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Recent Developments in Microbial Inulinases

Its Production, Properties, and Industrial Applications

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Abstract

Microbial inulinases are an important class of industrial enzymes that have gained much attention recently. Inulinases can be produced by a host of microorganisms, including fungi, yeast, and bacteria. Among them, however, *Aspergillus* sp. (filamentous fungus) and *Kluyveromyces* sp. (diploid yeast) are apparently the preferred choices for commercial applications. Among various substrates (carbon source) employed for their production, inulin-containing plant materials offer advantages in comparison to pure substrates. Although submerged fermentation has been universally used as the technique of fermentation, attempts are being made to develop solid-state fermentation technology also. Inulinases catalyze the hydrolysis of inulin to D-fructose (fructose syrup), which has gained an important place in human diets today. In addition, inulinases are finding other newer applications. This article reviews more recent developments, especially those made in the past decade, on microbial inulinases—its production using various microorganisms and substrates. It also describes the characteristics of various forms of inulinases produced as well as their applications.

Index Entries: Microbial inulinases; production; microorganisms; substrates; properties; applications.

Introduction

Inulin, a polyfructan consisting of linear β-2,1-linked polyfructose chains, terminated by a glucose residue attached through a sucrose-type

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linkage, is a reserve carbohydrate in the roots and tubers of plants such as Jerusalem artichoke, chicory, or dahlia. Such inulin sources have recently received much interest as renewable raw material for the production of fructose syrup (D-fructose) or other chemicals such as ethanol. Inulin is insoluble in water, and owing to variation in chain length, its molecular weight varies between ±3500 and 5500.

D-Fructose is occupying an increasingly important position in the modern world as a sweetener *(1)* because it offers several advantages, such as its natural occurrence both as a free sugar in the majority of fruits and as a polymer in several plants, its higher sweetening value, its physiological metabolism in the human body, and its insignificant insulinogenic effects.

Microbial inulinases (2,1-β-D-fructan fructanohydrolase [EC 3.2.1.7] are usually inducible and exo-acting enzymes, which catalyze the hydrolysis of inulin by splitting off terminal fructosyl units (D-fructose) by cleaving the glycosidic linkages in polymer moiety. Thus, microbial inulinases play an important role in the hydrolysis of inulin for its commercial exploitation. In a recent review, Storey and Schafhauser *(2)* termed microbial inulinases as an important class of industrial enzymes.

Microorganisms Employed for Inulinase Production

A number of fungal, yeast, and bacterial strains have been used for the production of inulinases (*see* Table 1). Among them, fungal strains belonging to *Aspergillus* sp. (filamentous fungus) and yeast strains belonging to *Kluyveromyces* sp. (diploid yeast) are apparently the most common and preferred choice. Viswanathan and Kulkarni *(3)* isolated several fungal and yeast strains from dahlia rhizosphere belonging to *Aspergillus*, *Penicillium*, *Sporotrichum*, *Cladosporium*, and *Streptomyces* sp. and reported *A. niger* as the highest inulinase producer (75 U/mL). When culture was grown in medium with inulin and corn steep liquor, the enzyme yields were 1.5-fold more than those obtained with synthetic medium *(4)*. In general, fungal strains produced higher extracellular inulinase in less time than the actinomycetes. On ultraviolet radiation, the *A. niger* strain resulted in 3.1 times more enzymes in comparison to the parent strain *(5)*. Nakamura et al. *(6)* also reported higher enzyme titers from a mutant strain of *A. niger* 817. Gupta et al. *(7)* compared the thermostable inulinase production by several strains of *Aspergillus* sp., namely, *A. fischeri*, *A. aureus*, *A. flavus*, *A. niger*, and *A. nidulans*. All five strains showed maximum production of 1000–1200 U/L of extracellular enzyme after 9 d of growth. Ongen-Baysal et al. *(8)* achieved very high yields of inulinases (4600 U/g) from a culture of *A. niger* A42. Korneeva et al. *(9)* reported a new strain of *A. awamori* that was capable of carrying out intensive inulinase synthesis. The enzyme carried out complete hydrolysis of inulin to fructose. Novo Industries, Denmark, has developed a commercial preparation of inulinase enzyme from *A. ficuum* (Novozym 230) *(10)*. Kim and Rhee *(11)* have also used *A. ficuum* inulinase for the production of fructose syrup. Elferink et al. *(12)* have used another strain of *Aspergillus* sp., *A. phoenicis*, for inulinase production.

