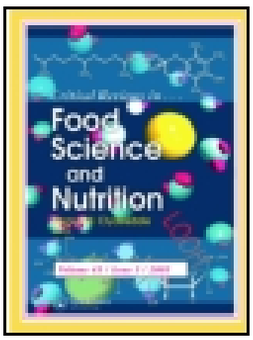

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Title page

Biotechnological potential of microbial inulinases: Recent perspective

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Abstract

Among microbial enzymes, inulinases or fructofuranosylhydrolases have received considerable attention in past decade and as a result a variety of applications based on enzymatic hydrolysis of inulin have been documented. Inulinases are employed for generation of fructose and inulooligosaccharides (IOS) in a single step reaction with specificity. The high fructose syrup can be biotransformed into value added products such as ethanol, single cell protein while IOS are indicated in nutraceutical industry as prebiotic. Myriad microorganisms produce inulinases and a number of exo and endoinulinases have been characterized and expressed in heterologous hosts. Initially predominated by Aspergilli, Penicillia and some yeasts (*Kluyveromyces* spp.), the

list of prominent inulinase producers has gradually expanded and now includes extremophilic prokaryotes and marine derived microorganisms producing robust inulinases. The present article summarizes important developments about microbial inulinases and their applications made in the last decade.

Keywords Inulin, exoinulinase, endoinulinase, fructose, fructooligosaccharide, immobilization

1. Introduction

Microbial inulinases are an important class of industrial enzymes, which are usually inducible and extracellular. Microbial inulinases belong to the members of glycosylhydrolases (GH 32 family). Inulinases hydrolyzing β-(2→1) fructosyl linkage are produced by many microorganisms (bacteria, fungi and yeast). Exoinulinase (EC 3.8.1.80; β-D-fructan fructohydrolase or β-D-fructosidase) liberates the terminal fructose unit of inulin linked by hydrolyzing β-fructofuranosidic bonds. High fructose syrup (upto 95%) generated by inulinase action can be utilized as a fermentable substrate. This process is preferable over starch or cellulose degradation as these require enzyme consortia for generation of feedstock glucose. The single step process involving one enzyme encoded in a single gene is therefore much attractive over use of enzyme consortia and has better prospects in terms of cloning and heterologous expression (Chi et al., 2011; Kango and Jain, 2011; Liu et al., 2013). Endoinulinase (EC 3.2.1.7; β-D-fructan fructanohydrolase) hydrolyzes the internal bonds of inulin and liberates inulooligosaccharides (IOS) including inulotriose, inulotetraose and inulopentaose as major products. Fructose is a low-calorie healthy sweetener and a readily fermentable feedstock sugar. IOS have similar structure and functions of fructooligosaccharides (FOS) which have been well known as functional food and nutraceutical (Ganaie et al., 2014; Tsurumaki et al., 2015; Singh et al., 2016).

Inulin, the second most abundant storage polysaccharide after starch, can be exploited as a substrate for the production of ultra-high fructose syrup (UHFS), IOS, bioethanol, single cell oil (SCO), citric acid, lipids, single cell protein (SCP), 2, 3-butanediol, lactic acid and other

important chemicals (Chi et al., 2009; Zhang et al., 2010a; Cui et al., 2011; Kuhn et al., 2012; Wang et al., 2012 and 2014; Rawat et al., 2015b). Most of the industrial processes using inulin as raw substrate employ microbial inulinases preceding its biotransformation. Among fungi, members of *Aspergillus*, *Penicillium* and *Kluyveromyces* spp. are reported as prolific inulinase producers (Liu et al., 2013; Rawat et al., 2015a). Some bacteria such as *Xanthomonas* and *Arthrobacter* are also known sources of microbial inulinases (Chen et al., 2009; Vijayaraghavan et al., 2009; Zhou et al., 2014a).

With recent explorations, the realm of microbial inulinase producers has expanded to extremophilic prokaryotes and marine derived yeasts (Chi et al., 2009). Heat, salt and cold active inulinases sourced from extremophilic microorganisms can be appropriately utilized in developing industrial bioprocesses. The present review covers the more recent developments on microbial inulinases in the last ten years. It discusses their properties, improved production *via.* parametric optimization, heterologous expression and biotechnological applications.

2. Microbial sources of inulinase

Inulin hydrolyzing enzymes are reported to occur in diverse and phylogentically different microorganisms including bacteria, filamentous fungi, yeasts and actinomycetes (Kango and Jain, 2011). More recently, the focus has been to identify novel inulinases exhibiting tolerance of extreme conditions to suit industrial applications. Halotolerant and cold active exoinulinase from *Arthrobacter* sp. MN8 was active at low temperatures (0 to 20°C) (Zhou et al., 2014a). In another study, *Arthrobacter* sp. S37 endoinulinase active over cold and acidic pH has been cloned in *Yarrowia lipolytica* (Li et al., 2012). Similarly, a novel exoinulinase tolerant to detergent, salt and protease has been reported from *Sphingomonas* sp. JB 13 isolated from

phosphate rock site (Zhou et al., 2015). *Lactobacillus paracasei* B41 isolated from Bulgarian traditional beverage 'boza' producing inulinase (232 U/ml) has been demonstrated to hydrolyze chicory flour inulin and convert it into lactic acid (Petrova et al., 2015). *Lactobacillus casei* produced distinct zone of clearing on inulin agar plate and inulinase gene (lev H1) has been deciphered (Kuzuwa et al., 2012). Thermophilic soil isolate, *Bacillus smithii* T7 produced 135.2 U/ml thermostable endoinulinase releasing fructose and oligosaccharides with optimum activity at 70°C (Gao et al., 2007). Exclusive endoinulinase production has been reported from *Bacillus safensis* AS-08. Some inulinase producing bacteria *Xanthomonas oryzae, Pseudomonas* sp., *Bacillus licheniformis* and some actinomycetes *Nocardiopsis* sp. DN-K15, *Streptomyces* sp. etc. are recent additions (Naidoo et al., 2009; Lu et al., 2013; Singh and Singh, 2014; Li et al., 2014).

