae (*Erythronium gradiflorum*), and Asteraceae (*Cichorium intybus*, *Dahlia pinnata*, *Helianthus tuberosus*, *Taraxacum officinale*) (Table 15.1) (Singh and Singh, 2010; Chi et al., 2011). Structurally, inulin is a fructan polymer having β-(2 → 1)-D-fructosyl link ended by sucrose residue. The function of inulin is proportional to the change in degree of polymerization (DP), which is also responsible for its prebiotic potential. Various factors such as environmental conditions, plant origin, plant age, harvesting period, growth and storage conditions, and so on play a major role in DP of plant inulin. Branching and DP of inulin define its physico-chemical and functional properties, for example, oligofructoses with long chains are less soluble and sweeter compared to short chain oligofructoses, and raw inulin has less DP than commercially available dahlia, Jerusalem artichoke, and chicory inulin (de Leenheer, 2007; Apolinario et al., 2014). A high DP indicates that the inulin has a good prebiotic potential in the food industry (van de Wiele et al., 2007). Inulin plays a significant role in inulin-rich plants to address issues of osmoregulation and tolerance of plants against drought, freezing, and abiotic stress (Apolinario et al., 2014).

Inulinase belongs to glycoside hydrolase (GH) family 32 produced by various sources like plants (Lefebvre et al., 1992), animals, and microorganisms (Neagu and Bahrim, 2011; Kango and Jain, 2011). Inulinase consists of two forms, namely exoinulinase (E.C. 3.8.1.80) and endoinulinase (EC 3.2.1.7). Based on the mode of action, exoinulinase breaks inulin polymer to generate high-fructose syrup (HFS) as a main product and glucose as a co-product, which is considered as impurity during industrial HFS production that needs to be removed. Meanwhile, endoinulinase breaks inulin polymer to produce FOS. Apart from this, inulinase is used in generating bioethanol (Faga et al., 2010; Wang et al., 2015), lactic acid (Ge et al., 2009), and so on. Plants and animals produce low levels of inulinase compared to microorganisms, which is advantageous from the viewpoint of easy handling, cultivation, and increased productivity. Efficient inulinase producers have been identified from different microbial sources such as bacteria (*Pseudomonas* sp., *Bacillus* sp., *Arthrobacter* sp.), yeasts (*Saccharomyces* sp., *Kluyveromyces* sp., *Candida* sp.), and fungi

https://doi.org/10.1016/B978-0-12-821007-9.00015-2

TABLE 15.1 Different plant families recognized as potential inulin producers.

Plant (common name)	Plant parts	Reference(s)
Amaranthaceae		
Gomphrena macrocephala	Roots	Vieira and Figueiredo-Ribeiro (1993)
Pfaffia glomerata (Brazilian ginseng or Suma)	Roots	Caleffi et al. (2015)
Amaryllidaceae		
Allium ampeloprasum var. *porrum* (Leek)	Bulbs	van Loo et al. (1995) and Tasar et al. (2015)
Allium cepa (Onion)	Bulbs	van Loo et al. (1995) and Ayyachamy et al. (2007)
Allium sativum (Garlic)	Bulbs	Mahmoud et al. (2011)
Asparagaceae		
Agave americana (Century plant)	Lobes	Partida et al. (1998)
Agave angustifolia (Caribbean agave)	Stems	Allsopp et al. (2013)
Agave atrovirens (Pulque agave)	Leaves	Leopoldo et al. (2011)
Agave durangensis (Agave Cenizo)	Pine heads	Orozco-Cortés et al. (2015)
Agave fourcryodes (Henequen)	Lobes	Villegas-Silva et al. (2014)
Agave salmiana (Aguamiel or Salm-dick)	Stems	Andrade et al. (2019)
Agave sisalana (Giant agave)	Leaves	Elisante and Msemwa (2011) and Kamble et al. (2018)
Agave tequilana (Tequila agave or blue agave)	Stems and leaves	Praznik et al. (2013)
Asparagus officinalis (Garden asparagus)	Root tubers	Gupta and Kaur (1997)
Asparagus racemosus (vernacular name in Hindi: *satavar, shatavari* or *shatamuli*)	Root tubers	Gupta and Kaur (1997)
Camassia sp. (Camas)	Bulbs	van Loo et al. (1995)
Asteraceae (or Compositae)		
Apopyros warmingii	Roots	Abdalla et al. (2016)
Arctium lappa (Burdock)	Roots	van Loo et al. (1995)
Atractylodes chinensis (Cangzhu)	Rhizome	Xu et al. (2016)
Cichorium intybus (Common chicory)	Roots	van Loo et al. (1995) and Gupta and Kaur (1997)
Cynara cardunculus (Artichoke thistle)	Leaves	de Leenheer (2007)
Cynara scolymus (Globe artichoke)	Heads	Leroy et al. (2010)
Dahlia pinnata (Garden dahlia)	Root tubers	Zubaidah and Akhadiana (2010) and Rawat et al. (2015)
Echinacea purpurea (Eastern purple coneflower or hedgehog coneflower)	Roots	Wack and Blaschek (2006)
Echinops ritro (Southern globethistle)	Roots	Vergauwen et al. (2003)
Helianthus annuus (Sunflower)	Root tubers	Chi et al. (2011)
Helianthus tuberosus (Jerusalem artichoke)	Root tubers	Ertan et al. (2003) and Sarchami and Rehmann (2015)
Ichthyothere terminalis	Roots	Abdalla et al. (2016) and de Almeida et al. (2017)
Lactuca sativa (Lettuce)	Roots	Hendry and Wallace (1993)
Matricaria maritima (False mayweed or sea mayweed)	Leaves	Cérantola et al. (2004)
Microseris lanceolata (Yam daisy; Murnong)	Roots	van Loo et al. (1995)
Polymnia sonchifolia (Yacon)	Root tubers	Cazetta et al. (2005)
Saussurea lappa (Costus or kuth)	Roots	Viswanathan and Kulkarni (1995) and Kuniyal et al. (2005)
Scorzonera hispanica (Spanish salsify)	Roots	Dolota and Dabrowska (2004)

TABLE 15.1 Different plant families recognized as potential inulin producers—cont'd

Plant (common name)	Plant parts	Reference(s)
Smallanthus sonchifolius (Yacon)	Roots	de Leenheer (2007)
Stevia rebaudiana(Sweet leaf)	Roots	Lopes et al. (2015)
Taraxacum officinale (Dandelion)	Leaves	van Loo et al. (1995)
Tithonia rotundifolia (Weed)	Stems	Kamble et al. (2018)
Tragopogon sp. (Goatsbeard or salsify)	Roots	Gupta and Kaur (1997)
Vernonia herbacea (Daisy)	Rhizomes	de Carvalho and Dietrich (1993) and Pessoni et al. (1999)
Viguiera discolor (Golden eye)	Root tubers	Isejima et al. (1991) and Itaya et al. (1999)
Campanulaceae		
Campanula rapnculus (Rampion)	Taproots	Vergauwen et al. (2000)
Dioscoreaceae		
Dioscorea esculenta (Lesser yam)	Root tubers	Zubaidah and Akhadiana (2010)
Fabaceae		
Pachyrhizus erosus (Jicama or Mexican turnip)	Root tubers	Zubaidah and Akhadiana (2010)
Iridaceae		
Trimezia juncifolia	Corm	Almeida et al. (2015)
Liliaceae		
Erythronium gradiflorum (Glacier lily)	Corm	Mullin et al. (1997)
Musaceae		
Musa acuminata (Banana)	Fruits	van Loo et al. (1995)
Poaceae		
Hordeum vulgare (Barley)	Grains	van Loo et al. (1995)
Phleum pratense (Timothy grass or Meadow cat's tail)	Leaves	Thorsteinsson et al. (2002)
Secale cereale (Rye)	Grains	van Loo et al. (1995)
Saccharum officinarum (Sugarcane)	Stems	Neagu and Bahrim (2011)
Triticum aestivum (Wheat)	Grains	Neagu and Bahrim (2011)
Polygonaceae		
Fagopyrum esculentum (Buckwheat)	Grains	Bonciu et al. (2012)
Rubiaceae		
Morinda officinalis (Indian mulberry)	Roots	Jiang et al. (2018)

(*Penicillium* sp., *Aspergillus* sp.) (Neagu and Bahrim, 2011). However, the current focus is more on fungal strains due to their advantages over other microbial sources. These include thermostability, growth on economical substrates, and occurrence of synergistic effect of both *exo*- and *endo*-inulinases. Various fermentation processes produce fungal inulinase. In addition, many reports exist on the production of recombinant inulinase from various microbial sources (Singh et al., 2016a,b; Liu et al., 2016).