Table 1
Microorganisms Employed for Production of Inulinases

Microorganism	Nature of enzyme and maximal activities[a]	Ref.
Fungi		
Aspergillus sp.	E, 75, 258[b]	*3,19,55,56*
A. aureus MTCC 151	E, 160	*7*
A. awamori 808	E	*9*
A. ficuum	E, 3000,[b] 3366[b]	*10,11*
A. fischeri MTCC 150	E, 1-1.2	*7*
A. flavus MTCC 277	E, 1-1.2	*7*
A. nidulans MTCC 344	E, 1-1.2	*7*
A. niger 817	E, 68.5[c]	*57*
A. niger A42	E, 4600,[b] 43.7	*8,58*
A. niger MTCC 281	E, 1-1.2	*7*
A. niger mutant 817	E, 160	*6*
A. niger mutant UV1	E, 374	*5*
A. niger van Teighem	E, 120	*4*
A. phoenicis CBS294.80	E	*12*
Cladosporium sp.	E	*3*
Chrysosporium pannorum	E, 10.9	*62*
Fusarium sp.	E	*19*
F. oxysporum NCIM 1072	E, I, 80,[c] 21,[d] 63.3[d]	*20–22*
Penicillium sp.	E	*3,13,19*
Penicillium sp. 91-4	E, 50	*15*
P. purpurogenum var. rubisclerotium	E, 3.74	*14*
P. purpurogenum var. rubisclerotium	E	*18*
P. rugulosum	E, 54	*17*
P. trzebinskii	E, 11	*16*
Streptomyces sp.	E, 32	*3*
S. rochei E87	E, 1	*23*
Bacteria		
Acetobacter sp.	E	*19*
Achromobacter sp.	E	*19*
Arthrobacter sp.	E	*39*
Bacillus sp.	E, I, 5.14	*41*
B. subtilis 430 A	E, I, 50-70	*47,48*
Clostridium acetobutylicum IFP 912	E, I, 43.7	*52*
C. acetobutylicum ABKn8	E, 6.06	*51*
C. pasteurianum var. I-53	E	*61*
C. thermoautotrophicum T1	E, I	*49*
C. thermosuccinogenes	E, I, 11[c]	*50*
Escherichia coli	I	*53*
Flavobacterium mulivorum	I, 456[c]	*54*
Pseudomonas sp. 65	E, 15[b]	*46*
Staphylococcus sp.	E, 634[c]	*42–45*
Yeast		
Candida sp.	E	*19*
C. kefyr DSM70106	E	*18*

(continued)

Table 1 *(continued)*

Microorganism	Nature of enzyme and maximal activities[a]	Ref.
Yeast		
C. pseudotropicalis IP513	E, 25,000[b]	*60*
Kluyveromyces fragilis	E, I, 58,[e] 7, 29,[f] 46[g]	*30,31,34,35*
K. fragilis ATCC 12424	E, I, 355, 1000,[b] 19[e]	*37,60,63*
K. fragilis CCY 51-1-4	E, I	*33*
K. lactis	E, I	*64*
K. marxianus	E, 43.7, 38,[e] 418[c]	*58,64,65*
K. marxianus	E, I, 56[e]	*27*
K. marxianus ATCC 36907	E, 260	*25*
K. marxianus ATCC 52466	E, 418[c]	*42–45*
K. marxianus CDBB-L-278	E, 82	*26*
K. marxianus var. marxianus CBS 6556	E, 3000	*29*
K. marxianus CBS 6556	E, I, 58[e]	*30*
K. marxianus UCD (FST) 55-82	E, I, 212	*28*
Pichia sp.	E	*19*

[a]Units/mL, unless otherwise specified. E, extracellular; I, intracellular.
[b]Units/gram.
[c]Units/liter.
[d]Specific activity.
[e]Units/milligram.
[f]Total activity from ground cell and medium in wild type.
[g]Total activity from ground cell and medium in mutant.

Several species of *Penicillium* have also been used for inulinase synthesis *(3,13–19)*. The enzyme produced by all the species was recovered from the culture broth, i.e., extracellular in nature. Wenling et al. *(13,15)* isolated a high-inulinase-yielding species of *Penicillium*, designated as 91-4, from soil. Inulinase preparation from *P. ruglosum* proved to be a better choice for preparation of high fructose syrup than *A. niger* or *Penicillium* sp. *(17)*. Gupta et al. *(20–22)* prepared inulinase from *Fusarium oxysporum* and found that the nature of enzyme, extra- or intracellular, varied with the substrate used for cultivation. Only 14% of the enzyme was present in the culture medium and the remainder was obtained from the mycelial mat.

Actinomycetes have also been used for inulinase production *(3,23,24)*. *Streptomyces* sp. required a longer cultivation time for optimal yields of enzyme *(3)*. Yokota et al. *(23)* isolated a culture of *S. rochei* E87 from a soil sample that produced extracellular inulinase. The enzyme was able to degrade inulin into inulotriose as the major end product.

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

Passador et al. *(25)* used a microtiter reader system for the screening of yeast belonging to the genera of *Kluyveromyces*, *Candida*, *Debaryomyces*, and *Schizisaccharomyces*. Four strains belonging to *K. marxianus* (CBS 6397, DSM 70792, ATCC 36907, and IZ 619) were found suitable; on the basis of the results obtained, strain ATCC 36907 was found to be the most suitable for inulinase production. A strain of *K. marxianus*, CDBB-L-278 (hyper-producing strain), gave a 3.3-fold increase in enzyme yields in comparison to the control strain, *K. marxianus* NCYC-1429 *(26)*. Enzyme produced by most of the strains was extracellular in nature but Rouwenhorst et al. *(27)* reported only about 50% enzyme in the culture liquid. Parekh and Margaritis *(28)* also reported only 25% of total inulinase activity of extracellular nature; the remaining 75% was cell associated.