Among filamentous fungi, members of *Aspergillus* and *Penicillium* are reported as prolific producers of inulin hydrolyzing enzymes (Kango and Jain, 2011). Several strains of *A. niger*, a prominent producer, sourced from a variety of ecological niches have been demonstrated to produce high titers of inulinase when subjected to growth on media containing inulin-rich vegetal extracts (Housseiny, 2014; Rawat et al., 2015a). Screening of 200 fungal strains isolated from Jerusalem artichoke fields resulted in identification of *Penicillium subrubescens* FBCC 1632 that efficiently produced inulinase (Mansouri et al., 2013). Such explorations have lead to the availability of a number of inulinase genes and present opportunity of developing industrial strains (Chi et al., 2011; Chen et al., 2013; Flores-Gallegos et al., 2015).

Recently, inulinases from *Aspergillus niger* F0602, *Aspergillus ficuum* JNSP5-06 and *Penicillium janthinellum* have been expressed in yeast, *Pichia pastoris* (Chen et al., 2013; He et al., 2014; Chen et al., 2015). A fungal strain, *Aspergillus tubingensis* CR16 isolated from chicory rhizospheric soil produced 257 U/gds yield of inulinase in SSF (Trivedi et al., 2012). *Penicillium* sp. NFCC 2768 has been isolated from dahlia rhizospheric soil and explored for inulinase (3.83 U/ml) production (Rawat et al., 2015b). *A. niger* AUMC 9375 originating from soil samples of Cairo city Egypt, has been reported to ba a prolific inulinase (440 U/ml) producer (Housseiny, 2014). Inulinase production is reported from *Aspergillus terreus, Aspergillus versicolor*, *Aspergillus parasiticus* and *Penicillium brevicompactum* originating from of marine-derived samples collected from Ismalia, Egypt (Abd El Aty et al., 2014). Some other fungi *Thermomyces lanuginosus*, *Fusarium oxysporum*, *Aspergillus awamori*, *Aspergillus fumigatus* are also reported as inulinase producers (Nguyen et al., 2013; Chen et al., 2015; Rawat et al., 2015a).

Diploid yeast, *Kluyveromyces marxianus,* is a known producer of exoinulinase while some of the marine derived yeasts such as *Meyerozyma guilliermondii*, *Cryptococcus aureus*, *Zygosaccharomyces cerevisiae* are recent additions (Kango and Jain, 2011; Chi et al., 2011; Liu et al., 2014). Among these, *Pichia guillermondii* sourced from sea water from China, produced 60 U/ml exoinulinase. Increase in NaCl concentration (up-to 2%) and addition of sea water to the culture medium enhanced inulinase production by two-folds (Gong et al., 2007). Novel yeast strains *Zygosaccharomyces*, *Gordonia*, *Hanseniaspora*, *Torulosopra*, *Saccharomyces*, *Metschnikowia*, *Lachancea* were screened for inulinase production, and *Zygosaccharomyces bailli* (Talf1) was identified as prominent inulinase

(8.67 U/ml) producer when Jerusalem artichoke juice was used as substrate (Paixao et al., 2013).

3. Production and optimization of inulinases

Cost-effectiveness of an enzymatic bioprocess is a prime requisite for realizing its industrial application. Use of low-cost inulin-rich plant extracts, strategic statistical optimization, over-expression of inulinase and high cell density fermentations are being explored in order to achieve this objective. *B. safensis* AS-08 produced 28 U/ml of endoinulinase in medium containing raw dahlia tuber extract for extracellular inulinase production (Singh and Singh, 2014). *L. paracasei* DSM 23505, a prolific exoinulinase producer strain has been used for saccharification of chicory flour (136 g/L) and its biotransformation into lactic acid (123.7 g/L) (Petrova et al., 2015). *Nocadiopsis* sp. DN-K15, novel alkali-tolerant and thermostable actinomycete strain produced 25 U/ml of inulinase using dahlia tuber extract (Lu et al., 2013).

Several reports suggest use of low-value particulate inulin-rich substrates in solid state fermentation (SSF) for inulinase production by filamentous fungi (**Table 1**). *A. niger* AUMC 9335 produced 300 U/gds of endoinulinase on sunflower tubers and lettuce roots mixed in 6:1 (w/w) ratio (Housseiny, 2014). Trivedi *et al*. (2012) have used wheat bran and corn steep liquor for production of inulinase (257 U/gds) by newly isolated *A. tubingensis* CR16 in SSF. Inulinase extraction from fermented bran has also been optimized leading to enhanced yield upto 193 U/gds (Chen et al., 2011).

Several inulin-rich substrates have been used for the production of microbial inulinases *viz*. dandelion, dahlia, asparagus and Jerusalem artichoke (Mansouri et al., 2013; Paixao et al.,

2013; Rawat et al., 2015a). Kango (2008) used dandelion tap root extract and yeast extract used for inulinase production from *A. niger* NK-126 and obtained 55 U/ml in 96 h. *Penicillium citrinum* isolated from Mexican desert produced 18.7 U/L inulinase at 30°C after 48 h (Adriana et al., 2012).

