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review

Production of Inulinases: Recent Advances

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Summary

Inulinases constitute an important class of enzymes for production of fructose and fructooligosaccharides, which are extensively used in pharmaceutical and food industry. The production of inulinases has been reported from various fungal, yeast and bacterial strains. The inulinases characterized until now show considerable variability with respect to biophysical and biochemical characteristics. High temperature optimum and thermostability are two important criteria which determine the suitability of these enzymes for industrial applications. Inulinases with high thermostability from strains of *Aspergillus* spp. and thermophilic bacteria have been reported. Molecular cloning of inulinase genes from different sources has revealed that beside conserved domains, the endo- and exo-acting inulinases show motifs which are distinct for the two classes of enzymes. The present article reviews some of the recent advances in the production and characterization of inulinases from different microbes and their possible applications.

Key words: endoinulinase, exoinulinase, enzyme purification, thermostability

Introduction

Fructose and fructooligosaccharides are emerging fast as important ingredients in the food and pharmaceutical industry. Fructose is considered as a safe alternative sweetener to sucrose because it has beneficial effects in diabetic patients, increases the iron absorption in children, and has a higher sweetening capacity (*1*). Sucrose, on the other hand, is known to cause problems related to corpulence, cariogenecity and artherosclerosis (*2*). In addition, fructose has higher solubility than sucrose, it is less viscous, and in low levels it can be metabolized without a need for insulin (*3*). Fructooligosaccharides have good functional and nutritional properties such as low calorie diet, *Bifidus* stimulating factor, and source of dietary fibre in food preparations (*4–6*). These oligosaccharides, therefore, are now widely used to replace sugars in many food applications such as in confectionery, chocolate and dairy products (*2,7*).

Both fructose and fructooligosaccharides can be produced from inulin, which consists of a linear β-2,1-linked polyfructose chain, terminated by a glucose residue through a sucrose-type linkage at the reducing end (*2,8,9*). Inulin is found as a carbohydrate reserve in the roots and tubers of several plants such as Jerusalem artichoke, chicory, dahlia and also in burdock, goldenrod and dandelion. Inulin can be hydrolysed by acid (pH=1.0–2.0 at 80–100 °C), but low pH results in degradation of fructose and the process also gives rise to formation of difructose anhydrides, which are coloured and have no sweetening capacity (*10*). The conventional production of fructose is based on amylolysis of starch with α-amylase and amyloglucosidase followed by glucose isomerase, which catalyzes the conversion of glucose to fructose. However, this process yields only about 45 % of fructose at best, the rest being glucose (50 %) and oligosaccharides (8 %). Though ion exchange chromatography techniques have been developed for enrichment of fructose, these techniques add to the cost of production (*2,11*). Thus the use of microbial inulinases has been proposed as the most promising approach to obtain pure fructose syrups from inulin. Inulin is degraded by inulinase, which cleaves glycoside bonds to form largely (95 %) D-fructose by a single-step process and is attrac-

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tive for the industrial production of high fructose inulin syrups. The production of inulinases by various microbial inulinases and their properties has been reviewed earlier (*2,12*). The present review highlights some of the recent advances in this field.

Sources of Inulinases

Yeast

Although inulin-hydrolyzing activity has been reported from various microbial strains (*2,12*), yeasts (*Kluyveromyces* spp.) together with *Aspergillus* spp. have proved to be the most versatile source of inulinases. The inulinase activity of yeasts has been primarily characterized in *K. fragilis* and *K. marxianus*. The partially purified inulinase reported in *K. fragilis* was optimally active at 45 °C and pH=5.0 (*13*). The inulinase (2,1-β-D-fructan fructanohydrolase, E.C. 3.2.1.7) of *K. fragilis*, which was purified to homogeneity and immobilized on 2-aminoethyl cellulose, showed good operational stability in the presence of inulin or the tuber extract of Jerusalem artichoke (*14*). Another isoform of inulinase in *K. fragilis*, purified by Workman and Day (*15*), was a glycoprotein, stable at 50 °C, optimally active at pH=4.5 and was inhibited by the cations Hg^{2+}, Ag^{+}, Cu^{2+} and Cd^{2+}. Compared to a single microorganism, mixed cultures of *K. fragilis* and *Saccharomyces cerevisiae* or the bacterium *Zymomonas mobilis* resulted in 2–12 % higher production of ethanol in Jerusalem artichoke tubers, thus demonstrating the possible biotechnological applications of inulinase producing yeast (*16*).

The inulinase activity produced by *K. marxianus* strain CBS 6556 was both cell wall-associated and extracellular, thus implying the presence of two isoforms (*17*). The enzyme was able to utilize sucrose, raffinose, stachyose and inulin as substrates and exhibited an S/I ratio of 15, with the enzyme activity being inversely proportional to the chain length of the inulin. Purification and characterization demonstrated that the extra- and intracellular forms of inulinase consisted of a similar subunit of 64 kDa but differed in size due to the difference in subunit aggregation, with the former being a dimer, and the latter a tetramer. Another inulinase-hyperproducing strain of *K. marxianus* CDBB-L-278, which was able to grow in a medium containing inulin as the sole carbon source in the presence of 2-deoxyglucose (glucose analog), produced up to 3.3 times the activity of the control strain *K. marxianus* NCYC-1429 in an inulin medium, and 3.6 times in a medium with glycerol as the sole carbon source (*18*). Since inulinase was produced in a glycerol medium without an inducer, it was proposed that the enzyme production was partially constitutive in *K. marxianus* CDBB-L-278 as well as in strain NCYC-1429. The inulinase of *K. marxianus* CDBB-L-278 showed an optimum pH=5.0, whereas optimum temperature was 50 °C for inulin and 70 °C for sucrose. The enzyme was stable at high temperature, with a half-life of 180 min at 50 °C. The K_m of inulinase on inulin was 3.0 and 40.18 mM on sucrose.