Hensing et al. *(29)* employed a strain of *K. marxianus* var. *marxianus*, CBS 6556, for the production of extracellular inulinase in high cell density fed-batch cultures when yields as high as 3,000,000 U/L were achieved. When this strain was grown in a continuous culture in a chemostat under various experimental conditions, a substantial portion of the enzyme was associated with the cell wall; however, this could be released from the cell wall by a simple chemical treatment *(30)*.

Gupta et al. *(31)* screened *K. fragilis* NCIM 3217, *K. marxianus* NCIM 3231, *Hansenula polymorpha* NCIM 3377, *Pichia fermentan* NCIM 3408, *P. polymorpha* NCIM 3419, and *D. castellii* NCIM 3446 for inulinase synthesis and found that only *K. fragilis* was suitable for enzyme production. An enzyme complex capable of lysing yeast cells was produced by *Arthrobacter* sp. containing live cells of *K. fragilis* as the sole nutrient source. Maximum lytic activity appeared after 29 h, but no lytic activity was observed when *Arthrobacter* sp. was grown in standard medium. The yeast cell hydrolysate was used as a source of nutrient for subsequent growth and inulinase production by *K. fragilis*. Thus, waste generated from inulinase fermentation might be used for further production of inulinase *(32)*. Abraham and Klaushofer *(33)* produced an inulinase from *K. fragilis* CCY-51-1-4 (*K. marxianus*) for production of ethanol from Jerusalem artichoke and reported that inulinase activity was not the limiting factor in fermentation. Guiraud et al. *(34)* isolated respiratory-deficient mutant strains of *K. fragilis* LM92 (treatment with ethidium bromide at 2.8 mg/L), and one of the mutants, S23, was characterized with improved inulinase activity (apart from other improved characteristics). Treatment with 0.5% ethylmethanosulfonate of another strain, *K. fragilis* LG, also resulted in mutants with enhanced activity of inulinase synthesis; one of them, D9, produced 1.5 times the amount of enzyme that the parent strain produced and secreted three times that amount into the medium *(35)*.

A comparative study involving a strain of *K. fragilis*, ATCC 12424, and that of *C. pseudotropicalis*, IP513, was conducted for inulinase production in liquid medium, and enzyme titers of 1000 and 25,000 inulinase U/g of dry cells, respectively, were obtained *(36)*. Thonart et al. *(37)* mutated the strain of *K. fragilis* (ATCC 12424) using nitrosoguanidine and obtained 50 mutants.

They found that the most important improvement was intracellular inulinase activity. This activity was notably increased with the KF28 mutant strain. In a study on recovery of extracellular inulinase from *C. kefyr* DSM 70106, which showed good capacity to synthesize the enzyme, it was found that the enzyme could be recovered directly from the culture broth. The presence of yeast cells did not cause any problems *(38)*.

There are reports on production of inulinases using different bacteria, e.g., *Acetobacter* sp., *Achromobacter* sp., *Arthrobacter* sp., *Bacillus* sp., and *Cladosporium* sp. (*see* Table 1). *Arthrobacter* sp. E.M. 278, isolated from chicory roots, was grown in a fermenter, and high amounts of the inulinases were secreted in the medium, which was suitable for fructose production from root explants rich in polyfructosans or sucrose *(39)*. Hauly et al. *(40)* collected microflora from the rotting dahlia tubers and recovered bacterial strains producing difructose anhydrides. They also found an inulinolytic mold (probably an *Aspergillus*) from the same source. Except for the mold, all other Gram (+) and (–) bacteria presented a flat and spreading growth with whitish colonies. The remaining isolates, forming gummy colonies, could not be differentiated within the first 48 h of growth. Baron et al. *(41)* also isolated bacterial strains from rotting dahlia tubers, one of which was characterized by formation of a yellow hue in agar slants (hence named "ŸLW"). The strain, apart from inulinolytic activity, also showed formation of difructofuranose anhydride. The biochemical characterization of the strain showed the presence of two cell-associated glycolipids whose carbohydrate moieties were galactose and mannose. The enzyme produced was successfully immobilized on controlled-pore silica.

Fontana et al. *(42)* proposed polymerase chain reaction protocol- and inulin catabolism–based differentiation on inulinolytic bacteria. They found that bacteria collected from rotting dahlia tubers, instead of degrading inulin to D-fructose, preferentially formed the known difructo furanose anhydride, inulobiose, higher inulo-oligosaccharides, and exoheteropolysaccharides.

One of the advantages of using bacterial strains for inulinase production is the thermophilic nature of the strains. Allais et al. *(43)* isolated four thermophilic strains belonging to *Bacillus* sp. that produced varying amounts of enzyme (73–282 U/L) in liquid culturing. The majority of this activity (76–100%) was extracellular. We, at the Regional Research Laboratory (RRL), have isolated nine bacterial strains belonging to the mesophilic and thermophilic range *(44–46)*. *B. circulans* was suitable for culturing at 45°C in shake flasks for optimal enzyme production *(46)*. Among the eight other strains, belonging to the mesophilic range, three were identified as *Staphylococcus* sp. *(44,45,47)*; this was the first report on the production of inulinase enzyme by *Staphylococcus* sp. Kim et al. *(48)* isolated a strain of *Pseudomonas* sp. that was strongly active for inulinase production at 45°C. A strain of *B. subtilis*, 430 A, also performed best at 45°C when grown in a fermenter. Most of the enzyme (70% of total enzyme) produced by the strain was secreted in the culture medium; that is, it was extracellular, and

all but 9% of the remainder was easily recovered from the cell-soluble fractions *(49,50)*.