K. marxianus NRRLY-7571 produced 586 U/gds on sugarcane bagasse in fed-batch mode (Astolfi et al., 2011). *K. marxianus* has been demonstrated to produce inulinase on dahlia tuber extract (1.51 U/ml) and asparagus root extract (1.49 U/ml) (Jain et al., 2012; Rawat et al., 2015a). Induced mutant, *Geotrichum candidum* MEU-5fc-6 showed 50-fold enhancement in exoinulinase titers (71.8 U/ml) when grown on sucrose (5 g/L) in 48 h in a fermentor (Mughal et al., 2009). Sheng et al. (2007) isolated marine yeast *Cryptococcus aureus* G7a producing 85 U/ml exoinulinase in shake flask after 42 h fermentation.

Optimization of medium components is very important in the development any bioprocess and helps in understanding interaction of parameters and identifying optimal range for higher yield. Several workers have reported effective optimization of inulinase production using fungal, bacterial and yeast strains and a variety of substrates such as Jerusalem artichoke, sugarcane baggasse, molasses in submerged or solid state cultivation. A comprehensive list of microorganisms and optimized experimental variables is presented in **Table 2**. Novel inulinases from *M. guilliermondii* and *Kluyveromyces cicerisporus* were expressed in *Saccharomyces cerevisiae* and *P. pastoris* X33 and produced 43.8 and 45.2 U/ml, respectively (Liu et al., 2014; Ma et al., 2015). High titers of inulinase (167.4 U/ml) were produced by alginate immobilized *P. guilliermondii* M-30 on Jerusalem artichoke tuber extract in 48 h (Zhao et al., 2011).

4. Heterologous expression and molecular regulation of inulinase

Heterologous expression in yeast and bacterial systems for efficient production of exo and endoinulinase sourced from bacterial and fungal strains has been a matter of intense research. Yeasts play important host for heterologous expression of inulinase and can grow to very high cell densities with established easy recovery methods (Chi et al., 2011; Liu et al., 2013; Zhou et al., 2014b). Characteristics of inulinase genes expressed in heterologous hosts are presented in **Table 3**. Most commonly used yeasts for heterologous expression of inulinase are *P. pastoris, Y. lipolytica, S. cerevisiae, Kluyveromyces lactis* (Zhang et al., 2010b; Yuan et al., 2013; Liu et al., 2014). Simultaneous inulin hydrolysis and fructose bioconversion to ethanol by engineered yeast presents an opportunity of consolidated bioprocessing (CBP) of inulin (Yuan et al., 2012). Endoinulinase (*inuA*) gene of *A. niger* was cloned and expressed in *S. cerevisiae* JZIC-J producing 3.1 U/ml inulinase and 55.3 g/L ethanol (Yuan et al., 2013). Chen et al. (2013) have expressed exoinulinase gene (exon I and exon II) of *A. ficuum* JNSP5-06 in *Escherichia coli*. The molecular weight of recombinant inulinase was 63 kDa with optimum temperature and pH 55°C and 5.0, respectively. Over expression of *P. guilliermondii Inu1* (pPlcZaA) gene in *P. pastoris* X-33 resulted in high level of inulinase titers (286.8 U/ml) in 120 h (Zhang et al., 2009a). Exoinulinase gene from *P. janthinellum* B01 was cloned in *P. pastoris* and high level of expression 272.8 U/ml was achieved which was 11 fold higher. The deduced amino acid sequence indicated highly conserved N-terminal region GH32 and 82% identity with *Talaromyces stipitatus* exoinulinase but was divergent from other microbial exoinulinases (Wang et al., 2011). Thermostable exoinulinase (T_{opt} 55°C) from *K. cicerisporus*

was expressesd in *P. pastoris* X33. Very high degree of sequence identity was noticed with *K. marxianus* and *C. aureus* exoinulinases (92-99%).

Studies on regulation of inulinase synthesis have been mostly limited to fungal and yeasts expression systems. Several such reports have established that synthesis of inulinase is subject to repression by glucose and fructose present in the medium. The transcriptional repressor Mig1 coded by *MIG1* gene, a C_2H_2 zinc finger protein, binds to the promoter of genes repressed by glucose (Zhou et al., 2014c). Exoinulinases have conserved motifs WMNDPNGL, RDP, ECP, SVEVF, FS and Q while those in endoinulinases are reported to be WMNEPNGL, RDP, EVP, SVEVF, FT and Q (Liu et al., 2013; Cao et al., 2013). The alignment studies with other exoinulinases showed WMNDPNG, RDP and ECP as three highly conserved motifs (Ma et al., 2015). The expression of *A. niger* 12 *inuE* gene is repressed by fructose and glucose (Yuan et al., 2006). Deletion of *MIG1* gene responsible for glucose repression resulted in enhanced exoinulinase production from *Saccharomycopsis fibuliegra* (Liu et al., 2011). *K. marxianus* strain with deleted *MIG1* gene produced 5.7 U/mg inulinase which was two-fold higher as compared to wild type (Liu et al., 2013). Mig1 binding site, however, is reported to be absent in *A. niger* endoinulinase gene. Thus the gene expression is independent from carbon sources (Liu et al., 2013). In another filamentous fungus *Penicillium* sp. TN 88 the expression of *inuC* and *inuD* was enhanced by 42 and 3260-fold when grown on inulin containing media, while sucrose and fructose showed no induction and glucose showed strong catabolite repression (Moriyma et al., 2006). Inulinolytic genes of *A. niger* have been shown to be co-regulated by inulin and sucrose through a positively acting transcription factor InuR. This factor is a member of fungal specific transcription factor of Zn(II)2Cys6. Expression of

inulinolytic genes (*inuE*, *inuA*) and sucrolytic gene (*sucA*) was found to be dependent on InuR (Yuan et al., 2008). Recently, *K. marxianus* derepressed mutant (KM-69) with disrupted *MIG1* gene produced 101.7 U/ml inulinase, while the native strain KM-0 was sensitive to glucose repression (Zhou et al., 2014c).

