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BIOSYNTHESIS OF INULINASES BY *Aspergillus terreus* USING ORANGE PEELS POWDER AS A POTENTIAL SUBSTRATE

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Abstract

Inulinases are an important class of enzymes used in many fields, especially in the food and pharmaceutical industries, to produce fructose syrups. Microbial inulinases are important in the hydrolysis of inulin to produce fructose syrup and FOS. These enzymes are produced by various strains of microorganisms, of which Aspergillus sp. and Kluyveromyces sp. are the most commonly used strains for inulinase production. The goal of the study was to biosynthesis inulinase using the Aspergillus terreus ICCF 262 strain, with inulin and orange peel powder as carbon and energy sources, the enzyme being isolated from the fermentation medium by fractional precipitation with ammonium sulfate followed by purification on DEAE-Cellulose using ion exchange chromatography. Within 7 days of cultivating the fungal strain on a mineral medium containing inulin and orange peel at a final concentration of 2% in the fermentation medium, yields of biotechnological interest were higher than those previously reported in the literature. Through the procedure of isolating and purifying inulinase from the fermentation medium results a specific activity of between 164.6 - 396.4 U / mg protein.

Key words*: inulinases, Aspergillus terreus, biosynthesis, inulin, orange peel powder.*

INTRODUCTION

In the past few years, due of an increase in metabolic problems (cardiovascular disease, obesity, diabetes, hypocalcemia, gout), researchers have turned their attention to creating natural polysaccharides. Thus, the production of high purity fructose syrup was studied by enzymatic hydrolysis of inulin to D-fructose, using immobilized inulinase. Fructose is useful to diabetics, obese people, boosts calcium absorption and bifidobacteria growth, and promotes iron absorption in children (Chi et al., 2009).

Inulinases are an important class of enzymes used in many fields, especially in the food and pharmaceutical industries, to produce fructose syrups. The inulinases characterized to date (five inulinases) show considerable variability in biophysical and biochemical characteristics. These enzymes hydrolyze the inulin chain's β-(2,1) links to create fructose and fructooligosaccharide (FOS) units. They are known as 2, 1-D-fructan fructanohydrolases and are divided as endo- or exo-inulinases based on the mode of action of the inulin. Endo-inulinases (fructano-hydrolases 2, 1-β-D-fructan; EC 3.2.1.7) are inulin specific and hydrolyze it by breaking the bonds between fructose units far from the polymer chain ends to create oligosaccharides. Exo-inulinases (β-D-fructohydrolases; EC 3.2.1.80), cleave successive terminal units at the non-reducing end of the inulin molecule.

In addition to inulin, exo-inulinases also hydrolyze sucrose and the rest of the fructose in raffinose (Henrissat, 1991; Henrissat & Bairoch, 1993; Pons et al., 1998). Microbial inulinases are important in the hydrolysis of inulin to produce fructose syrup and FOS.

These enzymes are produced by various strains of microorganisms (fungi, yeasts and bacterial strains), such as those of the species *Penicillium, Kluyveromyces, Streptomyces* and *Aspergillus*. Of these, strains belonging to the genera *Aspergillus* and *Kluyveromyces* are the most commonly chosen for inulinase production (Gao et al., 2007; Santisteban et al., 2009; Gern et al., 2001)

Because of their thermostability, bacterial strains are used to produce inulinase. Information on inulinase biosynthesis using

bacterial strains is limited, mainly referring to end-inulinases. *Streptomyces* spp. has been found to be a good producer of inulinase, for example Sharma et al. (2006) used garlic as a substrate to produce an inulinase with an activity of 524 IU/L.

Yeasts are used in the production of inulinase because they grow easily compared to bacteria. *Kluyveromyces* spp., *Pichia* spp., and *Candida* spp. are yeasts with a high potential for producing inulinase, as evidenced by high yields and activity. Gao et al. (2007) screened over 400 marine yeasts and discovered that some of them can secrete large amounts of inulinase.

In order to optimize the fermentation process, a number of researchers studied inulinase production parameters (agitation, aeration, carbon supply, fermentation medium composition, fermentation time) on *Kluyveromyces* spp. strains. Thus, Santisteban et al. (2009) investigated the effects of carbon and nitrogen sources, using sucrose at a concentration of 20 g/L as a carbon source and obtaining an inulinase activity of 208 U/ml. Inulinase purification from *Kluyveromyces marxianus* was studied using an ethanol precipitation method followed by ultrafiltration (Golunski et al., 2011), yielding a specific activity of 262.9 U/mg. By purifying the enzyme obtained from *Kluyveromyces marxianus* var. *bulgaricus* using ion exchange chromatography methods, Kalil et al. (2010) obtained an inulinase with an activity of 194.1 U/ml.