Several strains belonging to anaerobic bacteria, *Clostridium* sp., were isolated from different sources. *C. thermoautotrophicum* T1 was isolated from dahlia tuber *(51)*, which showed extra- and intracellular inulinase production. This strain was different from the type strain in many aspects, including optimum temperature for growth and substrate spectrum. Four other closely related thermophilic *Clostridium* strains were isolated from sugar-beet pulp from a sugar refinery, soil around a Jerusalem artichoke, fresh cow excrement, and mud from a tropical pond in a botanical garden. These strains were identified as new strains of *C. thermosuccinogenes (52)*. The enzyme produced by these strains was almost completely cell bound. Efstathiou et al. *(53)* examined 16 strains of *C. acetobutylicum* (isolated from sugar-beet pulp or soil on which dahlia was cultivated) for inulinase synthesis and finally selected one, designated as ABKn8, which produced enzyme with a specific activity of 3.03 U/mg of protein. Most of the enzyme activity was detected in culture supernatant. However, another strain, *C. acetobutylicum* IFP 912, produced higher inulinase activity (than the invertase activity) in the extracellular fraction *(54)*.

There are a few reports describing intracellular inulinase production by some bacterial strains. Yun et al. *(55)* produced intracellular inulinase using a recombinant strain of *Escherichia coli* that possessed endoinulinase genes. Allais et al. *(56)* isolated 32 bacterial strains from different soil samples, and 20 of these were identified as *Favobacterium multivorum*. In all strains, enzyme activity was cell bound and produced at the end of growth phase. Table 1 lists some other strains reported to produce inulinases *(57–68)*.

Substrates Used for Inulinase Production

Although inulin is the most commonly used substrate for the production of inulinase enzyme, a variety of substrates (carbon/energy source) have been used for enzyme production using different microorganisms (*see* Table 2). They include pure substances, naturally occurring inulin-rich materials, and mixed substrates.

Among the pure substrates (which are mostly sugars of mono-, di-, or polysaccharide nature), inulin and sucrose have been employed as the preferred carbon source. In general, if the microbial strain showed only inulinase activity, inulin served as the best substrate *(4,38,44,48,50,67,69–71)*. But, if the microorganism exhibited inulinase activity coupled with invertase activity, sucrose served as a better source for enzyme production. There are many reports describing the use of sucrose as the carbon source primarily for inulinase production *(6,29,30,62,72,73)*.

Poorna and Kulkarni *(4)* carried out a study on the use of various carbon sources, singly or in combination for inulinase production using fractional factorial design. The results suggested that inulinase production by fungal culture was probably inducible and subject to catabolic repres-

Table 2
Substrates Used for Production of Inulinases

Substrate and concentration	Microorganism	Ref.
Pure substrates		
Glucose (1–2%)	*C. thermoautotrophicum*, *K. marxianus*	*28,51,76*
Fructose (1%)	*K. marxianus*, *K. fragilis*	*28,65*
Sucrose (0.2–5%)	*A. niger*, *K. marxianus*, and several other yeasts strains	*6,28–30,59,62, 72,73*
Lactose (1%)	*K. fragilis*, *C. thermoautotrophicum*	*28,51,65*
Maltose (1%)	*C. thermoautotrophicum*	*51*
Fructan (3%)	*F. oxysporum*	*21*
Fructosan (3%)	*F. oxysporum*	*22*
Inulin (0.5–2%)	*A. niger*, *K. marxianus*, *C. kefyr*, *Staphylococcus* sp., *Pseudomonas* sp., and other soil isolates	*4,28,38,44,48, 50,67,69–71*
Caproyl-inulin (0.5%)	*K. marxianus*	*74*
Cholesteryl-inulin (0.5%)	*K. marxianus*	*74*
Natural substrates		
Dahlia rhizosphere (1, 3%)	Several bacterial yeasts and fungal strains	*3,17,46,50*
Jerusalem artichoke (0.2–8%)	*A. niger*, *P. purpurogenum*	*8,18*
Chicory roots (0.2–8%)	*P. purpurogenum*, *S. rochei*, *Arthrobacter* sp., *Staphylococcus* sp., *F. oxysporum*	*18,23,39,71,77*
Kuth roots (1%)	*A. niger*	*78,79*
Wheat bran (40%)[a]	Bacterial strain (16 U/gds)[b]	*71*
	Staphylococcus sp. (108 U/gds)[b]	*61*
	K. marxianus (123 U/gds)[b]	*61*
Mixed substrates		
Inulin (0.25–1.0%) + 2-deoxyglucose (0.01–0.1%)	*K. marxianus*	*26*
Glycerol (0.25–1.0%) + 2-deoxyglucose (0.01–0.1%)	*K. marxianus*	*26*
Chicory roots + fructans (1%)	*K. fragilis*	*31*

[a]Solid-state fermentation.
[b]U/gds, units per gram of dry substrate.

sion. Inulin was found to be the most suitable substrate for enzyme production.

