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Comparative study of fungal strains for thermostable inulinase production

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Fructose and fructo-oligosaccharides (FOS) are important ingredients in the food industry. Fructose is considered an alternative sweetener to sucrose because it has higher sweetening capacity and increases iron absorption in children, and FOS's are a source of dietary fiber with a bifidogenic effect. Both compounds can be obtained by enzymatic hydrolysis of inulin. However, inulin presents limited solubility at room temperature, thus, fructose and FOS production is carried out at 60°C. Therefore, there is a growing interest to isolate and characterize thermostable inulinases. The aim of this work was to evaluate the capacity of different fungal strains to produce potential thermostable inulinases. A total of 27 fungal strains belonging to the genera *Aspergillus*, *Penicillium*, *Rhizopus*, *Rhizomucor* and *Thermomyces* were evaluated for production of inulinase under submerged culture using Czapek Dox medium with inulin as a sole carbon source. Strains were incubated at 37°C and 200 rpm for 96 h. Crude enzyme extract was obtained to evaluate inulinase and invertase activity. In order to select the fungal strain with the highest thermostable inulinase production, a selection criterion was established. It was possible to determine the highest inulinase activity for *Rhizopus microsporus* 13aIV (10.71 U/mL) at 36 h with an optimum temperature of inulinase of 70°C. After 6 h at 60°C, the enzyme did not show any significant loss of activity and retained about 87% activity, while it only retains 57% activity at 70°C. According to hydrolysis products, *R. microsporus* produced endo and exo-inulinase.

[**Key words:** Inulin; *Rhizopus microsporus*; Exoinulinase; Endoinulinase; Thermal stability]

Inulin is a widespread, naturally occurring reserve of polyfructan found in plants. It consists of linear β-2,1-linked polyfructose chains, containing a terminal glucose unit. It can be hydrolyzed by two types of enzymes: exo-inulinase (β-D-fructan fructanohydrolase, EC 3.2.1.80) which split-off the terminal β-(2,1) fructofuranosidic bonds yielding fructose, and endo-inulinase (2,1-β-D-fructan fructanohydrolase, EC 3.2.1.7), which hydrolyze the internal linkages of inulin and release oligosaccharides (1). These oligosaccharides, called FOS, are made of 1–3 fructose units bonded to one molecule of sucrose, and they are classified as 1-kestose (GF2), nystose (GF3) and 1^F fructofuranosyl nystose (GF4) (2).

FOS are widely used in many countries as food ingredients since they have many health benefits such as promoting a good balance in the intestinal flora, inducing proliferation of intestinal bifidobacteria (probiotics). Currently, oligosaccharides have received GRAS status (generally recognized as safe) by the FDA (Food and Drug Administration) and have an approximated value of 200 US dollar per kg (3). Inulin has been proposed as a feedstock for production of inulo-oligosaccharides (IOS) through action of endo-inulinases; however, inulin is insoluble in cold water and only slightly (5%) soluble in water at 55°C. Unfortunately, only a few inulinases required for industrial applications have an optimum temperature of 50°C or higher (4–6). Therefore, the search for thermostable inulinase producers has received increasing attention.

Microbial inulinases have been reported from filamentous fungi *Aspergillus fumigatus*, *Aspergillus niger* NK 126 (7), *Penicillium* sp. TN-88 (8), *Rhizopus* sp. TN 96 (9), yeast *Kluyveromyces marxianus* (10), *Cryptococcus aureus* G7a (11) and bacteria *Streptomyces* sp. (5), *Bacillus* sp. (12), *Staphylococcus* sp. (10). However, among the diverse microbial strains, *K. marxianus* and *A. niger* are reported as the most common and preferred microorganisms for inulinase production (13–15). The aim of this work was to evaluate the potential of twenty seven Mexican fungal strains to produce thermostable inulinases.

MATERIALS AND METHODS

Microorganisms Twenty one fungal strains isolated from plants and soil and belonging to the Food Research Department-UAC collection (16) and six thermotolerant and thermophilic strains from the Chemical Engineering Department-CUCEI (17) culture collection were tested for inulinase production (Table 1). The strains were maintained on potato dextrose agar, while spores were mixed with a solution of skinned milk (9%) and glycerol (1%) and kept at −80°C.

Evaluation for inulinase production Screening experiments were carried out in triplicate using a rotary shaker and a 250 mL Erlenmeyer flask with 30 mL of culture medium, with the following composition (gL^{-1}): inulin 10.0, $NaNO_3$ 7.65, KH_2PO_4 3.04, $MgSO_4$ 1.52, and KCl 1.52. The pH of culture medium was adjusted to 5.0 before sterilization. Flasks were inoculated with a spore suspension containing 10^7 spores mL^{-1} and incubated in a rotary shaker at 37°C and 200 rpm for 96 h.