15.2 Inulinase production from various fungi

Filamentous fungi are favored over other microbial sources due to their capability of nurturing at low pH and cultivation on low-cost substrates and at high temperatures (Vandamme and Derycke, 1983; Kango and Jain, 2011). Growth at higher temperature and low pH reduces the chances of contamination and prevents secondary products

and color formation. Some filamentous fungi are reported to have a synergistic effect of both *exo*- and *endo*-inulinases during fructan hydrolysis. Different fungi reported as potential inulinase producers are listed in Table 15.2. Moreover, yeasts are also considered as resourceful sources of inulinase due to their unicellular forsssssm, easy cultivation, high growth rate, and good inulinase yield within a short span. *Kluyveromyces marxianus* and *Kluyveromyces fragilis* are the most commonly explored yeast strains (Singh and Gill, 2006). Other yeast strains including *Candida kefyr*, *Pichia* sp., *Cryptococcus aureus*, and others are widely reported for inulinase production (Vandamme and Derycke, 1983; Chi et al., 2009; Guo et al., 2009).

TABLE 15.2 Different fungi reported as potential inulinase producers.

Fungal source (family)	References
Aspergillus arachidicola (Trichocomaceae)	Jiang et al. (2019)
A. aureus (Trichocomaceae)	Gupta et al. (1994)
A. awamori (Trichocomaceae)	Arand et al. (2002) and Kulminskaya et al. (2003)
A. candidus (Trichocomaceae)	Kochhar et al. (1999)
A. ficuum (Trichocomaceae)	Lefebvre et al. (1992) and Jing et al. (2003a,b)
A. fischeri (Trichocomaceae)	Gupta et al. (1994)
A. flavus (Trichocomaceae)	Gupta et al. (1994)
A. foetidus (Trichocomaceae)	Fawzi (2011)
A. fumigatus (Trichocomaceae)	Gouda (2002)
A. kawachii (Trichocomaceae)	Chesini et al. (2013)
A. nidulans (Trichocomaceae)	Gupta et al. (1994)
A. niger (Trichocomaceae)	Uhm and Byun (1987), Nakamura et al. (1995), and Dinarvand et al. (2012)
A. niveus (Trichocomaceae)	de Souza-Motta et al. (2005)
A. ochraceus (Trichocomaceae)	Guimaraes et al. (2007)
A. oryzae (Trichocomaceae)	Gupta et al. (1998)
A. parasiticus (Trichocomaceae)	Ertan et al. (2003)
A. tamarii (Trichocomaceae)	Saber and El-Naggar (2009) and Abd Al-Aziz et al. (2012)
A. terreus (Trichocomaceae)	Coitinho et al. (2010)
A. tubingensis (Trichocomaceae)	Trivedi et al. (2012)
A. versicolor (Trichocomaceae)	Kochhar et al. (1998)
Penicillium sp. (Trichocomaceae)	Nakamura et al. (1997) and Singh and Shukla (2012)
P. citrinum (Trichocomaceae)	Flores-Gallegos et al. (2012), Lafuente-Castaneda et al. (2012), and Abd Al-Aziz et al. (2012)
P. expansum (Trichocomaceae)	Fernandes et al. (2012)
P. janthinellum (Trichocomaceae)	Wang et al. (2011)
P. janczewskii (Trichocomaceae)	Pessoni et al. (1999)
P. oxalicum (Trichocomaceae)	Singh and Chauhan (2017)
P. purpurogenum (Trichocomaceae)	Onodera et al. (1996), Sharma et al. (2005), and Muthuselvi et al. (2012)
P. rugulosum (Trichocomaceae)	Barthomeuf et al. (1991)
P. spinulosum (Trichocomaceae)	Ertan et al. (2003)
P. subrubescens (Trichocomaceae)	Mansouri et al. (2013)
P. trzebinskii (Trichocomaceae)	Murmatsu et al. (1992)
Rhizomucor pusillus (Mucoraceae)	Flores-Gallegos et al. (2015)

TABLE 15.2 Different fungi reported as potential inulinase producers.—cont'd

Fungal source (family)	References
Rhizopus sp. (Mucoraceae)	Ohta et al. (2002)
R. microsporus (Mucoraceae)	Flores-Gallegos et al. (2015)
R. oligosporus (Mucoraceae)	Mohamed et al. (2015)
Alternaria alternata (Pleosporaceae)	Hamdy (2002); Sanal et al. (2005)
Chrysosporium pannorum (Onygenaceae)	Xiao et al. (1988)
Cladosporium cladosporioides (Davidiellaceae)	Ferreira et al. (1991)
Fusarium oxysporum (Nectriaceae)	Gupta et al. (1988)
Mucor circinelloides (Mucoraceae)	Singh et al. (2018)
Rhizoctonia sp. (Ceratobasidiaceae)	Bonciu et al. (2012)
Rhizoctonia solani (Ceratobasidiaceae)	Ertan et al. (2003)
Scytalidium acidophilum (Incertae sedis)	Kim et al. (1994)
Thermomyces lanuginosus (Chaetomiaceae)	Flores-Gallegos et al. (2015)
Thielavia terrestris (Chaetomiaceae)	Fawzi (2011)
Ulocladium atrum (Pleosporaceae)	Mohamed et al. (2014)
Yeasts	
Candida guilliermondii (*Meyerozyma guilliermondii*) (Saccharomycetaceae)	Sirisansaneeyakul et al. (2007) and Liu et al. (2014)
C. kefyr (Saccharomycetaceae)	Hiroaki et al. (1991)
C. kutaonensis (Saccharomycetaceae)	Yuan et al. (2012)
Cryptococcus aureus (Tremellaceae)	Sheng et al. (2007)
Geotrichum candidum (Dipodascaceae)	Mughal et al. (2009)
Kluyveromyces marxianus (Saccharomycetaceae)	Parekh and Margaritis (1985), Singh and Bhermi (2008), and Sokolenko and Karpechenko (2015)
K. marxianus var. *bulgaricus* (Saccharomycetaceae)	Manzoni and Cavazzoni (1992), Kushi et al. (2000), and de Paula et al. (2008)
K. fragilis (Saccharomycetaceae)	Kim and Byun (1982) and Workman and Day (1983)
Pichia guilliermondii (Saccharomycetaceae)	Guo et al. (2009)
P. polymorpha (Saccharomycetaceae)	Chautard et al. (1981)
Rhodotorula glutinis (Incertae sedis)	Tasar et al. (2015)
Saccharomyces fragilis (Saccharomycetaceae)	Synder and Phaff (1960)
S. cerevisiae (Saccharomycetaceae)	Brevnova et al. (1998)
Zygosaccharomyces bailii (Saccharomycetaceae)	Paixão et al. (2013)

15.3 Production of inulinase

Since inulinase is an inducible enzyme, inulinase-producing microorganisms are cultured in an inulin-rich medium, where inulin acts as an inducer and carbon source for inulinase production (Singh and Singh, 2010). Various substrates of pure or naturally occurring inulin-rich plant materials and/or mixed substrates have been employed for inulinase production. Inulin, chemically modified inulin, fructose, lactose, and sucrose are some of the pure carbon sources used for inulinase production (Pandey et al., 1999). In addition, extracts of different plant parts like roots, leaves, tubers from plants like chicory, asparagus, dahlia, and Jerusalem artichoke, and others were also explored for inulinase production (Neagu and Bahrim, 2011). Among the organic and nitrogen sources, yeast extract, beef extract,

KNO_3, $NaNO_3$, $(NH_4)_2HPO_4$, $NH_4H_2PO_4$, and $(NH_4)_2SO_4$ were extensively used for inulinase production (Vandamme and Derycke, 1983; Vijayaraghavan et al., 2009). Trace elements like Co^{2+}, Zn^{2+}, Mg^{2+}, H_3BO_3, Na^+, Fe^{2+}, and Cu^{2+} are mandatory for enhancing inulinase activity (Li et al., 2011). Production of inulinase in stirred tank reactors is strategized by aeration, agitation, and type of impeller (Silva-Santisteban and Filho, 2005). For production of inulinase, different fermentations were employed including the following:

15.3.1 Submerged fermentation

Root tuber extracts from dahlia and *Asparagus officinalis* served as raw inulin substrates for inulinase production by *Aspergillus niger* and *Kluyveromyces marxianus*, respectively (Singh and Bhermi, 2008; Rawat et al., 2015). Yacon extracts were recently reported as suitable carbon sources for inulinase production from *Aspergillus kawachii* (Chesini et al., 2013). The different substrates used for fungal inulinase production by submerged fermentation (SmF) are listed in Table 15.3. The temperature and pH of production medium play a crucial role in fungal growth and inulinase production. Most of the fungal and yeast strains showed high inulinase production in the pH range of 4–6.5 (Kango and Jain, 2011). pH affects fungal growth by directly affecting cell surface and indirectly increasing nutrient absorption (Barnett and Ayers, 1981). A change in pH less than 4 and greater than 6 delays fungal growth. In general, *Aspergillus* sp. grows at alkaline pH, and *Penicillium* sp. are tolerant to acidic pH (Wheeler et al., 1991). The optimal cultivation temperature for most fungal species favoring inulinase production is 28–30°C (Kango and Jain, 2011). *Rhizopus microsporus* has been reported to produce a thermostable inulinase at 70°C (Flores-Gallegos et al., 2015). Under SmF conditions, microorganisms isolated from some extreme climatic zones have also been reported to give high inulinase yields. *Penicillium citrinum*, isolated from the Mexican semi-desert, is as an efficient inulinase producer (Flores-Gallegos et al., 2012).