5. Extremophilic inulinases

Although inulinase production from mesophilic counterparts has been reported by many workers, studies on extremophilic inulinase producing microorganisms are relatively less. However, several reports on cold-active and thermophilic inulinases indicate the recent spurt of interest in this area (**Table 4**). Zhou et al. (2014a) reported cold-adaptive *Arthrobacter* sp. MN8 bacterium isolated from lead-zinc rich soil and exploited for its ability for exoinulinase production in generation of fructose syrup at low temperature. Thermostable *Bacillus smithii* T7 strain has been reported to produce 135.2 U/ml thermostable endoinulinase in media containing inulin. The strain was stable over pH range of 4.0 to 8.0 and half life of inulinase 9 h at 70°C (Gao et al., 2009). A novel detergent, salt and protease tolerant exoinulinase from *Sphingomonas* sp. JB13 (rInuAJB13) was optimally active at 55°C and pH 5.0 and stable at 70°C (Zhou et al., 2015).

6. Biotechnological potential of inulinases

Inulin is one of the abundant storage polysaccharides that await exploitation. It can be used as a source of fructose and fructooligosacchrides which can further be utilized as feedstock for production of ethanol, gluconic acid, sorbitol and other important products (Chi et al., 2011; Kango and Jain, 2011).

6.1 Production of fructose and fructooligosaccharides

Fructose is a generally recognized as safe (GRAS) sweetener, sweeter than sucrose (up to 1.5 times), with lower calories and functional properties that enhance flavor, color and product stability. Furthermore, fructose metabolism bypasses the known metabolic pathway of glucose and therefore does not require insulin. High fructose syrup (HFS) produced by the action of inulinase can be beneficially used for diabetic patients. Thus it is widely used in many food, pharmaceutical and beverage preparations instead of sucrose (Ettalibi and Baratti, 2008; Barclay et al., 2010).

Fructose can be produced by one-step enzymatic hydrolysis of inulin. Exoinulinases from different microorganisms are used for production of ultra high fructose syrup (UHFS) from inulin and inulin-containing materials (Mansouri et al., 2013). Yeast inulinases are generally reported to be exo-acting enzymes and only fructose is released from inulin by their action. Such exoinulinases from marine yeasts *C. aureus* G7a (Zhang et al., 2009a) and *P. guilliermondii* strain (Zhang et al., 2009b) have been purified and applied for fructose generation. *A. niger* inulinase produced 37.5 g/L fructose from 100 g/L inulin in 20 h while the *Candida guilliermondii* TISTR 5844 inulinase produced 35.3 g/L fructose in 25 h under similar conditions (Sirisansaneeyakul et al., 2007). *Asparagus racemosus* raw inulin extract and pure inulin have been used to obtain fructose yields of 39.2 and 40.2 g/L, respectively in 4 h, using *K. marxianus* YS-1 exoinulinase (Singh et al., 2006). Exoinulinase from *A. ficuum* produced 98 mg/L fructose from inulin (g/L) at 50°C (Mutanda et al., 2009). Optimized kinetic parameters (K_m = 24.2 g/L/h and V_{max} = 0.108 g/L min) were proposed for complete inulin hydrolysis and fructose production from chicory using inulinase at 40°C, isolated from *A. ficuum* (Ricca et al., 2009). 40°C

A. niger inulinase was immobilized on Concanavalin-A attached cryogel (poly ethylene glycol dimethacrylate) for the production of high fructose syrup and the conversion efficiency with pure inulin was found to be 0.23 mg/mL after 5 min hydrolysis (Altunbas et al., 2013). Some other inulinase immobilization and fructose generation studies are described in **Table 5**. Zhao et al. (2011) reported direct conversion of inulin and Jerusalem artichoke extract into single cell oil (SCO) mediated by calcium alginate immobilized inulinase of *Rhodotorula mucilaginosa* TJY15a. The bioprocess yielded 56.6% (w/w) oil from inulin rich tuber extract with SCO density reaching 12.8 g/L in 48 h in a 2 *l* fermenter.

The immobilized endoinulinase of *A. niger* on chitosan was used for continuous production of oligofructose syrup generation from Jerusalem artichoke juice. End product analysis suggested that higher DP (DP8 and more) oligosaccharides were hydrolyzed into syrup containing DP3-DP7 (Nguyen et al., 2011). Inulin was hydrolyzed by the endoinulinases to produce IOS such as inulotriose and inulotetraose and other inulooligosaccharides (Ronkart et al., 2007; Mutanda et al., 2014). Fructo-oligosaccharides produced enzymatically by the action of two different enzymes *viz*. (i) transfructosylation of sucrose by fructosyl transferase (FTase) and (ii) hydrolysis of inulin by endoinulinase where it randomly cleaves β-2,1 linkages of inulin to yield inulo-oligosaccharides (Kim et al., 2008; Silva et al., 2013; Ganaie et al., 2014; Bali et al., 2015). Oligofructose have numerous applications in food industries like confectionary, milk desserts, chocolate, ice cream and sauces (Kuntz et al., 2013). The use of IOS in animal nutrition has also attracted considerable recent interests, primarily because they significantly change colonic bacterial populations and fermentation end-products (Kelly, 2009). Endoinulinase has been characterized from several species of filamentous fungi and bacteria (Kango and Jain, 2011; Li et