In their studies on microbial inulinase secretion, Fontana et al. *(74)* used chemically modified inulins for enzyme production. Caproyl and cholesteryl derivatives of native dahlia inulin were prepared from the respective chloride donors, and the light derivatization was monitored by ^{13}C-nuclear magnetic resonance and Fourier transform infrared spectroscopy (FTIR). These inulin derivatives were employed as carbon source and as inulinase inducers using different strains of the inulinolytic yeast, *K. marxianus*. Compared to the cholesteryl derivative, caproylated inulin was superior as an inulinase inducer, which gave a 6.8- or 4.9-fold increase in the inulinase titers in the presence or absence of ammonium phosphate, respectively. Fontana et al. *(74)* concluded that inulinase induction or the secretion process is affected by the presence of ammonium phosphate.

In a sucrose-limited fed-batch fermentation using a yeast culture of *K. marxianus (29)* when the temperature was 40°C (in comparison with 30°C), the formation of organic acids, particularly acetic acid, was pronounced. A nonstructured Monod-type equation, describing the relationship between specific growth rate and specific extracellular inulinase production rate, was incorporated in a model for the production of biomass and extracellular inulinase in a high cell density fed-batch culture of yeast strain (grown on sucrose). The model adequately described biomass production. The production of enzyme was slightly higher than predicted by the model *(72)*.

In another sucrose-limited chemostat study, Rouwenhorst et al. *(30)* found that production of the enzyme was negatively controlled by the residual substrate (sugar) concentration. High enzyme activities were seen during growth on nonsugar substrates, indicating that enzyme synthesis was a result of a depression/repression mechanism. A mutant fungal culture of *A. niger* produced high levels of inulinase, irrespective of the C-sources. When sucrose was used as the substrate, the addition of sucrose fatty acid ester in the fermentation medium as surfactant resulted in enhanced yields of the enzyme *(74)*.

Although inulinase synthesis is regulated by both induction and repression, reasonable amounts of enzyme were produced in the absence of inducer with cultures grown on glucose as the sole carbon source *(75)*. Espinoza et al. *(76)* evaluated yeast culture of *K. marxianus* for the simultaneous production of two enzymes (one being inulinase) using glucose as the carbon source. Notably higher activities of inulinases were produced in two-enzyme fermentation. A strain of *C. thermoautotrophicum* could also use glucose for its growth. Although this culture showed best inulinase synthesis using inulin as the C-source, a wide range of substrates supported bacterial growth. These included amygdalin, arabinose, cellobiose, esculin, fructose, galactose, $H_2 + CO_2$, inulin, lactose, maltose, mannitol, melewitose, melbiose, methanol, raffinose, salicin, starch, and sucrose *(51)*.

Tsang and Groot-Wassink *(65)* used fructose and lactose for inulinase production using yeast culture *K. fragilis* and found that inulinase yields were twice as high as the peak yields on lactose. Gupta et al. *(21,22)* cultivated *F. oxysporum* in fructan- and fructosan-containing media for production of inulinase. Both substrates supported the growth and activity of the culture. Of various sugars tested, sucrose showed the greatest inhibitory effect on enzyme synthesis and led to a significant increase in the formation of β-D-fructo furanosidase.

Roots and tubers of several *Compositae* and *Gramineae* appear to be a good source for either direct fermentation for inulinase production or the use of compounds such as fructan and inulin isolated from them. Dahlia (*Dahlia pinnata*) rhizosphere *(13,17,50)*, Jerusalem artichoke (*Helianthus tuberosus*) *(8,18,60,69)*, chicory (*Cichorium intybus*) roots *(18,23,39,77)*, and kuth (*Saussaurea lappa*) roots *(78,79)* have widely been used for this purpose. A new method was developed for the preparation of inulinase by using a mixture of roots of Jerusalem artichoke or chicory (in slurry or powder form at 0.2–4.0%, w/w) and maltose as C-sources. It was claimed that the cost of enzyme production by this method was cheaper than that by inulin *(18)*.

When a strain of *K. marxianus* was cultivated in a medium containing inulin as a unique C-source in the presence of 2-deoxyglucose, the enzyme yields were 3.3 times higher than the medium with only inulin. When glycerol was used as the sole C-source, the inulinase yields were 3.6-fold higher. Although the strain was able to produce enzyme in the presence of 2-deoxyglucose, it was shown that it was not a depressed strain, since enzyme production was reduced when the concentration of glucose or fructose was increased in the medium. Since inulinase was produced in a glycerol medium lacking an inducer, it was thought that enzyme production was partially constitutive *(26)*. Gupta et al. *(31)*, using *K. fragilis*, found that an aqueous solution of chicory roots with 1% fructan was a better C-source for inulinase production than inulin.

Fermentation Techniques

Although the entire commercial production as well as the reported work on microbial inulinases involved submerged fermentation (batch, fed-batch, or continuous mode) as the technique of fermentation, we, at the RRL, have successfully cultivated a bacterial strain in solid-state fermentation (SSF) using wheat bran as substrate *(61,71)*. It has been reported that SSF offers several advantages in comparison to liquid fermentation *(80–82)*. Different other sources, including chicory roots as substrate, have also been evaluated for the cultivation of RRL bacterial isolates, and a strain of *K. marxianus* and preliminary results showed reasonably good success (unpublished data). In these studies, when wheat bran was used as the C-source, 16 U of enzyme/g of dry fermented substrate were produced *(71)*. Further improvements in process parameters and nutritional conditions led to a 6.5-fold increase in enzyme yields by a strain of *Staphylococcus* sp. *(61)*.