TABLE 15.3 Fungal inulinase production using submerged fermentation.

Microorganism	Substrate	Inulinase production (IU/mL)	Reference
Filamentous fungi			
Aspergillus awamori	Asparagus root extract	8.21	Rawat et al. (2015)
A. ficuum	Inulin	25	Jing et al. (2003a,b)
A. fumigatus	Inulin	7.97	Flores-Gallegos et al. (2015)
A. kawachii	Yacon juice	110[a]	Chesini et al. (2013)
A. niger	Dandelion tap root extract	55	Kango (2008)
	Inulin	176	Kumar et al. (2005)
	Sucrose	3199	Dinarvand et al. (2012)
	Raw garlic juice	5.65	Mahmoud et al. (2011)
A. parasiticus	Jerusalem artichoke powder	2.90	Ertan et al. (2003)
A. tamarii	*Dahlia* tubers extract	57.07	Saber and El-Naggar (2009)
A. tritici	Raw asparagus inulin	25.01	Singh et al. (2016a,b)
A. tubingensis	Chicory roots powder	21.90	Trivedi et al. (2012)
Mucor circinelloides	Inulin	23.55	Singh et al. (2018)
Penicillium citrinum	Inulin	2.24	Lafuente-Castaneda et al. (2012)
P. expansum	Inulin	2.84	Fernandes et al. (2012)
P. oxalicum	Inulin	38.52	Singh and Chauhan (2017)
P. rugulosum	Inulin	54	Barthomeuf et al. (1991)
P. spinulosum	Jerusalem artichoke powder	1.60	Ertan et al. (2003)
Rhizopus microsporus	Inulin	10.71	Flores-Gallegos et al. (2015)
Rhizoctonia sp.	Buckwheat flour	1.87	Bonciu et al. (2012)

TABLE 15.3 Fungal inulinase production using submerged fermentation.—cont'd

Microorganism	Substrate	Inulinase production (IU/mL)	Reference
Thermomyces lanuginosus	Inulin	7.23	Flores-Gallegos et al. (2015)
Yeasts			
Cryptococcus aureus	Inulin	52.37	Gao et al. (2007)
	Inulin	85	Sheng et al. (2007)
Kluyveromyces marxianus	*Dahlia* tubers extract	25.30[b]	Jain et al. (2012)
	Sugarcane molasses and corn steep liquor	1139	Sguarezi et al. (2009)
	Asparagus racemosus root tubers extract	47.31	Singh and Gill (2006)
	Asparagus officinalis root tubers extract	50.20	Singh and Bhermi (2008)
	Dahlia tubers extract	140.02	Singh and Saini (2013)
	Sucrose	208	Silva-Santisteban et al. (2009)
K. marxianus var. bulgaricus	Yacon extract	4.10	Cazetta et al. (2005)
	Sucrose	15.29	Cazetta et al. (2010)
Pichia guilliermondii	Inulin	39.56	Gao et al. (2007)
Rhodotorula glutinis	Leek powder	30.89	Tasar et al. (2015)
Yarrowia lipolytica	Inulin	62.85	Gao et al. (2007)

[a] *Enzyme activity in mU.*
[b] *Enzyme activity in Kcat.*

Agitation and oxygen transfer rate are crucial factors under SmF conditions in bioreactors. For successful bioconversion in a fermenter, agitation is required for proper heat and mass transfer, homogenization, and gas dispersion. Its failure may cause production of undesirable products due to change in enzymatic organization. Agitation increases broth viscosity due to either increased cell concentration, morphological changes, or accumulation of extracellular products that directly affect the rheological characteristics of the broth during fermentation. All these changes together affect the oxygen transfer rate and further exhibit a profound effect on cellular growth, microbial biosynthesis, and nutrient uptake (Tang and Zhong, 2003). By improving oxygen supply, an increase in biomass yield and consumption of nitrogen sources can be achieved (Hajjaj et al., 1999). Alternatively, a decrease in oxygen partial pressure can decrease cell growth and increases the accumulation of extracellular compounds (Rau et al., 1992). In case of *Kluyveromyces marxianus* var. *bulgaricus*, rate of aeration and speed of agitation have significant effect on production of inulinase (Silva-Santisteban and Filho, 2005). Generally, agitation increases the production of inulinase, which is further linked with shear stress. Shear stress antagonistically acts on inulinase production. Hence, the impeller design should be appropriately chosen to reduce shear stress on microbial growth. In general, use of a pitched blade impeller is suggested, which generates less stress as compared to disk and marine impellers (Silva-Santisteban and Filho, 2005).

15.3.2 Solid-state fermentation

Primarily when fungal strains are used, solid-state fermentation (SSF) is the most recommended choice for inulinase production, since their morphology can be retained when grown on solid medium. At an industrial level, resurgence of SSF has gained attention because of its innumerable applications in bioremediation, biotransformation, biological detoxification, and biopulping. (Lafuente-Castaneda et al., 2012). SSF has several advantages over SmF, such as enhanced product concentration, firm support for fungal growth, better oxygen circulation, simple equipment, and inexpensive substrate requirements. A comparative study on inulinase production by *Penicillium citrinum* under SmF and SSF has also shown that SSF is a more suitable process. For successful SSF, parameters like substrate particle size, moisture, pH, relative humidity, substrate pre-treatment, incubation temperature, agitation and aeration, and age and size of inoculum are the important factors. In addition, heat and mass transfer play a significant role during SSF. In general, non-soluble matrices used for SSF have low thermal conductivity, resulting in heat accumulation that further impacts the final product formation. Substrate temperature also plays

an important role since it directly affects growth of fungal and/or yeast cells. Furthermore, both high and low moisture conditions are vulnerable for SSF. High moisture conditions cause poor oxygen penetration, whereas low moisture conditions cause poor nutrient absorption. Astolfi et al. (2011) reported maximum inulinase production from fed-batch mode with saturated air as compared to batch mode with unsaturated air. This can be attributed to the decrease in mean temperature of the solid medium because of evaporative cooling, displaying the significance of moisture in SSF. Water activity (a_w) is also important for the transportation of water and solutes through fungal and yeast cells (Pandey, 2003).

Many agro-residual substrates like soybean bran, wheat bran, coconut oil cake, and sugarcane bagasse are used for inulinase production by SSF. They provide good anchorage to cells or mycelia. The choice of the substrate in SSF primarily depends upon the availability and cost of the substrate. The different substrates used for fungal inulinase production by SSF are listed in Table 15.4. A previous study has demonstrated that as compared to other substrates, *Saccharomyces* sp. cultivation on wheat bran supported maximum inulinase production (78.29 ± 0.13 U/gds) (Onilude et al., 2012). In another study, copra waste was used as the sole carbon source for inulinase production by *Penicillium rugulosum* and showed 18% increase in inulinase production (239 U/gds), validating it as a good candidate for inulinase production (Dilipkumar et al., 2014). In addition, it is important to extract inulinase after SSF without loss in activity, which depends on the choice of buffer, agitation, and time of enzyme extraction (Yu et al., 2008).

TABLE 15.4 Fungal inulinase production employing solid state fermentation.