al., 2012). Inulobiose (F2), inulotriose (F3), inulotetraose (F4) and IOS were obtained by the action of *A. niger* NK-126 fungal inulinase (Kango, 2008). Some other *A. niger* strains are also reported to produce endoinulinases (Kango and Jain, 2011). Rawat et al. (2015a) screened 25 fungal strains, in which *A. fumigatus*, *P. citrinum* and *Penicillium rugulosum* produced mixture of endo and exo-inulinase. Combined action of endo and exoinulinase from *Penicillium* sp. NFCC 2768 and produced a mixture of oligosaccharides, GF2, GF3, and GF4 along with glucose and fructose. An overview of biotechnological applications of microbial inulinases for achieving various objectives are given in **Table 6**.

6.2 Production of ethanol and single cell oil (SCO)

Ethanol is one of the most employed biofuel either as a fuel or as a gasoline enhancer. Selective hydrolysis of inulin using exoinulinase produces fructose that can be selectively converted into ethanol (Chi et al., 2011). Mostly *S. cerevisiae* and *K. marxianus* are used for the production of bio-ethanol from feedstock sugars (Abe et al., 2009; Yuan et al., 2012). Different raw materials used for bioethanol production include sugarcane, starch and lignocelluloses. However, these materials require different pre-treatments and sometimes suffer several disadvantages such as competition for food-grade starch and sucrose, interfering lignin, availability of lignocelluloses etc. (Schmitt et al., 2012). Inulin, produced by large scale cultivation of Jerusalem artichoke or chicory can be utilized for generation of fructose feedstock. Fructose, a readily fermentable substrate can be utilized and converted into ethanol (Chi et al., 2009; Hu et al., 2012).

Kluyveromyces sp., although reported to be a prolific producer of inulinase, cannot tolerate high concentrations of ethanol during production compared to *S. cerevisiae* (Lane and Morrissey,

2010). *K. marxianus* exoinulinase gene was expressed in *S. cerevisiae* and the recombinant yeast was grown on Jerusalem artichoke tubers extract. The conversion efficiency of the inulin type sugar to ethanol was 70% (Lim et al., 2010; Yuan et al., 2013). 106.5% mg/ml of ethanol was produced from 300.0 g/L inulin within 120 h by using endoinulinase obtained from *Arthrobacter* sp. (Li et al., 2013). Liu et al. (2014) reported bioethanol production (126.3 mg/mL) from inulin (300 g/L) by using *M. guilliermondii* exoinulinase expressed in *Saccharomyces* sp. W0 (**Table 6**).

Fresh Jerusalem artichoke contains nearly 20% (w/w) carbohydrate of which 70-90% (w/w) is inulin. About 50-60 g/kg of fresh weight of Jerusalem artichoke is inulin type fructan and the crop yield is estimated to 5.4 ton/ha (Li and Chan-Halbrendt, 2009). Immobilized cells of inulinase producers *Pichia guillirmondii* M-30 were co-cultured with oleaginous yeast *R. muciligonasa* on Jerusalem artichoke tuber extracts. The oleaginous yeast utilized sugar liberated from Jerusalem artichoke tubers and 48.59% (w/w) oil was accumulated (Zhao et al., 2010b; Wu et al., 2011; Wang et al., 2014). *R. mucilaginosa* TJY15a yeast culture was grown on Jerusalem artichoke juice for single cell oil production and accumulated maximum 55.4% oil (Zhao et al., 2011). Wang et al. (2014) used *R. toruloides* 2F5 yeast strain for direct conversion of inulin into lipid (62.14% w/w) at flask level while 70.36% (w/w) lipid production was reported in optimized conditions in a 2 *l* fermenter. Lipid content obtained after fermentation was also used for generation of biodiesel (Zhao et al., 2010c).

7. Conclusion and future perspectives

Owing to prospects in food and nutraceutical industries, inulin hydrolysis and bioconversion using microbial enzymes has received attention by several workers. Newer raw

materials with high inulin content have been demonstrated to be useful in generation of bioethanol and single cell oil. Heterologous expression of novel inulinases has paved way for consolidated bioprocessing of inulin by ethanol tolerant yeast expressing high titers of exoinulinase. Robust inulinases sourced from extremophiles will allow newer applications particularly at low temperature or in high salt foods. Although a number of exoinulinases have been characterized from yeasts and overexpressed in suitable hosts, fewer endoinulinases and their lesser understood catalytic mechanism presents a greater challenge. Availability of inulin as raw-material from regular and horticultural crops and developing a self-sustainable bioprocess either for generation of high fructose syrup or oligosaccharides is a prime concern that needs to be worked out.

Critical analysis of current literature shows that microbial inulinase is one of the most promising group of enzymes to be employed in food industries. This review showed that many researchers worldwide direct their activities to the screening of new inulinase-producing microorganisms and, subsequently, on the optimization of the medium composition and operational variables. All these efforts are justified by the relevance of inulinase applications.