The production of endo- and exocellular inulinases was also reported for *Kluyveromyces* sp. strain Y-85 (*19*). The molecular mass of the endocellular enzymes, designated as EI and EII, was 42 and 65 kDa, respectively, whereas the exocellular enzyme (Eexo) was a 57-kDa protein. The EI, EII, and Eexo were optimally active at pH=4.6, 4.5, 4.6, and at 52, 52 and 55 °C, respectively. The activity of all the three enzymes was strongly inhibited by Ag^{+}, Hg^{2+} and *p*-chloromercuribenzoate (*19*). Immobilization of the partially purified intracellular inulinase from *Kluyveromyces* sp. Y-85 onto a macroporus ionic polystyrene beads resulted in a fructan hydrolysis of 75 % and the hydrolytic product was a mixture of 85 % D-fructose and 15 % D-glucose (*20*). Inulinase activity was also produced in the extracellular extract of *K. marxianus* var. *bulgaricus*, when grown on inulin, sucrose, fructose and glucose as carbon source (*21*). The optimum pH (4.75), temperature (55 °C), and molecular mass (57 kDa) were similar to the extracellular enzyme reported earlier from *Kluyveromyces* sp. strain Y-85 (*19*). The activity of the purified enzyme of *K. marxianus* var. *bulgaricus* was completely inhibited by ferric chloride while barium, zinc and sodium inhibited activity by 50 %. Although the enzyme was stable for 3 h at 50 °C, incubation at 55 and 60 °C resulted in rapid loss of activity (*22*).

The immobilization of inulinase producing *K. marxianus* cells in open pore gelatin matrix enhanced the stability of the enzyme at pH below 4.0 and above 7.0 without altering the optimum pH (6.0) (*23*). On the contrary, immobilization of inulinase on molecular sieve 4A caused a shift in the optimum pH of the enzyme from 6.0 to 5.0 (*24*). The immobilization of the cells on both supports also resulted in higher temperature optimum. In a batch reactor, about 93 % of hydrolysis by immobilized cells in 3 h was observed with artichoke tuber extract. The percentage of the hydrolysis of inulin by immobilized cells was almost constant for all 10 batch cycles (*25*). Immobilization of a different *K. marxianus* strain in barium alginate, however, resulted in retention of 85 % of residual activity after five runs of inulin hydrolysis (*26*). Besides inulin, the inulinase of *K. marxianus* was also induced by glucose, fructose and sucrose (*27*).

The extent of variability observed with respect to inulinases among different strains of *K. marxianus* is quite remarkable and may be due to translational or post-translational modifications or simply due to difference(s) in the gene sequence. However, the yeast inulinases show only modest thermostability, due to which their potential for commercial scale applications appears to be limited.

Filamentous fungi

The inulinase activity has been reported and characterized from diverse fungal sources. *Panaeolus papillonaceus* was the first basidiomycete from which an inulinase was purified (*28*). The enzyme consisted of two subunits with a total M_r of 116 kDa and it was more active on sucrose than on raffinose, stachyose and inulin. The purified inulinase was highly thermostable with an optimum temperature range of 60–65 °C and pH optimum of 6.0. Iodoacetate, azide, EDTA at 20 mM concentration and SDS (1 % mass per volume ratio) had no effect on enzyme activity, whereas Ag^{+} and Hg^{2+} at 2 mM were highly inhibitory. *Chrysosporium pannorum* AHU 9700, a

mould isolated from soil, was found to produce a very active inulin-hydrolyzing enzyme which was induced by inulin, but not by sucrose, glucose or fructose (*29*). On the contrary, the inulinase activity of *C. cladosporioides*, besides inulin, was also induced on sucrose (*30*). The crude enzyme preparation of *C. pannorum* consisted of both exo- and endoinulinase activity and catalyzed complete hydrolysis of 10 % inulin suspension at pH=5.6 and 50 °C in 8 h (*29*). The exoinulinase activity of *C. pannorum* AHU 9700 was associated with two glycoproteins of 84 and 70 kDa, having isoelectric points of 4.6 and 4.45, respectively. Relative to *C. pannorum* inulinase (*29*), the exoinulinase of *C. cladosporioides* displayed higher temperature optimum (60 °C) and was stable for 4.5 h at this temperature (*30*). Of the two species, *C. cladosporioides*, due to the high temperature optimum (60 °C) of its inulinase, appears to be more promising and thus warrants further screening for isolation of strains with better inulinase producing capabilities.

Inulinase has also been purified from acidophilic fungus, *Scytalidium acidophilum* (*31*). The maximum activity of the enzyme from *S. acidophilum* was observed between pH=3.0–3.5 and it retained about 95 and 85 % of activities at 60 and 65 °C, respectively, after 6 h of incubation. Although the inulinase of *S. acidophilum* is thermostable, immobilization studies are required to determine its industrial potential. Four different inulinase isoforms, with temperature and pH optima ranging from 34–45 °C and 5.5–6.5, respectively, were characterized from the extracellular extracts of *Fusarium oxysporum* (*32*), which produced maximum inulinase activity after 9 days of growth at 25 °C on a medium (pH=5.5) containing 3 % fructan and 0.2 % sodium nitrate (*33*). Immobilization of the enzyme on various supports, though, resulted in higher temperature optimum (45 °C) as compared to the free enzyme (37 °C), but it is still lower than the industrial requirement of 60 °C, thus limiting its practical application.