Microorganism	Substrate	Inulinase yield (U/gds)	Reference
Aspergillus ficuum	Corn steep liquor	205.63	Chen et al. (2011)
A. niger	Wheat bran	160	Narayanan et al. (2013)
	Rice bran	137.20	
	Banana peel	237	
A. parasiticus	Artichoke leaves	0.17	Aty et al. (2014)
	Banana leaves	0.32	
	Chicory roots	0.19	
	Garlic waste	0.58	
	Orange rinds	1.34	
	Sugarcane bagasse	1.77	
A. sclerotiorum	Artichoke tubers	5721.5	El-Naggar et al. (2016)
A. terreus	Artichoke leaves	4.43	Aty et al. (2014)
	Banana leaves	0.36	
	Chicory roots	0.13	
	Garlic waste	0.02	
	Orange rinds	0.77	
	Sugarcane bagasse	1.10	
A. tubingensis	Wheat bran and corn steep liquor	257	Trivedi et al. (2012)
A. versicolor	Artichoke leaves	0.17	Aty et al. (2014)
	Banana leaves	0.20	
	Chicory roots	0.17	
	Garlic waste	0.39	
	Orange rinds	1.91	
	Sugarcane bagasse	1.31	
Cryptococcus aureus	Wheat bran and rice husk	420.90	Sheng et al. (2009)

TABLE 15.4 Fungal inulinase production employing solid state fermentation—cont'd

Microorganism	Substrate	Inulinase yield (U/gds)	Reference
Kluyveromyces sp.	Wheat bran	409.80	Xiong et al. (2007)
	Wheat bran, rice bran, coconut oil cake and corn flour	122.88	Selvakumar and Pandey (1999)
	Sugarcane bagasse	445	Bender et al. (2006)
	Sugarcane bagasse and soybean bran	436.70	Mazutti et al. (2010)
	Sugarcane bagasse and soybean bran	463	Mazutti et al. (2010)
	Sugarcane bagasse and soybean meal	586	Astolfi et al. (2011)
	Pressmud	300.50	Dilipkumar et al. (2013)
	Artichoke leaves	1.77	Aty et al. (2014)
	Banana leaves	1.35	
	Chicory roots	0.22	
	Garlic waste	0.88	
	Orange rinds	0.44	
	Sugarcane bagasse	0.08	
Penicillium brevicompactum	Artichoke leaves	1.24	Aty et al. (2014)
	Banana leaves	1.21	
	Chicory roots	0.88	
	Garlic waste	0.66	
	Orange rinds	0.85	
	Sugarcane bagasse	1.21	
P. oxalicum	Corn bran	77.95	Singh et al. (2018a)
	Carrot pomace	322.10	Singh et al. (2018b)
P. rugulosum	Copra waste	239	Dilipkumar et al. (2014)
Pichia guilliermondii	Wheat bran and rice bran	455.90	Guo et al. (2009)

15.4 Production of recombinant inulinase

Molecular changes in the genetic make-up of an enzyme increase the enzyme yield and reduce the processing period. Some of the advantages of recombinant enzymes over native ones include control in production environment, product purity and consistency, and easy affordability due to high yield. Many recombinant strains have been successfully developed to produce inulinase. These recombinant inulinase are reported to be stable at varied pH and temperature as compared to the native strains.

15.4.1 Exo-inulinase gene from yeasts and filamentous fungi

Exo-inulinase genes have been reported from yeasts like *Pichia* sp., *Cryptococcus aureus*, and *Kluyveromyces marxianus* (Gong et al., 2008; Sheng et al., 2007). The first inulinase gene was cloned and characterized from *Kluyveromyces marxianus* (*INUI*), which encoded a precursor protein of 555 amino acids (Laloux et al., 1991) due to its ability of assimilation of inulin and lactose, thermotolerance, short generation time, and high secretory capacity (Lane and Morrissey, 2010). Sequence analysis signifies that both inulinase and yeast β-fructosidases have common evolutionary origin, as inulinase showed 67% similarity with invertase gene of *Saccharomyces cerevisiae* (Laloux et al., 1991). From *Klyuveromyces marxianus* CBS 6556, inulinase gene was cloned, and protein deduced from it has molecular

mass of 62.0 kDa and showed 59% and 95% similarity to *Saccharomyces cerevisiae* invertase and *Klyuveromyces marxianus* var. *marxianus* ATCC 12424, respectively. Repression of expression of inulinase by glucose is signified by 5′-flanking region, (TAAATCCGGGG) matching with consensus sequence Mig1 binding (Bergkamp et al., 1993). From *Kluyveromyces cicerisporus* CBS4857, *KcINU1*, an inulinase gene was cloned and encoded protein (555 amino acids) with signal protein (Wen et al., 2003). Likewise from *Pichia guilliermondii* strain 1, intronless inulinase gene was cloned and produced a protein of 58.04 kDa with signal peptide of 18 amino acids, containing the consensus sequence (WMNXPNGL) and (RDPKVF) having similarity with *Klyuveromyces marxianus* (Zhang et al., 2012). From marine yeast *Candida aureus* G7a, inulinase was cloned and purified having molecular weight of 60.0 kDa (Sheng et al., 2008). It also contained consensus motifs such as RDPKVFWH and WMNEPNG conserved among the inulinase from other microbes (Chi et al., 2009).

Further, from *Aspergillus niger* strain 12, *inuE* (*exo*-inulinase gene) was cloned and encoded protein of molecular weight of 57.25 kDa and signal peptide of 19 amino acids, showing identity (91%) with *Aspergillus awamori*. Inulin and sucrose favored the induction of transcription of the inulinase gene, while fructose and glucose repressed the rate of transcription (Moriyama et al., 2003). The *exo*-inulinase gene (*inuD*)-encoding protein and signal peptide of 677 and 25 amino acids, respectively, was isolated from *Penicillium* sp. strain TN-88 (Moriyama et al., 2003). From *Penicillium janthinellum* strain B01, *inuA1* (*exo*-inulinase gene) was cloned and encoded protein of 684 amino acids and signal peptide of 6720 amino acids and showed similarity (82%) with *Talaromyces stipitus* exoinulinase; however, it did not show any resemblance with inulinases of other species (Wang et al., 2011). Likewise, an exo-inulinase gene was cloned *Kluyveromyces marxianus*, and *Yarrowia lipolytica* was used as the expression system (Liu et al., 2016).

15.4.2 Bacterial exo-inulinase gene

As reported in *Pseudomonas mucidolens*, *inu2* (exo-inulinase gene) was cloned, which encoded polypeptide of 501 amino acids (Kwon et al., 2000). Likewise, *inu* gene of *Bacillus polymyxa* MGL21 was cloned and encoded protein of molecular weight 55.5 kDa, which showed similarity of 39% and 57% with *Aspergillus niger* and *Pseudomonas mucidolens*, respectively, containing conserved sequence (WMNDPNG) (Kwon et al., 2003). *Geobacillus stearothermophilus* KP1289 produces the most thermostable exo-inulinases and it encodes protein of molecular weight 56.7 kDa with no signal protein showing identity with *Pseudomonas mucidolens* exo-inulinase (Tsujimoto et al., 2003). Extracellular degradation of inulin was exhibited by *Lactobacillus casei* IAM1045. It encodes protein (LevH1) of molecular weight of 138.8 kDa (Kuzuwa et al., 2012) and has eight conserved motifs (I–VIII). It was revealed by site-directed mutagenesis of the motifs that D198, R388, D389, and E440 were vital for the mode of action of inulinase, as mutations of D502A and D683A in motif VI and VIII, respectively, showed noteworthy decline in inulinase activity. This analysis showed that for inulin degradation activity of *LevH1,* β-propeller module as well as β-sandwich module and variable domain were significant (Kuzuwa et al., 2012).

15.4.3 Endo-inulinase gene

Endo-inulinase encoding genes have been cloned and characterized from various microbial sources like *Aspergillus niger* (Ohta et al., 1998), *Aspergillus ficuum* (Uhm et al., 1998), *Penicillium purpurogenum* (Onodera et al., 1996), and bacterium *Arthrobacter* sp. S37 (Kim et al., 2005).

15.4.3.1 Endo-inulinase gene from fungi

Endo-inulinase gene of *Penicillium purpurgenum* has no introns and encodes protein (490 amino acids) and signal peptides (25 amino acids) (Onodera et al., 1996). Likewise, endoinulinase gene *inu2* from *Aspergillus ficuum* ATCC encodes protein of 55.8 kDa showing 93.9% and 73.3% identity with *Penicillium purpurgenum* and *Aspergillus ficuum endo*-inulinases, respectively. The *inuC* (endo-inulinase gene) from *Penicillium* sp. strain TN-88 encodes protein (490 amino acids) and signal peptides (25 amino acids) and showed 85% and 72% similarity with *Penicillium purpurgenum* and *Aspergillus niger endo*-inulinase genes, respectively (Akimoto et al., 2000). Further, the endophytic fungi like *Aspergillus* sp. was also identified as an inulin degrader. The *inu2* gene of *Aspergillus* sp. Asf1 encoded protein of 55.9 kDa and signal peptide (23 amino acids) and showed 22%, 69%, 96%, and 97% similarity with genes of different microbes such as *Kluyveromyces marxianus INU1*, *Penicillium purpurogenum* inulinase gene, *Aspergillus niger inuB*, and *Aspergillus ficuum inu2*, respectively. Likewise from *Aspergillus ficuum* strain JNSP5-06, *endo*-inulinase producing gene (endo I) was cloned and encoded protein showed 99.59%, 97.97%, and 59.17% identity to *Aspergillus ficuum* and *Aspergillus niger* CBS513.88 and *Aspergillus fumigatus* AF293, respectively (Chen et al., 2012).