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Table 1 Production of inulinase by microorganisms

SN	Microorganisms	Carbon source[a]	Nitrogen source[b]	Temp (ºC)	pH	RPM	Time (h)	Activity (U/ml)	Reference
1	*Aspergillus niger* GNCC 2655 *A. niger* GNCC	DTE DRE ARE	YE	28	-	150	72	12.2 4.07 8.21	Rawat et al., 2015a
2	*Penicillium* sp.	DTE	YE	30	5.0	150	120	3.87	Rawat et
3	*A. niger* AUMC	ST, LR	YE	30	5.0	-	168	290-	Housseiny,
4	*Penicillium subrubescense*	JA	-	28	6.0	180	120	7.7	Mansouri et al., 2013
5	*Aspergillus*	Yacon	-	30	-	250	68	80	Chesini et
6	*A. niger* ATCC	Suc	ME	30	6.5	150	96	319	Dinarvand
7	*Kluyveromyces*	DTE	YE	28	4.0	120	96	17.9	Jain et al.,
8	*K. marxianus* NRRL Y-7571	SCB, SCM	SB, CSL	30	-	SSF	24	436.7*	Mazutti et al., 2010a
9	*K. marxianus* NRRL Y-7571	SCM	YE, CSL	36	5.5	150	72	722*	Makino et al., 2009
10	*K. marxianus* NRRL Y-7571	SCM	YE, CSL	-	-	300	-	1317*	Treichel et al., 2009
11	*K. marxianus*	SCB	YE, CSL	-	-	-	-	1139*	Sguarezi et al., 2009
12	*Aspergillus tamarii* AR-IN9	DTE, JA	CSL	35	5.5	-	72	71.97	Saber and Naggar, 2009

1	*Xanthomonas*	Suc	Tryptone	37	7.0	150	18	22.09	Naidoo et
1	*Bacillus smithi*	Inulin	YE,	50	7.0	200	72	135.2	Gao et al.,
1	*A. niger* NK-126	DRE	YE	30	-	150	96	54	Kango,
1	*K. marxianus* YS-1	ARE	BE	30	6.5	200	60	50.2	Singh and Bharmi, 2008
1	*Cryptococcus aureus*	Inulin	YE	28	5.0	170	42	85	Sheng et al., 2007
1	*Debaryomyces hansenii*	Inulin	YE	28	5.0	170	72	52.5	Gao et al., 2007
1	*X. campestris*	Inulin	YE	37	7.0	150	120	17.4	Ayyachamy et al., 2007
2	*Yarrowia lipolytica*	Inulin	YE	28	5.0	170	72	52.3	Gao et al., 2007

[a]ARE- Asparagus root extract, DTE-Dahlia tuber extract, DRE-Dandelion root extract, JA- Jerusalem artichoke, LT- Lettuce root, SCB- Sugarcane bagasse, SCM- Sugarcane molasses, Suc- Sucrose, ST- Sunflower tuber,; [b]YE- Yeast extract, BE- Beef extract, SB- Soyabean bran, ME- Meat extract, CSL- Corn steep liquor, ADP- Ammonium dihydrogen phosphate, AS- Ammonium sulphate; Production of inulinase using SSF [*]U/gds

Table 2 Optimization of process parameters for inulinase production

S	Organism	Optimized experimental variables	Substrate	Type of Fermentation	Inulinase Activity	References
	Aspergillus terreus	Artichoke leaves- 3g, KH_2PO_4- 0.8%, Ca^{2+}- 6mM	Artichoke leaves	SSF	21.058 U/gds	Abd-El Aty et al., 2014
	Kluyveromyces marxianus var.	CSL- 0.06072 g/gds Urea- 0.01916 g/gds Beef extract-0.00957	Pressmud	SSF	288 U/gds	Dilipkumar et al., 2014a
	Bacillus safensis AS-	Raw inulin-1%, YE - 1%,	Dahlia tubers	SmF	28.67 U/ml	Singh and Singh,
	Penicillum rugulosum	K_2HPO4- 0.0047 g/gds, $ZnSO_4.7H_2O$- 0.02677 g/gds, Soya bean cake-	Copra waste	SSF	239U/gds	Dilipkumar et al., 2014b
	K. marxianus MTCC 188	Air flow rate- 0.82 L/min,	Pressmud	SSF	300.5 U/gds	Dilipkumar et al.,
	Thermomyces lanuginosus	Jerusalem artichoke extract- 1.8 % (m/v),	Jerusalem Artichoke	SmF	6.09 U/ml	Nguyen et al., 2013

	Aspergillus tubingensis	Period of fermentation- 103 h,	Wheat Bran	SSF	257 U/gds	Trivedi et al., 2012
	Rhizoctonia sp. I-PN4	Buckwheat- 6% YE- 5.82%	Buckwheat	SmF	1.87 U/ml	Bonciu et al., 2012
	Aspergillus ficuum JNSP5-06	Extraction solvent- 0.1 M sodium acetate buffer (pH 4.5)	Wheat bran	SSF	189.02 U/gds	Chen et al., 2011
	A. ficuum JNSP5-06	Inulin- 11.47%, $NH_4H_2PO_4$- 0.76%,	Wheat Bran	SSF	205.63 U/gds	Chen et al., 2011
	Candida guilliermondi	Inulin- 1%, NH_4Cl- 2.4%,	Inulin	SmF	473.26 U/ml	Songpim et al.,
	K. marxianus	Ethanol precipitation-	Sugarcane	SSF	86.1 %	Golunski
	Penicillium rugulosum	CSL - 0.05813 g/gds, $FeSO_4.7H_2O$- 0.00011	Garlic Powder	SSF	268 U/gds	Dilipkuma r et al.,
	K. marxianus NRRL	Inlet temp- 30°C, Air flow rate- 2.2 m^3/h,	Sugarcane Bagasse	SSF	436.7 J/gds	Mazutti et al., 2010b
	K. marxianus MTCC 188	CSL - 0.0560 g/gds, $ZnSO4.7H_2O$- 0.0084	Copra waste	SSF	372 U/gds	Dilipkuma r et al.,
	Pichia guilliermondi	Inulin- 20.0 g/L YE - 5.0 g/L	Inulin	SmF	127.7 U/ml	Yu et al., 2009