The *Aspergillus* spp. are among the best known producers of inulinase. Different studies carried out on the inulinase activity of *A. ficuum* revealed the presence of multiple isoforms of exoinulinases and endoinulinases with all the enzymes being glycoproteins with high sugar content (22–41 % mass ratio) (*34–36*). Compared to a molecular mass of 74 kDa for different forms of exoinulinases, the molecular mass of the endoinulinases was 64 kDa (*34*). The S/I ratios ranged from 0.34 to 1.16 and from 2.75 to 6.38 for endo- and exoinulinases, respectively. Differential glycosylation resulted in difference in the molecular mass of the two endoinulinases, (64±0.5) kDa and (66±1.0) kDa, purified from Novozyme 230 (commercial preparation of inulinase from *A. ficuum* from Novo A/S, Denmark), although they consisted of the same protein of 64 kDa (*37*). Immobilization of the commercial inulinase preparation from *A. ficuum* onto porous glass beads did not alter the enzymatic properties except for a shift in the optimum temperature from 60 to 70 °C. The hydrolysis of Jerusalem artichoke tuber extracts by chitin-immobilized inulinase from *A. ficuum* on chitin was 90 % (D-fructose/D-glucose: 86/14) in 10 h. At a fixed residence time of 2.6 h and at 40 °C, this could be operated for over two weeks with only a slight loss of activity (14.8 %) (*38*).

Of the various *Aspergillus* spp. the strains of *A. niger* have been most extensively investigated for inulinase production and characterization. An *A. niger* strain, isolated from *compositae rhizosphere*, produced both extra- and intracellular inulinases, which displayed identical pH and temperature optima with maximal activity observed at pH=4.3–4.4 and at 55–56 °C, respectively (*39*). Three different inulinase isoforms, *viz.* I, II, and III with isoelectric points of 4.5, 4.9 and 5.2, respectively, were purified from *A. niger*. The three isoforms displayed exoinulinolytic activity at an optimum pH and temperature of 5.0 and 62.5–65 °C, respectively. Compared to the S/I ratio of 0.85 for the crude enzyme (*39*), the S/I ratio for form I was 1.8, and 2.4 for forms II and III. These isoforms were glycoproteins with a similar native molecular mass of about 300 kDa and consisted of four identical subunits of 85 kDa each (*40*). The exo-type activity reported from another strain of *A. niger* was, however, associated with an 81-kDa purified protein (*41*), which also exhibited invertase activity and was activated up to 20-fold by Fe^{3+} and partially inhibited by Mn^{2+} and Mg^{2+}. The biophysical characteristics of multiple isoforms of exoinulinases isolated from still another strain of *A. niger* (*42,43*) were distinct from the earlier reported isoforms. The estimated molecular mass of the five different isoforms was 102.6, 97.9, 62.5, 36.5 and 28 kDa, respectively, with the isoelectric points being 4.15, 4.24, 4.48, 4.15 and 5.4, respectively (*42,43*). Highest inulinase activity of these isoforms was observed between 55–60 °C with the optimum pH ranging from 4.0 to 5.0.

A. niger has also been reported to produce endo-acting inulinase activity. The two endoinulinase isoforms (P-1A and P-1B), purified by Nakamura *et al*. (*44*) from extracellular extracts of *A. niger* mutant 817, were monomers of 70 and 68 kDa, respectively, whereas the endo-acting activity from a different strain was associated with a 53-kDa protein (*41*). The enzymes were active only toward inulin and lacked activity toward sucrose, raffinose or levan, with S/I ratio of 1.14. Although optimum pH of the endoinulinase isoforms (P-1A and P-1B) was the same (pH=5.0) as that for exoinulinases, the temperature optimum of the former was lower (40 °C) as compared to the latter (62.5–65 °C) (*40,44*). The immobilization of a partially purified enzyme preparation from *A. niger* mutant 817 onto Amino-Cellulofine, as contrary to the immobilized β-fructofuranosidases from *A. niger* ATCC 20611 and *Aspergillus japonicus* TIT-KJ1 on methacrylamide based polymeric beads (*45*), resulted in a shift in optimum pH and temperature. The immobilized enzyme was stable in the pH range of 4.5 to 6.5 at 30 °C and from 5.0 to 6.0 at 50 °C. It is thus evident that, as compared to the endoinulinases, the exo-acting inulinases characterized from *A. niger* show higher variability in molecular mass (28–300 kDa) and are also active at higher temperatures (up to 65 °C), a property suitable for production of fructose syrups at industrial scale. Furthermore, the fact that certain abiotic stress conditions enhance the inulinase production by *A. niger* mutants by up to 4.5-fold (*46*) can also be exploited for enhancing the enzyme production.

Inulinase activity has also been characterized from other *Aspergillus* spp., which include *A. versicolor* (MTCC 280) (*47*), *A. oryzae* (*48*), *A. candidus* (*49*), and *A. awamori*

var. 2250 (*50*). The inulinase enzymes from these organisms were exo-acting in nature with pH and temperature optima ranging from 4.5–5.5 and 45–55 °C, respectively. The molecular mass of the inulinase purified from *A. versicolor* (MTCC 280) (*47*) was (230±20) kDa, as compared to 38 kDa of *A. oryzae* inulinase (*48*). The molecular mass of inulinases from *A. candidus* (*49*) and *A. awamori* (*50*) corresponded to (54.4±4) kDa and (69±1) kDa, respectively. Although the commercial preparation of inulinase (Novozyme, Sigma Chemical Co., St. Louis, Mo, USA) is derived from *A. niger*, a strain of *A. fumigatus*, which produces thermostable extracellular inulinase and was isolated in our lab from rhizosphere of dahlia and chicory (*51*), appears to be the most promising. The crude inulinase of *A. fumigatus* was maximally active at 60 °C with maximum production observed at 34 °C (pH=6.5) on a medium containing inulin as the sole carbon source, and combination of peptone and $NaNO_3$ (*52*). Two different isoforms (I and II), present extracellularly and exo-acting, were purified from the culture filtrate of *A. fumigatus.* The two isoforms depicted distinct biophysical and kinetic properties (*53–55*). Gel filtration chromatography revealed a molecular mass of about 200 kDa for isoform I, which on SDS-PAGE analysis resolved into three closely moving bands of about 66, 62.7 and 59.4 kDa. The isoform II, on the contrary, was a monomer of about 62 kDa. The pI values of isoforms I and II were 8.8 and 4.5, respectively. As compared to exoinulinase isoform I and Novozyme, the isoform II was more thermostable. Immobilization of partially purified inulinase resulted in marked enhancement in the thermostability (*56*), thus signifying its potential for commercial scale of fructose production.