15.4.3.2 *Bacteria producing endo-inulinase gene*

Kang and Kim (1999) reported that endoinulinase gene from *Arthrobacter* sp. S37 was cloned and encoded protein (82.3 kDa) and signal peptides (53 amino acids) showing 15.3%, 15.3%, and 15.6% similarity with *Aspergillus niger*, *Aspergillus ficuum*, and *Penicillum purpurogenum* endo-inulinase. As compared to other inulinase, it has a 508 amino acid C-terminal catalytic domain and N-terminal domains (25 amino acids). It was revealed that this N-terminal domain was needed for enzyme catalysis and dimerization. Similarly, endo-inulinase gene of *Microbulbifer* sp. strain JAM-301 was cloned and encoded protein of molecular weight 80 kDa (Kobayashi et al., 2012).

15.5 Expression of different microbial inulinase gene

15.5.1 Yarrowia lipolytica *as expression host*

Yarrowia lipolytica is one of the most suitable yeast host systems for the production of heterologous proteins due to wide range of genetic markers, its ability of natural secretion of proteins, and molecular tools (Madzak et al., 2004). Optimization of some *Yarrowia lipolytica* strains was done for the production of proteins, for example, genes for extracellular alkaline and acid protease were deleted making it feasible for them to nurture on sucrose and molasses by producing recombinant invertase. From various origins, effective production of active proteins has been confirmed by *Yarrowia lipolytica* expression or secretion systems. Interestingly, *Yarrowia lipolytica* can efficiently secrete larger or complex proteins with no hyperglycosylation issues (Madzak et al., 2004) as its glycosylation pattern and secretion apparatus are more similar to mammalian ones as compared to *Saccharomyces cerevisiae*. To maintain the Generally Recognized As Safe (GRAS) status and stop the dispersal of antibiotic resistance genes in the surroundings, bacterial moieties from these vectors can be removed and used as "yeast expression cassette" for recipient strain transformation.

15.5.2 *Saccharomyces cerevisiae* as expression host

Saccharomyces cerevisiae's genetic background makes it safe and widely used to produce recombinant proteins. *Kluyveromyces marxianus INUI* gene was expressed in *Saccharomyces cerevisiae* and the encoded protein showed higher thermostability contributed by hyperglycosylation of recombinant enzyme. Furthermore, *suc2* mutant *cerevisiae Saccharomyces* was used as host for the expression of *endo*-inulinase gene (*inuB*) of *Aspergillus ficuum* for production of enzyme without an exoinulinase and invertase (Liu et al., 2013).

15.5.3 *Pichia pastoris* as expression host

Pichia pastoris is a commonly used methylotrophic yeast expression system for high productivity of heterologous proteins and enzymes. Many industrially important enzymes are efficiently produced by the methanol-induced AOX1 promoter of this yeast. It has advantages over others due to its easy handling, simple fermentation conditions, ability to produce soluble and rightly folded recombinant proteins that have underwent all post-translational modifications necessary for its function. Furthermore, it does not contain a large amount of intrinsic protein therefore making separation of foreign protein easy. *Aspergillus niger* strain 12 *inuE* gene was expressed in *Pichia pastoris* having high secretion efficiency and no invertase activity (Moriyama et al., 2003).

15.5.4 *Escherichia coli* as expression host

Escherichia coli is the most commonly used prokaryotic host for the expression of heterologus protein, for example, *Pencillium mucidolens inu2* (*exo*-inulinase) gene was expressed in *Escherichia coli* and enzyme (55 kDa) having a monomer was produced. In addition to inulin, it can degrade sucrose and raffinose. In addition, after enzymatic reaction there is no liberation of oligopolymers (Kwon et al., 2000). Likewise, *Bacillus polymyxa* MGL21 *inu* gene was expressed in *Escherichia coli* and the resultant recombinant in addition to inulin can degrade sucrose, raffinose, and levan to release fructose primarily (Kwon et al., 2003). *Geobacillus stearothermophilus inuA* gene was expressed in *Escherichia coli* HB101 and purified, having molecular weight of 54.0 kDa (Tsujimoto et al., 2003). *Aspergillus ficuum endo*-inulinase gene (endo I) was expressed in *Escherichia coli* and resultant recombinant endoinulinase (molecular weight of 60 kDa) was purified and the main hydrolyse product of inulin by this endoinulinase was FOS with 3–4 DP (Chen et al., 2012).

15.6 Fungal inulinase: Purification and characterization

Purification and characterization of an enzyme is mandatory for obtaining a good biocatalyst and to determine its physico-chemical characteristics. Different purification techniques have been employed individually or in combination for inulinase purification from various fungal organisms. Purification results in the separation of different types and isoforms of inulinase. Inulinases can differ in their modes of action and structural conformation, depending on the source and growth conditions. Ettalibi and Baratti (1987) separated two types of endoinulinases, namely Endo-I and II, and five types of exoinulinases, namely Exo-I, II, III, IV, and V, from *Aspergillus ficuum* using fast protein liquid chromatography (FPLC), ammonium sulphate precipitation, and ion exchange chromatography. Recently, purification of three active forms of inulinase, namely INI, INII, and INIII from *Ulocladium atrum*, was done by ion exchange chromatography and ammonium sulphate precipitation. Further, chromatographic separation resolved INI into INIa, INIb, and INIc with a 3.43 purification fold (El-Souod et al., 2014). Thermal and pH stability of fungal inulinase are more important properties considering industrial requirements (Kango and Jain, 2011). The optimal temperature and pH range of inulinases from most fungal strains are 45–55°C and 4.5–7, respectively. Extracellular inulinase from *Aspergillus niger* was stable at 50°C and pH 5.5 (Viswanathan and Kulkarni, 1995). Chen et al. (2009) reported that *endo*-inulinases from *Aspergillus ficuum*, namely Endo-I and II, and *exo*-inulinases, namely Exo-I, II, and III, exhibited stability at wide range of pH (4–8) and temperature less than 50°C.

Metal ions such as Fe^{3+}, Mn^{2+}, Cu^{2+}, Mg^{2+}, Co^{2+}, Na^{+}, and Ag^{+} play a symbolic role in the activation and inhibition of inulinase (Pandey et al., 1999). Fe^{2+} exhibited a stimulatory effect on exoinulinase, whereas Mg^{2+} had an inhibitory effect on exoinulinase of *Kluyveromyces marxianus* expressed in *Pichia pastoris* host system (Zhang et al., 2005). Inulinase from *Cryptococcus aureus* was stimulated by Zn^{2+}, Ca^{2+}, Cu^{2+}, Na^{+}, and K^{+}, whereas it was inhibited by Mg^{2+}, Hg^{2+}, and Ag^{+} (Sheng et al., 2008). Al^{3+} and Fe^{2+} showed stringent inhibition of inulinase from *Aspergillus ficuum*, while Ag^{+} inhibited it completely (Chen et al., 2009). To display enzyme per molecular organization and reaction towards the substrate, molecular weight, K_m, and V_{max} are the vital characteristics to consider. K_m and V_{max} predict the relationship between reaction rate and substrate concentration. Therefore, it is important to govern the complexity between enzyme and the substrate. Molecular weight of fungal inulinase mainly ranged between 30 and 175 kDa. *InuA* (endoinulinase) and *inuI* (exoinulinase) from *Aspergillus niger* and *Aspergillus awamori*, respectively, were cloned into *Penicillium canescens*, which exhibited inulinase having molecular mass of 56 and 60 kDa, respectively (Volkov et al., 2012). *Aspergillus ficuum* inulinase expressed in *Escherichia coli* showed a high affinity towards inulin (Chen et al., 2013). To comprehend the phylogenetic relationship and genetic constitution of inulinase, molecular characterization of inulinase from various microorganisms has been done. Employing multiple sequence alignment, some conserved motifs like RDPKVF, WMNEPNGL, WGHATS, and SVEVFGGQGE were decrypted and found to similarly prevail in bacterial and fungal inulinases (Singh and Gill, 2006) Sequence analysis of *Aspergillus* sp. showed that the two enzymes, *exo*- and *endo*-inulinase, were evolved separately (Adawiyah et al., 2011). In addition, a molecular phylogenetic study also revealed the independent evolution of exo- and endo-inulinases, while invertases from fungal GH32 glycoside hydrolases were reported to be most ancient (Flores-Gallegos et al., 2015). Molecular docking of exo-and endo-inulinase from *Penicillium* sp. strain BAC16218 and *Penicillium* sp. strain BAA19132, respectively, was conducted to understand the interaction between substrates and different amino acid arrangements of inulinase. Docking with 1-ketose and fructose-6-phosphate showed that only fructose-6-phosphate showed a significant interaction with exoinulinase and conserved amino acids such as ASP 22, ASP 128, ASP 179, and SER 64 were responsible for this interaction (Singh and Shukla, 2012). The tertiary structure of exoinulinase of *Aspergillus awamori* revealed, based on X-ray crystallographic data, that the enzyme had two folds, namely N-terminal with a β-propeller fold and C-terminal with β-sandwich-like fold (Nagem et al., 2004).