Of the sixteen fungal strains of *Aspergillus* spp. (Gern et al., 2001), a favorite species for inulinase production, a maximum inulinase activity of 100 U/ml was obtained with the *Aspergillus niger* DSM 2466 strain, using sucrose S-770 added to the fermentation medium in a concentration of 6 g/L as substrate. Kumar et al. (2005) obtained a maximum inulinase activity of 176 U/ml with the stem *Aspergillus niger* using a fermentation medium containing 5% (w/v) inulin. Other *Aspergillus* spp. strains described for inulinase production in the literature include A. *fumigatus* (Gill et al., 2006), A. *awamori* (Nagem et al., 2004), A. *ochraceus* (Guimaraes et al., 2007), A. *ficuum* (Chen et al., 2011), and A. *parasiticus* (Ertan et al., 2003b).

The mentioned microorganisms use natural substrates from pure inulin to agro-industrial residues as carbon sources, registering different activities and properties, depending on the producing strain and substrate. Inulin can be found as a reserve of carbohydrates in the tubers and roots of plants such as chicory, turnips, dahlias, dandelions, garlic, onions, rye, barley, bananas, wheat, etc.

As microbial inulinase is an inducible enzyme, in the biosynthesis process a substrate rich in inulin can be used resulting from agro-industrial residues (cassava, corn cobs, oats, rice straw, sugar cane, wheat bran), which leads to streamlining the enzyme biosynthesis process (Singh & Chauhan, 2016).

Inulin is a natural fructose polyglucide with functional properties related to the length of the molecular chain, and it belongs to the fructans class of carbohydrates. Inulin is a generic term for all linear (2-1) fructans with polymerization levels ranging from 2 to 60. Because inulin is not recognized by digestive enzymes in the small intestine, tiny molecules can pass through the cell wall, whereas long-chain molecules (10-65 monomers) reach the colon intact, resulting in bacterial fermentations (Sarote et al., 2007)

Natural materials rich in inulin are preferred as substrates for obtaining inulinase, but lately, agro-industrial residues have gained the attention of researchers. In nature, inulin can be found in the tubers and roots of plants such as chicory, turnips, dahlias, dandelions, garlic, onions, rye, barley, bananas, wheat, and others as a carbohydrate reserve (Chi et al. 2011).

As a result, agro-industrial wastes and plant extracts appear to be a good source of inulinase. Glucose, sucrose, cassava flour, corn cob, oatmeal, rice straw, sugar cane and wheat bran were used to test the effect of carbon source on inulinase biosynthesis by the *Aspergillus ochraceus* strain (Guimaraes et al., 2007). When cane was used as a carbon source, the highest amount of extracellular inulinase activity (108 units of activity) was produced. Sharma et al. (2006) used various substrates to produce inulinase, namely: rye, barley, bananas, garlic, wheat, pure inulin, chicory, onion and dahlia.

The focus of this research is on inulinase production by *Aspergillus terreus* ICCF 262, including bioprocess optimization and inulinase purification. In order to optimize enzyme biosynthesis, we tested various substrates (inulin and orange peels) and optimization strategies

(nitrogen sources, temperature, pH, bioprocess duration).

MATERIALS AND METHODS

The microorganism *Aspergillus terreus* belongs to the Collection of Microorganisms of Industrial Importance of the National Institute for Chemical-Pharmaceutical Research - Development, ICCF. *Aspergillus terreus* ICCF 262, was cultivated on specific media and maintained at 4ºC in vegetative preserves. During the experiments, weekly/monthly passages were made to maintain their viability. Reagents used for the research, such as: organic solvents, analytical reagents and mineral salts, were purchased from Merck and Sigma-Aldrich.

Media culture and cultivation conditions

Preinoculum phase. The strain was grown in tubes on solid agar medium, PDA (potato dextrose agar), and incubated for 24-48 hours at 28-29°C.