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TABLE 1. Fungal strains tested for inulinase production.

Code	Strain	Collection
Mesophili		
Aa20	*Aspergillus niger*	UAC
GH1	*Aspergillus niger*	UAC
PSH	*Aspergillus niger*	UAC
GS	*Aspergillus fumigatus*	UAC
ESS	*Penicillium citrinum*	UAC
NH4	*Aspergillus oryzae*	UAC
AN1	*Aspergillus niger*	UAC
AN5	*Aspergillus aculeatinus*	UAC
AN9	*Aspergillus niger*	UAC
AN13	*Aspergillus niger*	UAC
AN15	*Aspergillus niger*	UAC
AN22	*Aspergillus aculeatus*	UAC
AN45	*Aspergillus niger*	UAC
AN49	*Aspergillus homomorphus*	UAC
AN63	*Aspergillus niger*	UAC
AN103	*Aspergillus aculeatus*	UAC
Thermotolerant		
4a	*Rhizomucor pusillus*	UAC
4b	*Rhizomucor pusillus*	UAC
4c	*Rhizomucor pusillus*	UAC
4d	*Rhizomucor pusillus*	UAC
5a	*Aspergillus fumigatus*	UAC
19aIV	*Rhizopus microsporus* var. *tuberosus*	CUCEI
23aIV	*Rhizomucor pusillus*	CUCEI
13aIV	*Rhizopus microsporus* var. *rhizopodiformis*	CUCEI
43aIV	*Rhizopus microsporus* var. *chinensis*	CUCEI
Thermophilic		
T1.10	*Thermomyces lanuginosus*	CUCEI
5S-2	*Thermomyces lanuginosus*	CUCEI

UAC, Universidad Autónoma de Coahuila; CUCEI, Centro Universitario de Ciencias Exactas e Ingenierías.

Samples for analysis were filtered using filter paper (Whatman no. 1) and then centrifuged at 3000 $\times g$ for 5 min. Supernatants were used as crude enzyme throughout the experiments for enzyme assays.

Enzymatic assay Enzyme activities were assayed by measuring the concentration of reducing sugars released from inulin and sucrose. The reaction mixture containing 0.1 mL of diluted crude enzyme and 0.1 mL of inulin or sucrose (1% w/v in 0.1 M acetate buffer pH 5.0) was incubated for 15 min at 50°C. Reaction was stopped by boiling the sample for 5 min (18). Reducing sugars were subsequently analyzed by 3,5-dinitrosalicylic acid-DNS reagent (19). One unit of inulinase/invertase (U) was defined as the amount of enzyme which produced 1 μmol min^{-1} of fructose/glucose under the assay conditions mentioned above. The ratio between these two activities was expressed as inulin/sucrose (I/S) (20).

Protein determination Protein content was determined as previously reported (21), using bovine serum albumin as a standard.

Statistical analysis All experiments were established under a randomized block design with three replications. The data were analyzed using an analyses of variance (ANOVA) procedure and when needed means comparison was performed using the Tukey's range procedure ($p \leq 0.05$) employing the SAS software version 6.1.7600.

Effect of temperature and thermal stability The effect of temperature on inulinase activity was determined by incubating 0.1 mL of enzyme and 0.1 mL of inulin (1% w/v in 0.1 M acetate buffer, pH 5.0) for 15 min at different temperatures. To determine the thermal stability, enzymes were incubated at different times (0–6 h). Residual activity was estimated under a standard assay conditions after each incubation level, and expressed as relative activity (%).

Determination of hydrolysis products In order to analyze the end products from inulin hydrolysis, a mixture of 100 uL of enzyme and 100 uL of inulin (1% w/v in 0.1 M acetate buffer pH 5.0) were incubated at 50°C. Samples were withdrawn at different time intervals. Products were analyzed using thin layer chromatography (TLC) on silica gel 60 plates. Plates were developed at room temperature with a solvent system containing propanol: water: butanol (12:4:3, v/v/v) (22). Sugar spots were revealed by spraying the plates with diphenylamine-aniline-phosphoric acid reagent and by heating them at 100°C for 5 min (23,24) . High purity 1-kestose, nystose and 1-fructofuranosylnystose (Sigma–Aldrich) were used as standards for identification of low molecular weight oligomers, as well as, fructose and glucose.