15.7 Immobilization of inulinase

Immobilization of an enzyme has an advantage over the free enzyme due to its increased stability, easier separation of product, and cheaper cost of operation. Depending upon the type of substrates used for the reaction, immobilized enzymes are used in various reactors like packed columns and stirred tank reactors. The different matrices used for fungal inulinase immobilization are listed in Table 15.5. Inulinase from different microbial sources has been immobilized in/on various matrices like gamma-alumina, diatomaceous earth, titanic, activated granular carbon, glass beads, and granular (Trytek et al., 2015). The number of cycles of the biocatalyst decides the stability (mechanical and functional) of the immobilized biocatalyst. Paula et al. (2008) reported the immobilization of inulinase from *Kluyveromyces marxianus* var. *bulgaricus* on various matrices like activated carbon, amberlite, hen eggshell,

TABLE 15.5 Fungal inulinases immobilized on different matrices.

Source	Substrate	Immobilization matrix/support	Reference
	Jerusalem artichoke tubers extract	Chitin	Kim and Rhee (1989)
Aspergillus ficuum	Pure inulin	Porous glass beads	Ettalibi and Baratti (1992)
Aspergillus sp.	Pure inulin (Chicory roots)	Sepabeads (Methacrylic polymer) (Mitsubishi Chem. Corp, Milano, Italy)	Ricca et al. (2010)
	Pure inulin (Chicory roots)	Polyvinyl alcohol particles	Anes and Fernandes (2014)
A. niger	Pure inulin (Chicory roots)	Sol-gel	Kim et al. (1997a,b)
	Pure inulin (Chicory roots)	Magnetic sol-gel particles	Santa et al. (2011)
	Pure inulin (Dahlia tubers)	Amino-Cellulofine beads	Nakamura et al. (1995)
	Pure inulin (Dahlia tubers)	Chitosan	Nguyen et al. (2011)
	Pure inulin (Chicory roots)	Silicone tube activated by APTES	Ribeiro and Fernanades (2013)
	Pure inulin (Chicory roots)	Octadecyl substituted nanoporous silica	Karimi et al. (2016)
	Pure inulin	Magnetite chitosan microparticles	Paripoorani et al. (2015)
	Pure inulin	Magnetite nanoparticles functionalized with soy protein isolate and bovine serum albumin	Torabizadeh et al. (2018)
	Pure inulin (Chicory roots)	Fe_3O_4 magnetic nanoparticles functionalized with wheat gluten hydrolysates	Torabizadeh and Mahmoudi (2018)
	Kuth (*Saussurea lappa*) root powder	Chitosan beads	Yewale et al. (2013)
A. fumigatus	Pure inulin	Chitin	Gill et al. (2006)
	Pure inulin	ConA-linked silica beads	Gill et al. (2006)
	Pure inulin	QAE-Sephadex	Gill et al. (2006)
A. tamarii	Pure inulin	Kaolinite clay	Garuba and Onilude (2018)
A. terreus	Pure inulin	Alginate-CMC gel beads	Awad et al. (2017)
Debaryomyces phaffi	Pure inulin	DEAE-cellulose	Guiraud et al. (1981)
Fusarium oxysporum	Chicory roots tubers extract	DEAE-cellulose	Gupta et al. (1992)
Kluyveromyces fragilis	Jerusalem artichoke tubers extract	2-Aminoethyl-cellulose	Kim and Byun (1982)
Kluyveromyces marxianus	Jerusalem artichoke tubers extract	Agar gel	Bajpai and Margaritis (1985)
	Raw *Asparagus racemosus* inulin	Duolite A568 (Macroporous resin) (Rohm and Haas, France)	Singh et al. (2007, 2008)
	Pure inulin	Alginate beads	Risso et al. (2010)
	Pure inulin	Chitosan beads	Singh et al. (2017)
Kluyveromyces sp. Y-85	Pure inulin	Macroporous ionic polystyrene beads	Wenling et al. (1999)
Penicillium chrysogenum P36	Pure inulin	Grafted alginate beads	Elnashar et al. (2009)
P. funiculosum P36	Pure inulin	Grafted alginate beads	Danial et al. (2010)
P. oxalicum BGPUP-4	Pure inulin	Carbon nanotubes	Singh et al. (2019)
Urocladium atrum	Pure inulin	Chitosan/polyester nonwoven fabrics	Mohamed et al. (2014)

and gelatine. Polyethylenimine and glutaraldehyde were reported; however, the disadvantage underlying the use of polyethylenimine and glutaraldehyde is that the enzyme is loosely bounded and can be liberated during the reaction because the forces connecting the enzyme and carrier are very fragile (Trevan, 1980; Fiedurek et al., 1986; Wójcik et al., 1987; Gupta et al., 1992; Yun et al., 2000; Paula et al., 2008). Due to this, groups that had higher affinity enhanced the strength of the bond between enzyme and carrier. Interestingly, the epoxy groups had higher affinity; however,

the consequence is that too many bonds can lead to change in the spatial structure of the enzyme (Katchalski-Katzir and Kraemer, 2000). Using nanomaterials like nanopolymers, nanofibers, and nanoparticles for the immobilization of enzymes has gained attention in recent years. For example, Fe_3O_4 magnetic nanoparticles were used for inulinase from *Aspergillus niger*. Advantages of magnetic nanoparticles over other nanomaterials is that they make it easy and cheap to collect enzymes by using magnetic field strategies, they are more stable in non-favorable environments (oxidation/acidic), they reduce nanoparticle accumulation to each other, and they have more reactive surface for immobilization (Torabizadeh and Mahmoudi, 2018).

15.8 Applications of inulinases

Inulinases find application for the production of FOS (Singh and Singh, 2010; Chi et al., 2011; de Moura et al., 2014; Mutanda et al., 2014; Apolinario et al., 2014), HFS (high fructose syrup) (Pandey et al., 1999; Ricca et al., 2007; Kango and Jain, 2011; Singh, 2011; Chi et al., 2011) and many other industrially important metabolites like bioethanol (Szambelan et al., 2004; Faga et al., 2010; Zhang et al., 2010; Hu et al., 2012; Hong et al., 2015; Wang et al., 2015; Villegas-Silva et al., 2014), citric acid (Liu et al., 2010), pullulan (Shin et al., 1989), single cell protein (Cui et al., 2011), and sorbitol (Wei et al., 2001).

Among all these applications, FOS have attracted a renewed attention from an industrial and nutritional perspective because of their prebiotic potential and health benefits. FOS are used as prebiotics mainly because they are indigestible in the GI tract and undergo colonic fermentation. They possess bifidogenic property. Endoinulinases cleaves the β-D-(2 → 1) glycosidic linkages in inulin, which generates FOS with a DP of 1–9. Immobilized and free inulinase from *Kluyveromyces marxianus* and *Aspergillus niger*, respectively, were used for the generation of FOS from inulin (Silva et al., 2013). Primarily, short-chain FOS, namely 1-kestose, nystose and 1F-fructosylnystose, were the major products, although at a later stage 6-kestose, levanohexose, levanopentose, and oligolevans (medium-chain FOS) became most dominant (Tian et al., 2014). Structurally, FOS are polymers having fructose units with β-glycosidic linkages and terminal sucrose residue at the reducing end of the oligosaccharides.

15.9 Production of fructooligosaccharides

Industrially FOS can be enzymatically produced for large-scale production by either synthesis or degradation. The two processes are enzymatic synthesis using fructosyl tranferases from sucrose, and enzymatic degradation of inulin by *endo*-inulinase through controlled hydrolysis. Endoinulinases randomly cleaves β-D-(2 → 1) linkages of inulin to generate FOS (Sangeetha et al., 2005c). From different microbial sources, endo-inulinases were purified for the yield of FOS (Yokota et al., 1995; Kim et al., 1997a,b; Yun et al., 2000; Cho et al., 2001; Zhengyu et al., 2005; Naidoo et al., 2009).

15.10 Optimization of fructooligosaccharide production

Plackett Burman design was employed for the analysis of important physiological and nutritional parameters affecting FOS production. Various parameters like nitrogen source, substrate, and fermentation time affects the enzyme production, whereas pH and time of the reaction affects the generation of FOS (Sangeetha et al., 2002). Further, response surface methodology (RSM) based on shell design was used to increase FOS yield. By using the Doehlert experimental shell design, synergistic combination of all the parameters needed for the high yield of FOS was analyzed. Statistical analysis of the parameters showed that high productivity of FOS can be achieved via optimal fermentation time of 90 h, reaction time of 18 h, pH 5, sucrose (10%), and KH_2PO_4 (0.9%) (Sangeetha et al., 2005c).