As is evident, the inulinases produced by different strains of *Aspergillus* spp. show variability with respect to temperature optima and thermostability. The thermostability of enzymes may be due to increase in salt bridges and/or hydrogen bonds, a tighter packing of the hydrophobic core, or a higher percentage of amino acids incorporated into helices and sheets (*57*). Comparative analysis of amino acid sequence of different *Aspergillus* inulinase proteins may elucidate the underlying molecuar basis of thermostability of these enzymes, thus allowing protein engineering for enhancing the thermoresistance.

The inulin hydrolytic activity produced by different *Penicillium* spp. also shows variabilty with respect to various enzyme charateristics. Two proteins of 81 and 87 kDa, respectively, which showed exo-inulinase activity, were purified from the culture broths of *P. trzebinskii* (*58*) and *Penicillium* sp. TN-88, with the latter also producing an endo-acting inulinase of 68 kDa (*59*). The exo-acting activity purified from the extracellular extract of *P. janczewskii*, on the contrary, was associated with two isoforms of 48 and 66 kDa, respectively, which also differed in their K_m values for inulin (*60*). The temperature and pH optima for both isoforms were, however, identical, *i.e.* 55 °C and 5.0, respectively. Of the different inulinases reported from other *Penicillium* spp., *viz. P. aculeatum*, *P. digitatum*, and *P. cyclopium*, the inulinase from *P. aculeatum* was more thermoresistant, whereas the inulinase from *P. cyclopium* was the most labile, particularly at 65 °C (*61*). Recently, *Rhizopus* sp. (*62*) and *Alternaria alternata* (*63*) have also been studied for inulinase production. The inulinase activity reported from both strains was produced extracellularly. Whereas the purified inulinase of *Rhizopus* sp., which was an 83-kDa protein, was maximally active at pH=5.5 and 40 °C, respectively, the partially purified inulinase of (115±5) kDa from *A. alternata* showed highest activity at pH=4.5 and 55 °C. Relative to the K_m value of 66 mM for the *A. alternata* inulinase (*63*), the lower K_m value of 9.0 mM for *Rhizopus* inulinase (*62*) implied greater affinity of the latter for inulin.

Bacteria

The production levels of inulinases in bacteria are not comparable to those of yeast and fungi. However, due to the ability of many bacteria to survive at high temperatures, attempts have been made to isolate bacterial strains which can produce high quantities of thermally stable inulinase. Tanaka *et al*. (*64*) and Nakayama (*65*) isolated several mutants of *Arthrobacter ureafaciens* showing varying thermostability of inulinase. The inulinase produced by some strains was stable up to 70 °C and it was activated only at a temperature between 45 and 55 °C. A β-fructofuranosidase purified from *Arthrobacter* sp. was exo-acting at an optimal pH and temperature of 6.0 and 50 °C, respectively (*66*). Takahashi *et al*. (*67*) purified to homogeneity an exoinulinase of 83 kDa from *Streptococcus salivarius* (β-D-fructan fructohydrolase, E.C. 3.2.1.80). The pH optimum of this enzyme was 7.0 with an isoelectric point of 4.7. The purified enzyme preparation hydrolyzed levan, inulin and several β-(2-1)--linkage-containing oligosaccharides such as sucrose and raffinose, but not melezitose, dextran and pseudonigeran. The fructosidase was inhibited by Fe^{3+}, Cu^{2+}, Hg^{2+} and Ag^{+}, at a concentration of 1 mM, while Mn^{2+} stimulated the activity at the same concentration.

A thermophilic *Bacillus* strain, which produced an inulin-inducible inulinase, was isolated by Allais *et al*. (*68*). The partially purified inulinase from *B. subtilis*, which displayed higher specificity for inulin (K_m=8 mM) than for sucrose (K_m=56 mM), was inhibited by the end product, fructose, at 14 mM (*69*). A thermophilic soil isolate *B. stearothermophilus* KP1289 that grew at temperatures ranging from 41 to 69 °C produced inulin-inducible extracellular inulinase, the molecular mass and pI of which were 54 kDa and 5.0, respectively (*70*). At 69 °C and pH=7.0 the half-life of the enzyme was 10 min. Zherebtsov *et al.* (*71*) studied the production of extracellular inulinase by *Bacillus polymyxa* 29, *B. polymyxa* 722, and *B. subtilis* 68. The maximum production of enzyme was observed between 33 and 35 °C after 72 h of incubation at pH=7.0. The presence of reduced mineral nitrogen or organic nitrogen was necessary for the enzyme biosynthesis. While *B. polymyxa* 722 and *B. polymyxa* 29 displayed highest activities on a culture medium containing starch, the maximum activity of *B. subtilis* 68 was observed in the presence of sucrose. Immobilization of a thermostable exoinulinase from growing cells of thermophilic *Bacillus* sp. 11 resulted in 1.5–2.0-fold higher enzyme yields (inulinase and invertase activities) than those of free cells (*72,73*).