Purification and Properties of Inulinases

Most of the reports on purification of extracellular inulinases produced by fungi, yeast, and bacteria deal with the conventional method of centrifugation and/or ultrafiltration, salt, or solvent precipitation, followed by column chromatography *(13,17,22,31,38,49,50,58,73,83)*. Intracellular inulinases needed the usual step of cell wall destruction and then followed the similar procedures. Table 3 summarizes the properties of some inulinases, isolated from different sources, as reported by many authors.

Inulinases of fungal origin have mostly been extracellular and have generally been exo-acting. However, Gupta et al. *(22)* found only 14% of total inulinase activity in a strain of *F. oxysporum* as extracellular; the remaining activity was mycelial bound. Both inulinases (extra- and intracellular) were purified by column chromatography on a Sephadex G-100 and yields between 65 and 75% were obtained. Both inulinases also hydrolyzed sucrose, raffinose, and stachyose and were primarily exo-acting. Wenling et al. *(13)* purified and characterized an extracellular inulinase from *Penicillium* sp. culture broth using ammonium sulfate precipitation, ion-exchange chromatography on DEAE-Sepharose and DEAE-Sephacel, ultrafiltration, and Sephadex G-100 gel filtration. Two forms of inulinase were obtained, which were further purified by preparative polyacrylamide gel electrophoresis. These were distinct from each other in several characteristics (*see* Table 3). The enzyme was a glycoprotein with a higher sugar content (from 21 to 26%). Both sucrose and raffinose could be hydrolyzed, and both forms of the enzyme were exo-acting. Barthomeuf et al. *(17)* purified *P. rugulosum* inulinase from the supernatant by centrifugation, ultrafiltration, and acetone precipitation with a yield of 32%. The enzyme was stable for 2 h at 50°C, lost 12% of its activity after 2 h at 55°C, and was totally inactivated after 30 min at 60°C. A crude inulinase preparation from *Aspergillus* sp. was dialyzed for 48 h and the lyophilized enzyme powder was loaded onto a CM-Sepharose column for ion-exchange chromatography. This step was selective for separating two forms of the enzyme. After high-performance gel permeation chromatography, endo- and exo-inulinases were obtained in 5 and 13.9% yields, respectively. Both forms exhibited invertase activity as well *(58)*. Two forms of inulinase were also reported from the culture broth of *A. niger* by chromatography on a DEAE-cellulofine A-500 column and then further separated on a Q-Sepharose HP column with yields of 18.2 and 18.9% for P-IA and P-IB, respectively *(73)*. Xiao et al. *(64)* purified and characterized an endoinulinase from *C. pannorum*. The enzyme was a glycoprotein and had a pI at pH 3.8. The enzyme was active on inulin but not on levan or sucrose, and catalyzed the production of inulotriose, inulotetraose, and inulopentaose.

Inulinases obtained from yeasts showed a mixed form; that is, they could be extra-, or extra- and intracellular. Pessoa et al. *(38)* studied purification of an extracellular inulinase produced by yeast cells by adsorption of culture broth on ion-exchange adsorbents in the absence and presence of

Table 3
Properties of Inulinases Produced by Some Microorganisms

Source	Forms	Mol wt	pH range	pH optima	Temperature range (°C)	Temperature optima (°C)	K_m (mmol/L)	Specific activity (U/mg)	Purification fold	Metal inhibition	Ref.
Penicillium sp.	EI	60,000	4.0–7.4	4.5	Below 50	50	1.12/0.47[c]	—	30.0	Ag^+, Cu^{2+}	*13*
	EII	65,000					1.63/0.75[d]				
Penicillium sp.	—	—	5.0–7.0	5.6	Below 45	45	—	—	—	Mn^{2+e}	*15*
K. marxianus	—	—	—	4.4	—	55	11.9[c], 3.92[d]	49.0	—	—	*64*
K. fragilis	—	250,000	—	—	—	55	—	—	26.0	—	*31*
F. oxysporum	—	—	—	5.5–6.5[b]	—	37, 45[b]	0.25–0.44[c,d]	—	—	—	*20*
P. rugulosum	—	—	—	5.5–5.6	—	55	—	—	—	—	*90*
B. subtilis	—	—	—	6.0–7.0	—	45–50	—	—	—	Cu^{2+}, Hg^{2+}, Ag^+, Mg^{2+}, Co^{2+}, Na^+	*48*
F. oxysporum	—	—	—	6.0, 6.2[b]	—	37, 45[b]	—	—	—	—	*21*
Chrysopannorum	—	58,000	4.5–8.5[a]	6.0–7.0	—	50	—	—	—	—	*62*
Aspergillus sp.	Endo-	53,000	—	—	—	—	570[d]	150.0	18.5	Fe^{3+e}	*66*
	Exo-	81,000					60[d]	258.0	31.9	Mn^{2+}, Mg^{2+}	
F. oxysporum	Extra-	300,000	—	6.2	—	37	—	63.3	—	—	*22*
	Intra-	300,000		5.8		30		21.0			
C. acetobutylicum	Extra-	—	—	5.5	—	47	32[c], 6[d]	—	—	—	*52*
	Intra-						44[c], 7[d]				
C. acetobutylicum	—	—	—	4.8	—	—	0.012[d]	—	—	—	*51*
A. niger	P-IA	70,000	—	5.0	—	40	0.48[d]	350.0	177.0	—	*71*
	P-IB	68,000		5.0		40	0.50[d]	340.0	170.0		