15.10.1 Submerged fermentation (SmF) for fructooligosaccharide production

In the present market, production of FOS by transfructosylation is via two stages. Initially enzyme production was by SmF followed by enzymatic bioconversion of inulin substrate to produce FOS (Singh and Singh, 2010). Concentration, cultivation time, agitation, rate of aeration, and carbon and nitrogen sources play an important role in deciding physiological and nutritional parameters for FOS production (Dominguez et al., 2013). Addition of mineral salts, amino acids in small amounts, polymers, and surfactants also plays a vital role in FOS production (Maiorano et al., 2008). Based on experimental results, yeast extract and sucrose at 25% (*w*/*v*) were evaluated as the best sources

for nitrogen and carbon, respectively (Chen and Liu, 1996). Other researchers reported that the highest FTase activity was at 35% (w/v) sucrose concentration, whereas biomass production remain unchanged from 5% (w/v) to 35% (w/v) sucrose concentration (Antošová et al., 2002). Chen and Liu (1996) inferred that inorganic salts and additives like magnesium sulphate and disodium hydrogen phosphate effected the fungal morphology from filamentous to pellet form without hindering or accelerating FTase production. Therefore controlling cell morphology is an important aspect from an industrial perspective. Subsequently, other research groups showed that varied concentrations of Mg^{2+} and K^{2+} directly affect the permeable properties of the cell wall; their optimal concentrations were 0.03% (w/v) (Sangeetha et al., 2005a,b) and 0.02% (w/v) (Balasubramaniem et al., 2001). It was reported that transfructosylation production of enzymes from fungi remained constant while maintaining nitrogen concentrations between 0.02% (w/v) (Lim et al., 2005) and 2.5% (w/v) (Dhake and Patil, 2007). A medium pH of 5.5 was reported optimal for FTase production and microbial growth in the case of *Aspergillus oryzae* CFR202 (Sangeetha et al., 2005a,b), *Aspergillus japonicus* JN19 (Wang and Zhou, 2006), and *Penicillium purpurogenum* (Dhake and Patil, 2007). The percentage of FOS productivity with respect to sucrose for different fungi was 24–70% for *Aspergillus niger* (Madlová et al., 1999; Nguyen et al., 1999), 44–62% for *Aspergillus pullulans* (Shin et al., 2004; Yoshikawa et al., 2008), 53–57% for *Aspergillus oryzae* (Sangeetha et al., 2005a,b), and 55.8–61% for *Aspergillus japonica* (Chiang et al., 1997; Wang and Zhou, 2006).

15.10.2 Solid state fermentation (SSF) for fructooligosaccharide production

SSF requires reduced size of the fermenter, less downstream processing, less stirring, cheap sterilization costs, and less water consumption (Sangeetha et al., 2005c; Mussatto and Teixeira, 2010). It has high volumetric production and increased concentration with less contamination, which makes it a perfect low-cost capital investment for production of FOS. The inherent drawbacks of less consumption of water for maintaining concentration, temperature, pH, and dissolved O_2 pose a major engineering hurdle (Pandey, 2003; Hölker et al., 2004). In SSF, the capital investment and operating costs are low due to extensive use of agricultural residues and byproducts when compared to SmF. SSF experiments conducted using *Aspergillus japonica* on agricultural residues wherein the nutrients were derived from the agricultural residues and thereafter fermented by SSF resulted in an increased yield in FOS production (Mussatto et al., 2012).

15.10.3 Production of fructooligosaccharide using recombinant enzymes

Yun et al. (1997) compared the production of FOS by batch process using both soluble and immobilized *Pseudomonas* sp. *endo*-inulinase. In a follow-up study, its gene was cloned in *Escherichia coli* and used for production of FOS in both batch and continuous systems (Yun et al., 1999). Studies on immobilization of *Pseudomonas* sp. endoinulinase on polystyrene were also carried out (Yun et al., 2000). Further, *Pseudomonas* endo-inulinase was used as whole cell in both continuous and batch systems for the production of FOS (Yun et al., 1999). In recombinant *Saccharomyces cerevisiae* strain, 90% increase in yield of FOS was accomplished by expressing endo-inulinase gene and suppressing the inherent *SUC2* invertase gene (Wang et al., 2016). Transfructosylation by means of ceramic membranes with various pore sizes and forced flow membrane reactor was investigated and resulted in high yield of FOS (560 times higher) than that produced in the batch process (Nishizawa et al., 2000).

15.10.4 High-content fructooligosaccharide production

FOS production was inhibited by coproduction of glucose (Yun, 1996), as a result of which maximum hypothetical yield based on initial substrate concentration was 50%–60% (Sangeetha et al., 2005c). Reports on high productivity of FOS using glucose oxidase showed that reduced glucose content for fermentation resulted in the formation of gluconic acid (Yun and Song, 1993; Sheu et al., 2001; Lin and Lee, 2008). Some approaches demonstrated for the isolation and purification of FOS from the product mixture (Nobre et al., 2015) included the use of a microfiltration module-equipped bioreactor that was designed to remove glucose from the medium (Sheu et al., 2002), nanofiltration membrane for selective permeation (Nishizawa et al., 2001; Kuhn et al., 2010), fixed-bed column chromatography packed with zeolites (Kuhn and Filho, 2010a), ion exchange resins (Shiomi et al., 1991; Gramblicka and Polakovic, 2007; Vankova and Polakovic, 2010; Nobre et al., 2014), activated charcoal (Hidaka et al., 1988; Kuhn and Filho, 2010b; Nobre et al., 2012; Kuhn et al., 2014), and as simulated moving bed chromatography (Nobre et al., 2016). Further, Crittenden and Playne (2002) used immobilized *Zymomonas mobilis* cells to exclude inhibitors from the mixtures of oligosaccharides. Alternatively, an increase in production of FOS was achieved by using microbial treatment during the enzymatic production of FOS; however, it implied an extra step of removal of common carbohydrates such as glucose, fructose, and galactose formed during the fermentation to ensure a final product with less contaminants.

Doing this incurred increased production costs. Recently, *Saccharomyces cerevisiae* (baker's yeast) treatment was adopted for removal of small saccharides in the product mixture and to favor efficient improvement in the purity of the FOS (Nobre et al., 2016; Jiang et al., 2019).

15.11 Fructooligosaccharides as prebiotics

The prerequisites for any food material to qualify as a prebiotic are as follows: (a) resistant to fermentation and absorption in GI tract, (b) colonic fermentation by resident bacteria, (c) promotes the growth of good microbiota in the gut, and (d) confers overall benefit to host health. FOS ferment in the colon and produce short-chain fatty acids like acetate, propionate, and butyrate, which promote the growth of good microbiota by eliminating harmful bacteria from the gut environment (Bornet et al., 2002; Scholz-Ahrens et al., 2007). Studies on animal and human models showed that FOS help in the absorption of minerals and reduction of colon tumors by stimulating high concentration pf effectors of the immune system and short-chain fatty acids. Thus consumption of FOS helps in reducing colorectal cancer, type II diabetes, and osteoporosis, maintains lipid metabolism, and stimulates the overall immune system (Dominguez et al., 2013).

15.12 Functional properties of fructooligosaccharides

FOS are low in calories as well as sweetness, which makes them suitable for diabetic patients. Prebiotics resist digestion by resident bacteria and help in the growth of the probiotics, which further prevent the pathogenic colonization of the human gut (Roberfroid, 2000b; Mishra and Mishra, 2013). As compared to short-chain FOS, the prebiotic effect of long-chain FOS is more distant in the colon (Manning and Gibson, 2004). FOS have various beneficial physiological properties that help in maintaining good health. We discuss these beneficial functions in the sections that follow.

15.12.1 Dietary fiber

Dietary fiber consists of non-digestive fiber, which is resistant to digestion by the human alimentary enzymes and helps in the various physiological functions of the body. Likewise, FOS are reserve fructans present in many fruits and vegetables, which makes them non-digestible in the small intestine. Fecal as well as colonic contents of rats fed on FOS showed an increased amount of lactate. Furthermore, the chances of gastrointestinal diseases is also reduced by strengthening mucosal protection and intestinal epithelial cells due to the fermentation properties of FOS, which makes them amenable as dietary fiber (Cherbut, 2002).

15.12.2 Immunomodulatory property

Functional FOS supplements are reported to improve immunity. FOS byproducts produced by fermentation could regulate gut-associated lymph tissue (GALT) and overall immune system as they work in close proximity (Seifert and Watzl, 2007). GALT consists of 60% lymphocytes in the human body and is the largest tissue in the immune system (Delgado et al., 2011; Saad et al., 2013). Dietary carbohydrates with FOS additives favor growth of commensal bacteria in the colon, which results in lower colonic pH, better barrier properties, and improved short-chain fatty acids, mucus, and production of cytokines (Korzenik and Podolsky, 2006). Medical trials have reflected decreased clinical inflammations and reduced cytokine levels (Furrie et al., 2005). The diet with FOS additives in mice showed an increase in yield of β-lymphocytes. On the contrary, T-lymphocytes remained unchanged after following the same diet with FOS additives (Manhart et al., 2003). A significant increase in CD4 + cells was observed with respect to CD8 + cells thus increasing CD4:CD8 ratio. Numerous other studies have also highlighted the immunomodulation benefits of FOS (Guigoz et al., 2002; Swanson et al., 2002; Hosono et al., 2003; Bunout et al., 2004; Roller et al., 2004; Morrison et al., 2006; Osman et al., 2006; Delgado et al., 2011). The benefits of oral administration of prebiotics have widely been recognized by researchers across the globe. Inulin and FOS showed promising immunomodulatory activity in experimental studies on mice (Hosono et al., 2003).