The inulin-inducible inulinase activity of *Clostridium acetobutylicum* was produced both extra- and intracellularly (*74*). The inulinase activity was higher than invertase activity in the extracellular preparation, whereas the opposite was observed for the cellular preparation. The pH and temperature optima of 5.5 and 47 °C, respectively, of inulinase of *C. acetobutylicum* (*74*) differed from that of *C. thermoautotrophicum* inulinase, which was maximally active at 60 °C and neutral pH (*75*). *Bifidobacterium longum*, *B. infantis* and *B. angulatum* were also reported to produce chicory fructooligosaccharides metabolizing activity (*76*). The β-fructofuranosidase of *B. infantis*, which was purified 47-fold, was a monomeric protein of 70 kDa and possessed both inulinase and invertase activities (*77*). The purified inulinase showed an isoelectric point of 4.3, while the optimum pH and temperature were 6.0 and 37 °C, respectively. The enzyme activity was inhibited by Hg^{2+} and *p*-chloromercuribenzoic acid.

Park *et al.* (*78*) reported a novel inulinolytic strain of *Xanthomonas* sp., which produced an endoinulinase. The endoinulinase was optimally active at 45 °C and pH=6.0. An extracellular endoinulinase of 139 kDa, which converted inulin into inulooligosaccharides, was later purified from *X. oryzae* (*79*). The enzyme activity was maximum at pH=7.5 and 50 °C, and it was stable over a pH range of 6.0–9.0. Selvakumar and Pandey (*80*) and Pandey *et al.* (*81*) studied the production of inulinase activity by different strains of *Staphylococcus* sp. Wheat bran, rice bran, coconut oil cake and corn flour, individually or in combinations, were tested for their efficacy to be used as the solid substrate. Under optimized conditions, the extracellular enzyme concentration peaked in 48 h. Under submerged conditions, inulin at 0.5–1.0 % concentration was the most favourable substrate for inulinase synthesis. Optimum pH for enzyme synthesis by the bacterial strain was 7.0–7.5. Maximum enzyme activities were obtained when fermentation was carried out at 30 °C for 24 h with a medium containing 0.5 % of inulin as a sole carbon source and 0.5 % of soybean meal as the nitrogen source.

The gene encoding for one of the most thermostable bacterial inulinases, which retained 85 % of its initial activity after 5 h at 80 °C and pH=7.0, was cloned from *Thermotoga maritima* (*82*). *Thermotoga maritima* is a strictly anaerobic heterotroph with a maximum growth temperature of 90 °C. The optimum temperature for activity of the enzyme was 90–95 °C. The gene encoding for a 432-residue polypeptide of about 50 kDa protein was expressed in *Escherichia coli* and the recombinant enzyme hydrolysed inulin quantitatively in an exo-type fashion. Although inulinase production by actinomycetes, *viz. Actinomyces longisporus*, *Acyanoalbus lavendotoliae*, *Streptomyces* spp., has also been reported (*83–86*), more elaborate studies are required to assess their potential for large scale applications.

The extracellular inulinase enzyme from *Pseudomonas* sp., after immobilization on anion exchange resin and a polystyrene carrier material (UF93®), was used for continuous production of inulooligosaccharides (IOS) from pure inulin (*87,88*). Immobilization of endoinulinase from *Pseudomonas* sp. on the polystyrene carrier material resulted in a shift in the optimal pH from 5.0 to 4.5, whereas optimal temperature (55 °C) was unaffected. Continuous production of inulooligosaccharides from chicory juice for 28 days at 55 °C using the polystyrene-bound endoinulinase did not result in any significant loss of initial enzyme activity (*89,90*).

Thermostability

Higher temperature optimum of inulinases is an extremely important factor for the application of these enzymes for commercial production of fructose or fructooligosaccharides from inulin, since high temperatures (60 °C or higher) ensure proper solubility of inulin and also prevent microbial contamination (*2*). Higher thermostability of the industrially important enzymes also brings down the cost of production because lower amount of enzyme is required to produce the desired product. Inulinases from yeasts, fungi and bacteria have been studied but only a few of these enzymes have an optimum temperature of 60 °C or higher, as required for industrial applications (*2,12,30,34,47,49,50,53–56,72,91–93*) (Table 1). Between yeasts and *Aspergillus* spp., which are the most versatile sources of inulinases, the inulin hydrolytic activity from the latter is more thermostable. Among the different fungal strains reported, the exoinulinase isoform II of *A. fumigatus* (*54–56*), due to its higher thermostability, appears to be more suitable for commercial hydrolysis of inulin than inulinases from *A. niger* (*93*), *A. ficuum* (*94*) and *Scytalidium acidophilum* (*31*). Although the inulinase from the thermophilic bacterium *T. maritima* is the most thermostable (*82*), its low production may be a limiting factor for inulin hydrolysis at industrial level.

The thermostability of the inulinases can be enhanced by immobilization (*20,24,38,49*) as it provides a more rigid external backbone for the enzyme molecules due to which the effect of higher temperatures in breaking the interactions responsible for the proper globular, catalytic active structure becomes less prominent (*95*). Irrespective of the support used, the thermal stability (~70 % up to 48 h) of the immobilized inulinase from *A. fumigatus* at 60 °C was considerably higher than that reported for immobilized inulinases of some other microbes, *viz. F. oxysporum* (50 % activity at 50 °C after 45 min), *A. niger* (stable for 30 min at 60 °C) and *A. candidus* (stable for 60 min at 55 °C) (*49,96,97*). The addition of stabilizing additives (polyethylene glycol 6000, ethylene glycol, isopropanol, dextran, sorbitol and glycerol), which enhance the shelf life of the enzyme products (*98*), can also be exploited to increase thermostability of inulinases (*54,99,100*). Enhancement in thermostability of the inulinase isoforms in the presence of polyols may result from reduced competition by water molecules for essential hydrogen bonds within the inulinase or from the increased strength of these bonds in an environment of low dielectric constant (*101*).