[a]For 24 h.
[b]Immobilized enzyme.
[c]K_m for sucrose.
[d]K_m for inulin.
[e]Enzyme activation.

cells. The favorable adsorption pH was 6.5–7.0 using the weak anion exchanger DEAE, and pH 4.0 for the strong cation exchanger (Streamline SP). Inulinase could be purified 93% directly from the culture broth without cell removal with enrichment and concentration factors 5.8 and 2.8, respectively. *K. marxianus* inulinase was extracellular, which was fractionated from the fermented broth using acetone, yielding greater than 81% enzyme activity *(66)*. An enzyme preparation from *K. fragilis* was purified from the fermented broth by ethanol precipitation and chromatography on Sephadex G-200, DEAE-cellulose, and CM-cellulose. The enzyme was primarily exo-acting. Purification of an endo-inulinase (isolated from a commercial preparation) was carried out at 4°C by CM-Sepharose CL-6B and DEAE-cellulose column chromatography, followed by dialysis in 1% glycine. After isoelectric focusing and gel filtration on a high-performance liquid chromatography Bio-Sil SEC-250 column, high-purity enzyme was achieved *(83)*.

Like yeast inulinases, extra- as well as extra- and intracellular bacterial inulinases have been reported. Vullo et al. *(49)* also used acetone (40%, v/v) for partial purification of inulinase (precipitation after ammonium sulfate fractionation [85%]) from a bacterial source. About 70% of the total activity was extracellular and all but 9% of the remainder was easily recoverable from cell-soluble fractions. Enzyme activity decreased 20% after 7 h at 45 or 50°C and was rapidly lost at 55°C. Total enzyme activity was inhibited by a fructose concentration of 14 m*M*. Looten et al. *(54)* reported extra- and intracellular inulinase from a bacterial strain of *C. acetobutylicum*. Enzyme showed pH and temperature optima as 5.5 and 47°C, respectively. Extracellular inulinase showed high specificity for long-chain inulofructions.

There are reports on characterization of immobilized inulinases obtained from various fungal, yeast, and bacterial sources. Kim and Rhee *(11)* characterized the immobilized inulinase from *A. ficuum*. The immobilized enzyme exhibited 23% of initial enzyme activity and was best active at pH 4.5. Gupta et al. *(31)* immobilized *K. fragilis* inulinase using the metal-link chelation method on cellulose. Only 40% of inulinase could be immobilized, which showed a half-life of 5 d at 25°C. Baron et al. *(41)* studied immobilization of a fungal inulinase-I and bacterial inulinase-II using controlled-pore silica. Immobilized enzymes showed a different pattern of inulin monomerization as well as different pH and temperature optima.

From the literature, it can be seen that although much work has been done on the purification of fungal inulinases, as well as on inulinases from yeast sources (but not as much as for fungal), not much work has been done on the characterization of bacterial inulinases. Selvakumar and Pandey *(47)* have reported a new source of bacterial inulinases, which was claimed to be a good source of enzyme. Work on its purification and characterization is ongoing. In general, there has not been much difference in several properties such as pH and temperature optima of inulinases obtained from different microbial sources, i.e., fungi, yeasts, and bacteria. Several fungal strains showed a pH optima between 4.5 and 7.0, for yeast strains it was between 4.4 and 6.5, and for bacterial strains it was between 4.8 and 7.0

(Table 3). In the case of temperature optima, it was in the mesophilic as well as thermophilic range for fungal inulinases. Bacterial and yeast inulinases generally showed higher temperature optimas (Table 3).

Applications of Inulinases

Industry uses large quantities of natural polysaccharides, and new sources of polysaccharides are being sought. In recent years, attention has been directed toward producing extracellular polysaccharides by microbial fermentation. The sugar industry has recently faced intense competition from high-fructose syrup, which is used as a low-cost alternative sweetener. Inulinase offers interesting perspectives in view of the growing need for the production of pure fructose syrups and may present an alternative way to produce the so-called Ultra High Fructose-Glucose Syrups—not from starch, but from inulin. Chemical (acid) hydrolysis of inulin to fructose displays several drawbacks. Acid hydrolysis has always resulted in an undesirable coloring of the inulin hydrolysate. Changes in taste and aroma are minimal on enzymatic hydrolysis. Acid hydrolysis also results in the formation of ~5% difructose anhydride, which has practically no sweetening properties. This compound cannot be further hydrolyzed enzymatically into fructose. Consequently, a forced interest has developed toward the microbial inulinase enzyme and its production.