	K. marxianus NRRL Y-7571	Molasses- 250 g/L , CSL – 80 g/L, YE – 6 g/L,	Molasses	SmF	1317 U/ml	Treichel et al., 2009
	Geotricum candidum	Incubation period- 48 h, Sucrose conc- 5.0 g/L,	Sucrose	SmF	71.85 U/ml	Mughal et al., 2009
	K. marxianus NCYC 587, NRRLY-7571	Molasses CSL YE pH — NCYC: 90 g/L 45 g/L 4.0 g/L 4.5 — NRRL Y: 5-4.5 L 5.7	Molasses	SmF	735 U/ml (NCYC) 722 U/ml (NRRL Y)	Makino et al., 2009
	K. marxianus	Sugarcane molasses-	Sugarcane	SmF	1,139	Sguarezi

Table 3 Heterologous expression of inulinase genes

	Source of	Host/vector	Properties of cloned gene	Reference
1	*Kluyveromyces cicerisporus*	*Pichia pastoris* X-33	Exoinulinase, MW- 79.5 kDa, Activity- 45.2 U/ml pH- 4.5, Temp- 55°C	Ma et al., 2015
2	*Sphingomonas* sp. JB 13	*Escherichia coli*, BL 21 (DE3), *pEASY*-T1, *pEASY*-E2	Exoinulinase, MW- 55.6 kDa, Enzyme – rInuAJB13 (408 bp), pH- 5.5, Temp- 55°C Inu Lev Suc Raff Stac K_m (mg/mL) 10 11 6.5 5.9 7.3 V_{max} (µmol/min/mg) 294 22 1666 213 164 K_{cat} (s^{-1}) 274 21 1552 198 153 K_{cat}/K_m (mLmg/s) 27 2.0 239 34 21 Activator- SDS, CTAB, Tween-80, Triton X-100, $FeSO_4$, $MnSO_4$,	Zhou et al., 2015

			β-ME (Retained activity 76.5%), Inhibitors- $HgCl_2$	
3	*Aspergillus fumigatus* Cl1	*P. pastoris* *E. coli* DH5α	Endoinulinase, ORF- 1561bp, Code- 507aa MW- 60 kDa, Activity-3860 U/ml pH- 6.0, Temp- 55°C, K_m - 2.18 mM, V_{max} - 1590 μmol/min/mg, Metal ion- Activation- $MnSO_4$, $CaCl_2$, $FeSO_4$, Inhibition- $AgNO_3$, SDS, EDTA, $CuCl_2$	Chen et al., 2015
4	*Aspergillus niger* CICIM F0620	*P. pastoris*, *E. coli* JM109	Endoinulinase Gene- *EnInu*, MW- 59 kDa,	He et al., 2014

5	*A. niger*	*S. cerevisiae* JZ1C-J, *E. coli* DH5α, pMD19-	Endoinulinase Gene- *inuA* Activity- 3.1 U/L	Yuan et al., 2013
6	*Aspergillus ficuum* JNSP5-06	*E. coli* JM 109 and BL 21 (DE3) pET-28 a (+), PMD 19	Exoinulinase MW- 63 kDa Metal ion- Activation- Mn^{2+} Inhibition Fe^{3+}, Fe^{2+}, Ni^{2+}, Zn^{2+}, Mg^{2+}	Chen et al., 2013
7	*A. ficuum* JNSP5-06	*E. coli* JM 109 and BL 21 (DE3) pET-28 a (+),	Endoinulinase ORF- 1482bp MW- 60 kDa Metal ion- Activation- Zn^{2+}	Chen et al., 2012
8	*Lactobacillus casei* IAM 1045	*E. coli* XL 1-Blue	Exoinulinase MW- 85 kDa	Kuzuwa et al., 2012
9	*Arthrobacter* sp. S37	*Y. lipolytica* pINAI317, pMD 19-T	Endoinulinase MW- 78.9 kDa, Gene- Enl A K_m- 37.1 mg/mL, V_{max}- 3.9	Li et al., 2012
1	*Kluyveromyces marxianus*	*P. pastoris E. coli* DH5α, pMD18-T,	Exoinulinase MW- 60 kDa Gene- *rKmInu*	Zhang et al., 2012
1	*K. cicerisporus*	*K. lactis*	*Kcinu* Activity- 391 U/ml	Yu et al., 2010

1	*K. marxianus* CBS 6556	*Y. lipolytica*	Gene- *INU1* Activity- 22.6 U/mg	Liu et al., 2010
1	*Pichia guilliermondii*	*P. pastoris* X-33	pPICZaA-Inu1 MW- 57.6 kDa	Zhang et al., 2009a
1	*P. guilliermondii*	*P. pastoris* X-33	pPICZalphaA ; MW- 60 kDa Activity- 58.7 U/ml	Zhang et al., 2009b

Table 4 Production and properties of thermostable, cold active and halotolerant inulinases