15.12.3 Diabetes and obesity

Due to changing lifestyles and urbanization, there has been consistent increase in cardiovascular diseases, kidney failures, blindness, and premature death due to obesity-related type II diabetes. In this regard, FOS can be used as

alternative sweeteners due to their low caloric value. A small dosage of FOS on a daily basis can decrease hepatic glucose production without altering insulin-triggered glucose metabolism (Luo et al., 2000). Evaluation of high dosage ingestion of FOS on plasma lipid, basal hepatic glucose production, and resistivity to insulin showed no change in fasting plasma blood glucose, serum triacylglycerol production, and insulin resistance. It was also observed that there was no significant change in HDL cholesterol, free fatty acids, and concentrations of apolipoproteins A1 and B. The use of FOS in the form of various dietary supplements was favorable for their use as an alternativesweeteners without hindering insulin resistance and basal hepatic glucose production in the body. As an example, rats that were given additives of 5% and 10% FOS in their diet did not show any increase in hyperglycemia and glucosuria, even in the case of diabetic animals (Mabel et al., 2008).

15.12.4 Absorption of minerals

Developing countries are struggling in mitigating basic health issues, which include inefficacy to absorb minerals in body. Absorption of various chemicals like Mg^{2+}, Fe^{2+}, and Ca^{2+} in the colon could be increased by decreasing colon pH and fermentation of FOS. Calcium and magnesium are very important for bone health, and loss of calcium can cause osteoporosis. Low intake of iron causes anemia, which is a big health issue facing women of developing countries. The requirement of zinc for growth and development is also well documented. Resident microbiota ferments FOS and releases short-chain fatty acids, which decreases luminal pH and thus creates an acidic environment. Ohta et al. (1998) carried out a gastrectomized experiment in which FOS were included in the diet, which led to an increase in absorption of iron thereby helping in recovery from anemia. Benefits of FOS on Ca^{2+} and Mg^{2+} absorption were documented in detail by various research groups (Morohashi et al., 1998; Ohta et al., 1998; Takahara et al., 2000). An Mg^{2+}-deficient diet in rats led to hyperemia and hemorrhage among them (Ohta et al., 1995). Experiments were carried out to demonstrate the protein-digesting properties of FOS in rats fed with 0.5% FOS additive in total diet. Analysis showed that body weight gain and body excretion significantly decreased as compared to other rats on a normal diet. FOS intake has contributed by fermenting anerobically in the colon by resident bacteria to produce short-chain fatty acids thus showing desirable effect on mineral's bioavailability.

15.12.5 Lipid and cholesterol mechanism

In hypercholesterolemia, FOS help by decreasing the levels of triglycerol and low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) cholesterol by producing short-chain fatty acids by fermenting itself in colon (Mabel et al., 2008). In many animal models, hepatic metabolism is modified by systemic effect of FOS (Delzenne et al., 2002). FOS reduce the secretion of LDL causing hepatic lipogenesis resulted from coordinated reduction of activity and mRNA of lipolytic enzyme. Postprandial insulinemia is reduced by 26% when male Wistar rats were fed 10% FOS additive in their diet for 30 days. Meanwhile, after overnight fasting, a glucose tolerance test showed no change in glycemic response as compared to rats with a controlled diet (Daubiol et al., 2000). In animal studies, it was proved that secretion of TAG-rich VLDL was inhibited by FOS (Parks, 2002). Furthermore, there was no modification in concentration of free fatty acids in serum, but there was a decrease in phospholipid and triglyceride concentration of serum in animals fed 10% FOS additives in their diet. In comparison to healthy subjects, in non-insulin-dependent diabetic patients, total serum and LDL cholesterol were lower as a result of FOS (Roberfroid, 2000a).

15.12.6 Anti-cancerous effect

The second most prevalent cancer is colorectal cancer. Studies revealed that FOS aid in prevention of colon cancer by reducing chemically induced aberrant crypts and fermenting itself into short-chain fatty acids by resident bacteria. Short-chain fatty acids like butyrate play an important role in resisting tumor growth, cell division, and regulation of apoptosis (Kim and Byun, 1982). According to Bugaut and Bentéjac (1993), epithelial cells of colon mucosa use butyrate as an energy source, and based on pre-clinical studies in carcinogenesis, it also functions as a chemopreventer (Scheppach and Weiler, 2004). Likewise, the anti-inflammatory functions of propionate on colon cancer cells is documented (Munjal et al., 2009). In experiments, F344 rats were fed an oligofructose diet and showed decline in azoxymethane (AOM)-induced colon cancer (Femia et al., 2002). Furthermore, immunomodulation was observed in Peyer's patches, which were reported for anti-cancerous effect of FOS (Roller et al., 2004). Pool-Zobel et al. (2002) reported that anticancer effects are due to prebiotic effects in rats, responsible for the growth of probiotic microflora, which resulted in the formation of metabolites like acetate and propionate. In one experiment, a

group of animals were fed on inulin/oligofructose (15%), which resulted in (1) a decrease in mammary tumous by methylnitrosourea in Sprague Daley rats, (2) inhibition of malignant tumor (which could be transplanted) growth in mice, and (3) decreased risk of lung metastases in intramuscularly implanted malignant tumors in mice (Taper and Roberfroid, 2002).

15.12.7 Enhancing efficacy of *Salmonella* vaccine

Protection against *Salmonella* infections and the effect of a mix of FOS with inulin on murine response to *Salmonella* vaccine was reported by Benyacoub et al. (2008). Experiments were carried out on Balb/C mice that were fed an inulin mix diet containing 5% FOS and who were administered a minimal dose of *Salmonella typhimurium* vaccine. After adequate time intervals, the value of *Salmonella* immunoglobulin G from blood and immunoglobulin A from feces increased in the mice whose diet consisted of FOS and inulin in contrast to the results observed in control mice. After one week of immunization, it was observed that the mice fed with FOS and inulin showed a significant increase in phagocytic activity in peritoneal macrophages as compared to control mice. The survival rate of mice fed with FOS showed a considerable increase when challenged with virulent *Salmonella* and was naturally accompanied by improved response to the *Salmonella* vaccine. This infers that diet consisting of FOS and inulin improved the response to an oral vaccine and triggered mucosal immunity.

15.13 Applications in food industry

15.13.1 Fortification of fruit juices

Partial substitution of sucrose with FOS can be done during fortification of fruit juices like pineapple, mango, and orange without comprising overall quality and thereby reducing the calorific value of these beverages. Based on studies carried out by the partial substitution of sucrose, it was found that the color, sensory properties, and total dissolved solids (TDS) were unchanged during the storage process resulting in acceptance of FOS-fortified juices (Renuka et al., 2009).

15.13.2 Confectionary industry

FOS is highly hygroscopic, viscous, and low in calories as compared to sucrose, which makes it suitable to use as an alternate sweetening agent for various confectionaries items like jam products. Organoleptic characteristics of FOS have made them amenable for use in replacing fat and more sugary components enabling its use in the ice cream industry. The use of FOS in all confectionary items like hard candies, gums, and marshmallows reduced their calorific value without compromising their quality and taste (Murphy, 2001).

15.14 Conclusions

FOS are an important emerging component of the functional food market in response to growing demands for healthy lifestyles. As such, significant research on production of FOS by microbial inulinase has been undertaken. Inulinases from various microbial sources were explored, and fungal inulinases were found to have more advantages than bacterial inulinases due to their high productivity. Industrial production of FOS has increased dramatically by the development of methods like membrane reactor systems, immobilized cells, enzymes, and bioreactors based on microfiltration. The purity of FOS could be improved considerably by removing contaminating mono and disaccharides. In order to gain better understanding of the inulinase enzyme's properties and kinetics, comprehensive studies towards its purification and characterization need to be done.

Recent advances in recombinant technology have led to the cloning and expression of inulinase genes from various fungal sources further aiding the commercial production of FOS. The developed recombinant yeast strains containing inulinase genes have shown a great potential for application in generation of bioethanol from inulin. FOS have shown great therapeutic efficacy towards ailments like diabetes, colorectal cancer, obesity, mineral absorption, and immunomodulation. FOS have also found applications in food, including dairy products, chocolates, and bakery products. With given health benefits and no side effects observed, FOS have global potential to be used in diverse arenas of the healthcare industry.

Acknowledgments

The authors acknowledge the financial support in the form of Inspire fellowship (DST), New Delhi, India, provided to Indrani Paul. IICT manuscript communication number: IICT/Pubs./2019/013.

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