Thus, the production of fructose syrup from inulin or inulin-rich materials *(6,11,19,49,84)* is the major area of application for inulinases. Conversion of starch to fructose requires three different enzymes (and reaction parameters): alpha amylase, glucoamylase, and glucose isomerase. The yields obtained are ~45% fructose at the maximum. Fructose formation from inulin is a single-step reaction (enzymatic hydrolysis using inulinase). It hydrolyzes the inulin into practically pure fructose, and yields as high as 95% fructose are easily achievable. Fructose is attributed to be a safe sweetener compared to sucrose, which causes problems related to corpulence, carcinogenicity, atherosclerosis, and diabetes.

Nakamura et al. *(6)* developed a process for the continuous production of fructose syrups from inulin by using immobilized inulinase from *A. niger*. Production was carried out in a packed-bed column reactor, and the volumetric productivity was 410 g of reducing sugars/(L · h). Kim and Rhee *(11)* also used an immobilization technique for the production of fructose production from Jerusalem artichoke. They evaluated the batch and continuous processes using *A. ficuum* inulinase immobilized on chitin. In a packed-bed column reactor, the volumetric productivity was 61 g/(L · h). Inulinases from various bacterial, fungal, and yeast sources were used for fructose production from the roots and tubers of Jerusalem artichoke and chicory *(19)*. Vullo et al. *(49)* also described a bacterial inulinase that was suitable for fructose production. Recently, Partida et al. *(84)* described a method for the production of fructose syrup from agave plant pulp, which involved hydrolysis of polyfructose extract of the pulp with inulinase.

The syrup was claimed to be of high purity with desirable color, stable over time, and suitable for human consumption in a wide variety of foods and beverages.

Inulinases have been used for the production of ethanol as well. The process generally involves simultaneous saccharification and fermentation *(ssf)* using inulinase or an inulinase-producing microorganism in combination with other microorganisms from different substrates *(34,59,85–91)*. Guiraud et al. *(34)* used a respiratory-deficient *K. fragilis* mutant for the production of ethanol from Jerusalem artichoke. Under anaerobic conditions, ethanol production was always higher by the mutant than the wild-type strain. Nakamura et al. *(59)* carried out *ssf* of Jerusalem artichoke using *A. niger* and *S. cerevisiae*. Ethanol concentrations of 10.4, 15, and 20.1% were obtained from ground tubers, juice concentrate, and flour, after 15, 72, and 120 h. Pejin et al. *(85)* prepared the juice of tubers of Jerusalem artichoke and treated it with inulinases. The treated juice was fermented to ethanol using *S. cerevisiae*. Untreated juice was fermented using *K. marxianus*. Ethanol productivities of 0.46 and 0.47 g/g were obtained, respectively. The process with *K. marxianus* required less energy because it did not require inulin degradation in the juice by inulinase. Ohta et al. *(86)* reported a process for the production of high concentrations of ethanol from inulin by *ssf* using *A. niger* and *S. cerevisiae*. The maximal volumetric productivities of ethanol were 6.2 and 6.0 g/(L · h) for chicory and dahlia inulins, respectively. The theoretical yields in this case were 83 to 84% in comparison with 95% using *S. diastaticus (87)*. Kim and Rhee *(88)* reported ethanol production from Jerusalem artichoke in *ssf* by coimmobilizing inulinase and *Z. mobilis* on colloidal chitin. The maximal ethanol productivity of the continuous *ssf* process was 55.1 g/(L · h) with 55% conversion yield. At a conversion yield of 90%, productivity was 32.7 g/(L · h). Allais et al. *(89)* also used inulinase to hydrolyze the juice of Jerusalem artichoke and fermented it with *Z. mobilis* for ethanol production. They achieved a volumetric productivity of 84 g/(L · h). Park and Baratti *(90,91)* used inulinase for the hydrolysis of sugar-beet molasses to prevent levan formation, which was then fermented to ethanol by *Z. mobilis*.

Inulinases also find their application for the production of inulo-oligosaccharides that have macrophage-activating and lipid-removing activities *(18,23,83,91)* as well as products such as gluconic acid, pullulan, and acetone-butanol *(30,63,92)*. Kim and Kim *(92)* reported continuous production of gluconic acid and sorbitol from Jerusalem artichoke and glucose using a strain of *Z. mobilis* and inulinase. Shin et al. *(93)* reported production of exopolysaccharide pullulan from inulin by a mixed culture of *Aureobasidium pullulans* and *K. fragilis*. Oiwa et al. *(63)* isolated a strain of *C. pasteurianum* var. I-53 (FERM P-9074) that showed extracellular inulinase activity and was able to produce acetone and butanol from inulin simultaneously.

Chung et al. *(94)* constructed expression and secretion vectors to study the use of inulinase signal peptide for the secretion of human recombinant

proteins from *S. cerevisiae*. New secretion signal peptides for the secretion of foreign proteins were also derived by using inulinase genes of *K. marxianus (95)*. Baron et al. *(41)* prepared difructose anhydride from an inulinase-producing bacterial strain that was a natural but rare anhydro form of inulobiose *(42)*. The compound attained importance because of its less polar structure and ability to interfere in fructose metabolism. These features brightened the possibilities of its application in diet as well as a substrate for glycoderivatization *(96)*.

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