SN	Extremophilic microorganism	Type of enzyme	Extremophilic conditions				Reference
			Temp	pH	Half-life inactivation period($\tau 1/2$)	Characteristics	
	Sphingomonas	Exo	10 to	5.5	135-163%	MW- 55.6	Zhou et
	Arthrobacter sp. MN8	Exo	0 to 20°C	-	More than 60 min at 50°C, rInuAMN8	Relative activity % at 0°C, 10°C and 20°C is	Zhou et al., 2014a
	Sphingobacterium sp. GN25	Exo	0 to 0°C	-	More than 60 min at	55.8% maximum retained	Zhou, et al., 2014b
	Bacillus licheniformis ATCC 14580	Levanase, inulinase	50°C	-	2,3-butandiol in 30 h	103.0 g/L 2,3 butanediol production	Li et al., 2014
	Nocardiopsis sp. DN-K15	Exo	60°C	8.0	81% active at 60°C for 1h over	25.1 U/ml	Lu et al., 2013

					wide pH 5.0-11.0 range		
	Microbulbifer sp.	Inulinase, Fructofuranosida	0 to 35°C	-	More than 50 min at	Relative activity % at	Kobayashi et al.,
	Bacillus smithii T7	Endo	70°C	4.0 -	9h/ 2.5h at 70/80°C	Inulinase yield of 135.2	Gao et al., 2009

Table 5 Use of immobilized inulinase for efficient production of industrially important products

SN	Organism	Immobilization properties	Carrier used	Product formed	Reference
1	*Aspergillus niger*	Hydrolysis of sucrose,	Sepabeads EC-EA and EC-HA	Glucose	Fernandez-Arrojo et
2	*A. niger* 20 OSM	Continuous hydrolysis of	Eupergit C	Fructose and Fructooligosaccharide	Trytek et al., 2015
3	*A. niger* AUMC 9375	Continuous hydrolysis of inulin,	Sodium alginate	Fructooligosaccharides and Fructose	Housseiny, 2014
4	*A. niger*	Continuous hydrolysis of inulin, pH - 5.0,	Concanavalin A-attached poly (ethylene glycoldimethacrylate	Fructose syrup	Altunbas et al., 2013
5	*A. niger* (Fructozyme)	Hydrolysis of inulin and sucrose	Montmorillonite	Fructooligosaccharides	Kuhn et al., 2012
6	*A. niger* (Megazyme)	Continuous hydrolysis of	Chitosan	Oligofructose syrup	Nguyen et al., 2011

SN	Microorgansim	Biotechnologically important end product[§]	Enzyme type	Fermentable sub

Table 6 Potential uses of inulinases for generation of industrially and nutritionally important products

1.	*Aspergillus* spp., *Penicillium* spp., *Fusarium* sp., *Arthrinium* sp.,	F, IOS, FOS	Exo, Endo	Chicory inulin, sucrose
2.	*Kluyveromyces marxianus*	Ethanol	Inu	JA
3.	*Lactobacillus paracasei* DSm 23505	LA	Inu	Chicory flour
4.	*Penicillium* sp. NFCC 2768	F, IOS	Endo, Exo	Dahlia inulin
5.	*Meyerozyma guilliermondii*,	Ethanol	Exo	Inulin
6.	*Rhodosporridium toruloides* 2F5	SCO	Inu	Inulin
7.	*Aspergillus niger* CICIM F0620	IOS	Endo	Inulin
8.	*Arthrobacter* sp., *Saccharomyces* W0[*]	Ethanol	Endo	JA
9.	*A. niger*, *S. cerevisiae*[*]	Ethanol	Invertase	Inulin
10	*Pichia guilliermondii* Pcla22	SCO	Inu	Inulin
11	*K. marxianus*	Ethanol	Inu	JA
12	*Aspergillus niger* (Fructozyme)	FOS	Inu	Inulin, Sucrose
13	*K. marxianus*, *S. cerevisiae*[*]	Ethanol	Inu	JA
14	*K. marxianus*, *Yarrowia lipolytica*[*]	SCP	Exo	Inulin
15	*A. niger* (Megazyme)	Oligofructose syrup	Endo	JA
16	*Rhodotorula mucilaginosa* TJY15a	SCO	Inu	JA
17	Fructozyme L	Tequila	Endo, Exo	*Agave tequilana*
18	*Cryptococcus aureus* G7a	SCP	Inu	Yacon
19	*K. marxianus*, *Y. lipolytica*[*]	Citric acid	Exo	Inulin
20	*P. guilliermondii*, *Saccharomyces* sp.[*]	Ethanol	Inu	JA
21	*K. marxianus*, *Y. lipolytica*[*]	SCO	Exo	JA , Inulin
22	*Pichia pastoris* X-33[*], *Saccharomyces*[*]	Ethanol	Exo	JA , Inulin
23	*Paenibacillus polymyxa* ZJ-9	2,3-Butanediol	Inu	JA
24	*R. mucilaginosa* TJY15a	SCO	Inu	Cassava
25	*Klebsiella pneumonia*	2,3-Butanediol	Inu	JA
26	*S. cerevisiae* KCCM50549	Ethanol	Inu	JA

27	*A. niger* SL-09, *Lactobacillus* sp.G-02	LA	Inu	JA
28	*A. niger* NK 126	F, IOS	Exo, Endo	Chicory inulin
29	*A. niger*	IOS	Endo	Inulin
30	*K. marxianus* YS-1	F	Exo	*Asparagus offici*

[§]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; JA- Jerusalem artichoke; [*]Cloned or expressed or recombinant inulinase