Molecular Characterization

The complete nucleotide sequence encoding an inulinase, which was endo-acting, was first reported from *Penicillium purpurogenum* (*102*). Since then, genes for inulinases have been cloned from various fungal and bacterial sources with the smallest and largest open

Table 1. Optimum temperature and thermostability of inulinases from different sources

Source	Optimum temperature °C	Thermostability	Ref.
Yeasts and moulds			
Kluyveromyces marxianus var. *bulgaricus*	55	Stable at 40 °C for 3.5 h, half-life at 50 °C for 40 min	(*21*)
Chrysosporium pannorum	50	Stable for 10 min at 50 °C	(*29*)
Fusarium oxysporum	45	Stable for 10–15 min at 50 °C	(*32*)
Panaeolus papillonaceus	60–65	Stable for 1 h at 50 °C	(*28*)
Scytalidium acidophilum	–	Retained 95 and 85 % activity after 6 h of incubation at 60 and 65 °C, respectively	(*31*)
Aspergillus niger	55	Retained 60–65 % after 2 h at 65 °C	(*93*)
Aspergillus ficuum	60	Retained 74 and 22 % activity for 6 h at 60 and 70 °C, respectively	(*36*)
Aspergillus oryzae	55	Retained over 90 % activity after 2 h incubation at 70 °C	(*48*)
Aspergillus versicolor	55–60	Retained 28 and 39 % activity at 60 °C	(*47*)
Aspergillus awamori	60	Retained 90 % activity after 24 h at 50 °C	(*50*)
Alternaria alternata (Fr.) Keissler	55	Stable up to 80 % for 1 h at 50 °C	(*63*)
Aspergillus fumigatus			
Isoform I	60	Retained 59 % activity after 3 h at 60 °C	(*55*)
Isoform II	60	Retained 100 % activity after 3 h at 60 °C	(*55*)
Immobilized inulinase (partially purified)	60	Retained 82–96 % activity after 12 h at 60 °C	(*56*)
Bacteria			
Xanthomonas oryzae	50	Stable for 1 h at 45 °C	(*79*)
Geobacillus stearothermophilus	60	Retained 90 % activity after 1 h at 60 °C	(*92*)
Bacillus stearothermophilus	60	Stable for 10 min at 60 °C	(*70*)
Thermotoga maritima	90	Active at 80 °C for 60 min	(*82*)
Arthrobacter sp.	50	Stable for 30 min at 50 °C	(*66*)
Clostridium acetobutylicum	47	Half-life of 1 h at 52.5 °C	(*74*)

reading frames (ORF) being observed for *T. maritima* exoinulinase and an endo-inulinase of *Arthrobacter* sp., respectively (Table 2). ClustalW analysis of the deduced amino acid sequences (Fig. 1) reveals the presence of se-

Table 2. Comparative analyses of inulinase genes from different sources. Exo (exo-acting) and Endo (endo-acting) refer to hydrolysis of inulin

Source No.	Accession No.	ORF (bp)	Source	Type	Reference
1	BAC16218	2106	*Penicillium* sp. TN-88	Exo, *inu*D	(*103*)
2	CAA48500	1668	*Kluyveromyces marxianus*	Exo, *INU1*	(*104*)
3	AAF44125	1503	*Pseudomonas mucidolens*	Exo, *inu*2	ds
4	EAL86248	1584	*Aspergillus fumigatus*	Endo	ds
5	NP695334	1554	*Bifidobacterium longum*	Exo	(*105*)
6	AJ001073	1296	*Thermotoga maritima*	Exo	(*82*)
7	AAU24331	2031	*Bacillus licheniformis*	Exo	(*106*)
8	BAC45010	1479	*Geobacillus stearothermophilus*	Exo	(*92*)
9	AF234992	1536	*Bacillus* sp. Snu7	Exo	ds
10	AA182575	1455	*Bacillus polymyxa* MGL21	Exo	ds
11	AF366292	1479	*Bacillus subtilis*	Exo	ds
12	CAL44220	1611	*Aspergillus awamori*	Exo	(*50*)
13	AAF24999	2328	*Pseudomonas mucidolens*	Endo, *Inu*1	ds
14	CAA07345	1548	*Aspergillus ficuum*	Endo, *inu*2	(*107*)
15	AJ131562	2436	*Arthrobacter* sp. S37	Endo	(*108*)
16	BAA33797	1548	*Aspergillus niger*	Endo, *inuA*	(*109*)
17	BAA33798	1551	*A. niger*	Endo, *inuB*	(*109*)
18	BAA19132	1545	*Penicillium* sp. TN-88	Endo, *inu*C	(*110*)
19	AAL34524	1548	*Aspergillus niger*	Endo	ds
20	BAA12321	1545	*Penicillium purpurogenum*	Endo	(*102*)