RESULTS AND DISCUSSION

Fungal production of inulinase A total of twenty seven fungal strains were screened for inulinase production. Although inulin-hydrolyzing activity has been reported from various microbial strains, yeast (*Kluyveromyces* spp.) and (*Aspergillus* spp.) have showed the best inulinase activity (14). However, in the present study it was observed that thermotolerant and thermophilic strains belonging to *Rhizomucor*, *Rhizopus* and *Thermomyces* genera showed higher inulinase activity than mesophilic strains belonging to *Aspergillus* and *Penicillium* (except for *A. fumigatus*). Inulinase activity ranged from a minimum of 1.52 U/mL (*Penicillium citrinum* ESS) to a maximum of 4.90 U/mL (*A. fumigatus* 5a) (Fig. 1A). *A. fumigatus* 5a also showed high amounts of specific activity (1.37 μmol of fructose released per minute per milligram of protein), followed by *Rhizomucor pusillus* 4a (1.24) and *Thermomyces lanuginosus* 5S-2 (1.23) (Fig. 1B).

Moreover, studies by Fawsi (25) tested the capacity of different fungal genera (*Aspergillus*, *Clodobotryum*, *Curvularia*, *Fusarium*, *Mucor*, *Penicillium*, *Rhizomucor*, *Rhizopus*, *Talaromyces* and *Thielavia*) for inulinase production finding that *Thielavia terrestris* NRRL 8126 (thermophile), and *Aspergillus foetidus* NRRL 337 (mesophile) were the best extracellular inulinase producers (8.42 and 8.36 U/mL respectively), after being grown on a basal medium containing chicory root extract 2% (w/v) as a sole carbon source for six days. Nandogopal and Kumari (26) mentioned that *Aspergillus niveus* and *Penicillium purpurogenum* when grown on chicory root produced the highest inulinase activity at levels of 7.0 and 9.0 U/mL respectively. Gern et al. (27) tested 16 fungal strains reported as endo-inulinase producers. The strain identified as *A. niger* DSM 2466 was selected as the best endoinulinase producer. Ge and Zhang (28) also used an *A. niger* strain and obtained a maximum inulinase activity of 100 U/mL in the presence of S-770 sucrose ester as nutritive substrate added into the fermentative medium. Kumar et al. (29) obtained a maximum inulinase activity of 176 U/mL at a 5% (w/v) inulin concentration in the medium, using a soil isolate fungal strain identified as *A. niger*.

As many microbial preparations of inulinase possess remarkable invertase (S) activity accompanying the inulinase activity (IN), their catalytic activity is described in terms of I/S or S/I ratios (30). When I/S ratio is higher than 10^{-2}, the enzyme complex has a preponderant inulinase activity, while for invertase activity the I/S ratio is lower than 10^{-4} (31). In this study, I/S ratios varied from 0.57 to 2.49, thus, concluding that inulinase activity is dominant in all strains. However, higher I/S ratios were observed for *Aspergillus* strains confirming that they have a stronger preponderant inulinase activity (Fig. 1C). A range of I/S ratio between 0.02 and 7.9 for various microbial inulinases has been previously reported by different authors (32). Ertan and Ekinci (33) also reported I/S ratios of 1.22 for *Alternaria alternata* and *A. niger*, and 1.04 for *Trichoderma harzanium*.

With the aim to select the fungal strains with the highest potential for inulinase production, a selection criterion was established. The response variables [specific activity (SA), inulinase activity (IN) and I/S ratio (IS)] were weighted according to their importance in the process [$S = 4.0$ (SA) + 2.0 (IN) + 1.0 (IS)]. The highest value was given to the specific activity (U/mg protein) as an indicator of purity of the obtained enzyme, and the lowest value to the preponderance of inulinase over invertase activity.

Through the use of ANOVA and later Tukey's multiple range tests for treatment means, it was possible to select *A. fumigatus* 5a, *Rhizopus microsporus*13aIV, *Thermomyces lanoginosus* T1.10 and 5S-2 and encouraged further investigation (Fig. 2).

Kinetic evaluation of inulinase production The four selected strains were evaluated for kinetic production of inulinase using the same medium mentioned above and incubating at 37°C for 96 h at 200 rpm. *R. microsporus* 13aIV showed a maximum inulinase activity of 10.71 U/mL at 36 h of fermentation while *A. fumigatus* SOC-5A showed its maximal enzymatic activity (7.97 U/mL) at 60 h (Fig. 3). On the other hand, *T. lanoginosus* T1.10