ds: direct submission

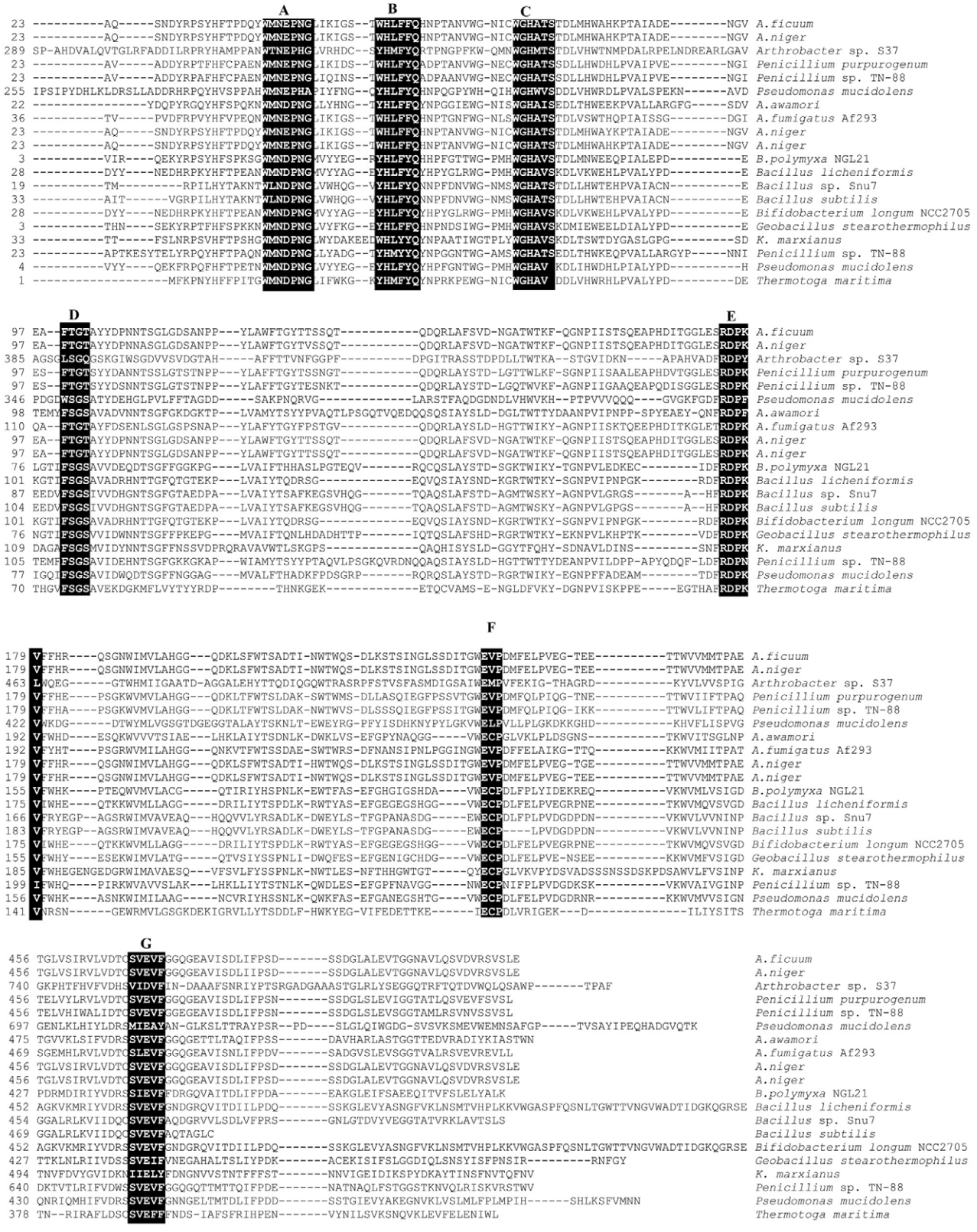

Fig. 1. Multiple alignment of deduced amino acid sequences of inulinases from different sources, *viz. Aspergillus ficuum* (CAA07345), *Aspergillus niger* (AAL34524), *Arthrobacter* sp. S37 (AJ131562), *Penicillium purpurogenum* (BAA12321), *Penicillium* sp. TN-88 (BAA19132), *Pseudomonas mucidolens* (AAF24999), *Aspergillus awamori* (CAL44220), *Aspergillus fumigatus* (EAL86248), *Aspergillus niger* (BAA33798), *A. niger* (BAA33797), *Bacillus polymyxa* (AAL82575), *Bacillus licheniformis* (AAU24331), *Bacillus* sp. Snu7 (AF234992), *Bacillus subtilis* (AF366292), *Bifidobacterium longum* (NP695334), *Geobacillus stearothermophilus* (BAC45010), *Kluyveromyces marxianus* (CAA48500), *Penicillium* sp. TN-88 (BAC16218), *Pseudomonas mucidolens* (AAF44125), *Thermotoga maritima* (AJ001073). The numbers in parentheses refer to the accession numbers for the reference sequences. The ClustalW analysis was performed with the MegAlign tool of Lasergene's DNASTAR programme using Gonnet series, slow/accurate matrix with default parameters

veral conserved motifs, *viz.* WMN(E/D)PNG (Block A), WHLFFQ (Block B), WGHATS (Block C), F(T/S)G(T/S) (Block D), RDPKV (Block E), E(V/C)P (Block F) and SVEVF (Block G) among the inulinases. The carboxyl groups of Asp residue [in the motif WMN(E/D)PNG (Block A)] and the Glu residue [in the motif E(V/C)P (Block F)] are involved in the catalytic activity of β-fructofuranosidases (*111*). Although all exoinulinases carry Asp in the motif WMN(E/D)PNG (Block A), the endoinulinase genes cloned so far, however, show Glu instead of Asp residue. The Glu residue within another conserved region Glu-(Cys/Val)-Pro (Block F) may be acting as a proton donor in the catalytic reaction, as reported for invertase from *S. cerevisiae* (*111*). The motif E(V/C)P (Block F), which is conserved between both fungal and bacterial exoinulinases, shows divergence between endoinulinases of bacteria and fungi. The fungal endoinulinases are characterized by the presence of Val instead of Cys in this motif, whereas bacterial endoinulinases contain Leu (*P. mucidolens*)/Met (*Arthrobacter* sp.) (Fig. 1). The Asp residue, present as part of a motif RDP (Block E), which is conserved in all the inulinases, has been implicated in substrate recognition since it provides hydrogen bonds (*112*). The exo- and endoinulinases of fungi also show divergence in another conserved motif, F(T/S)G(T/S) (Block D) (Fig. 1). Whereas all exo-acting inulinases contain Phe-Ser-Gly-Ser (FSGS), the endoinulinases show divergence between fungi and bacteria in this motif. The endo-inulinases from fungi are characterized by the presence of Phe-Thr-Gly-Thr (FTGT) as conserved sequence, whereas the bacterial endoinulinases (*Pseudomonas mucidolens* and *Arthrobacter* sp.) retain only Gly as the conserved residue in this motif. The precise functional significance of this sequence warrants further characterization by *in vitro* mutagenesis studies. The bioinformatics analysis (in this article) also suggests that the sequence (Acc. No. EA186248) reported for the putative inulinase of *A. fumigatus* is likely to correspond to an endo-acting enzyne since it contains the motifs WMNEPNG (Block A) and FTGT (Block D), which appear to be characteristic features of fungal endoinulinases (Fig. 1). The conserved motif, SVEVF (Block G), present in the C-terminal half of majority of the inulinases (both exo- and endo-acting), has been reported to be present only in those enzymes which attack inulin and levan but not sucrose and raffinose (*113*). This sequence, therefore, may be important for binding of the high M_r fructans.

To establish the evolutionary relationship among the inulinase genes cloned so far, a phylogenetic tree was constructed from the deduced amino acid sequences (Fig. 2). The fungal endoinulinases are distinct from the bacterial endoinulinases and fungal exoinulinases. The deduced amino acid sequence from the genes encoding endoinulinases of *A. niger* (*109*), *A. ficuum* (*107*), *P. purpurogenum* (*102*), and *A. fumigatus* (EA 186248) revealed approximately 62–99 % identity as compared to 18–20 % identity of their bacterial counterparts (*Arthrobacter* sp. and *P. mucidolens*). The phylogenetic analysis suggests an evolutionary divergence of endoinulinase genes between *A. fumigatus* and *A. niger/A. ficuum* (Fig. 2) with the latter showing common origin with the *Penicillium* endoinulinase genes. A common origin between *Penicillium* sp. and *A. awamori* is also observed for exo-acting inulinases with an identity of about 58 % at the amino acid level. However, the gene for cell wall inulinase of *K. marxianus*, which showed 67 % similarity with the SUC2 invertase of *S. cerevisiae* at amino acid level (*114*), is only 27 and 29 % identical with the exoinulinase genes of *Penicillium* sp. TN-88 and *A. awamori*, respectively. The exoinulinase genes of *B. licheniformis* (*106*) and *B. longum* show almost 100 % identity, thus suggesting conservation of this gene during evolution.

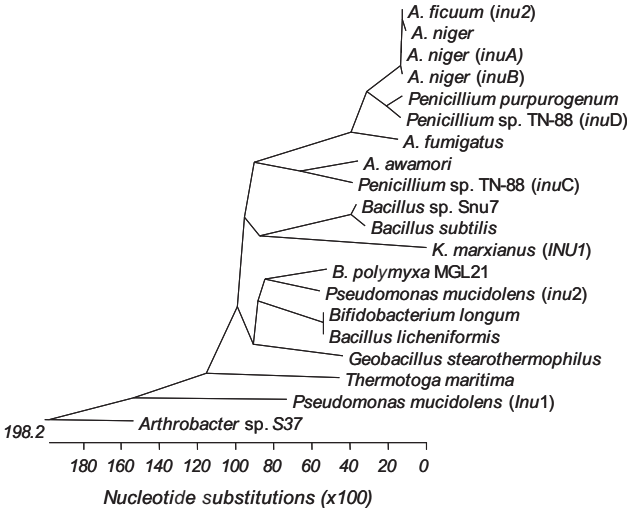

Fig. 2. Rooted tree showing phylogenetic relationship among deduced amino acid sequences of inulinase from *Aspergillus ficuum* (CAA07345), *Aspergillus niger* (AAL34524), *Arthrobacter* sp. S37 (AJ131562), *Penicillium purpurogenum* (BAA12321), *Penicillium* sp. TN-88 (BAA19132), *Pseudomonas mucidolens* (AAF24999), *Aspergillus awamori* (CAL44220), *Aspergillus fumigatus* (EAL86248), *Aspergillus niger* (BAA33798), *A. niger* (BAA33797), *Bacillus polymyxa* (AAL82575), *Bacillus licheniformis* (AAU24331), *Bacillus* sp. Snu7 (AF234992), *Bacillus subtilis* (AF366292), *Bifidobacterium longum* (NP695334), *Geobacillus stearothermophilus* (BAC45010), *Kluyveromyces marxianus* (CAA48500), *Penicillium* sp. TN-88 (BAC16218), *Pseudomonas mucidolens* (AAF44125), *Thermotoga maritima* (AJ001073). The phyllogenetic tree was generated by ClustalW analysis with the MegAlign tool of Lasergene's DNASTAR programme using Gonnet series, slow/accurate matrix with default parameters. The length of each pair of branches represents the distance between sequence pairs

Although two-thirds of the fungal genes sequenced so far have been shown to contain short introns (*115*), endoinulinase genes of *A. niger* (*109*), *A. ficuum* (*107*), and *P. purpurogenum* (*102*) lack introns, as contrary to the gene of *A. awamori* exoinulinase, which contain a short intron (*50*). Of the two endoinulinase genes of *A. niger*, *inuA* and *inuB*, which differ only by 23 nucleotides resulting in a change of eight amino acids (*109*), the transcripts were detected only for *inuB*, thus indicating that *inuA* may be a pseudogene or is expressed under specific conditions. Furthermore, the transcription of *inuB* starts at multiple points which are specifically regulated by the different substrates, *viz.* inulin, fructose and glucose, thus implying that complex regulatory mechanisms are involved in the synthesis of this enzyme.

Conclusion

The availability of thermostable inulinases is a prerequisite for production of fructose and inulooligosaccharides by enzymatic hydrolysis of inulin. Although the inulinases produced, in particular by the fungi, *viz. A. fumigatus, A. niger*, *S. acidophilum* and *A. ficuum*, and by the bacterium *T. maritima* are highly thermostable, further studies are required before their potential can be exploited at the industrial level. Furthermore, the inulinases characterized so far show substantial variability in their biophysical and biochemical characteristics which can be attributed to post-translational modifications as well as differences at the gene level.

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