Inoculum phase. After washing the preinoculum tubes with 2 ml of sterile inoculum medium, they were inoculated into 500 ml Erlenmeyer flasks containing 100 ml of liquid medium. For 24 hours, *Aspergillus terreus* ICCF 262 was grown on an inoculum medium containing 1 percent glucose, 0.3 percent malt extract, 0.3 percent yeast extract, and 0.5 percent peptone, with stirring at 220 rpm.

The bioprocessing phase. As shown in Table 1, the inoculum was used at a final concentration of 2% to inoculate 500 ml Erlenmeyer flasks containing 100 ml of the fermentative medium, which consisted of M2 or M3 (fermentation media) medium supplemented with 2% (g/v) inulin or/and orange peels as inductive substrates.

Table 1. Composition of the fermentation media utilized to create the experimental model

No.	Component	Concentration
M2		
1	Inulin/Orange peels	2.0%
2	Yeast extract	2.0%
3	NH_4NO_3	0.3%
4	$(NH_4)_2$ HPO4	0.4%
5	KH_2PO_4	0.1%
6	$MgSO_4$	0.05%
M3		
1	Inulin/Orange peels	2.0%
2	Corn steep liquor	2.0%
3	NH_4NO_3	0.3%
4	$(NH_4)_2$ HPO4	0.4%
5	KH_2PO_4	0.1%
6	$MgSO_4$	0.05%

The cultivation conditions were 28-29°C, an initial pH of 6.5, and 7 days of agitation on a rotary shaker with 220 rpm and a 2 cm agitator eccentricity. The mycelia were then separated by centrifugation and the supernatant was tested for enzyme activity.

Enzyme Purification

The final fermentation medium is centrifuged at 8000 rpm, for 20 minutes at 9°C. To extract inulinase, the supernatant is fractionally precipitated with ammonium sulfate. This phase is accomplished by adding 60% $(NH_4)_2SO_4$, centrifuging, and extracting the precipitate containing ballast protein; $(NH_4)_2SO_4$ is then added to the supernatant from the first fractionation process until saturation is reached at 80%. The precipitate containing 95% inulinase is centrifuged and stored at 4ºC. The precipitate is dissolved in distilled water and dialyzed for 24 hours against water before being dialyzed for another 24 hours against 20 mM phosphate buffer, pH 7.

The purification of inulinase is performed through the use of ion exchange chromatography. The dialysate precipitate is applied to a column of DEAE-Cellulose that has been pre-equilibrated with 20 mM phosphate buffer at pH 7. The enzyme is eluted with a NaCl solution in a gradient of concentrations ranging from 0.1 to 1.0 M in the same buffer, at a flow rate of 60 ml/h, in fractions of 3 ml. The fractions with enzyme activity were collected and lyophilized after they were analyzed. (Singh & Chauhan, 2016; Pessoni et al., 2007)

Analytical Methods

Miller (1959) described the following method for determining inulinase activity: the enzyme solution (0.1 ml) was mixed with 2% inulin (0.9 ml) in a 0.1 M acetate buffer, pH 5.5; at 50°C, the process takes 15 minutes. The reducing sugars were determined using the dinitrosalicylic acid method. 2 ml DNSA reagent was added to each tube and placed in boiling water for 5 minutes to stop the enzyme activity. Each sample's temperature was brought to room temperature. A spectrophotometer was used to measure the absorbance at 540 nm. Under test circumstances, one activity unit is defined as the quantity of enzyme necessary to create one micromole of reducing sugar per minute

(Petrescu & Eremia, 2018). Using BSA as a standard, the protein content was measured using the Lowry technique. (Lowry et al., 1951).

RESULTS AND DISCUSSIONS

1. Inulinase microbial biosynthesis and cell growth in conventional media

In previous research, the fungal strains *Aspergillus* sp. were reported to have the maximum amount of gas created in the cell formation process by metabolizing inulin from the environment in the Durham experiment (Durham, 1952). *Aspergillus awamori, Aspergillus niger, Aspergillus nigricans,* and *Aspergillus terreus* strains had higher cell activity and growth than the other strains examined, indicating that these strains may be able to use inulin as their only carbon source (Eremia & Petrescu, 2021).

The *A. terreus* strain was chosen to continue the research into the type of inoculum (Ig - inoculum with a single carbon source of glucose and Ii - inoculum with a single carbon source of carbon inulin) and the preferred source of organic or inorganic nitrogen to optimize inulinase biosynthesis conditions (Figure 1).

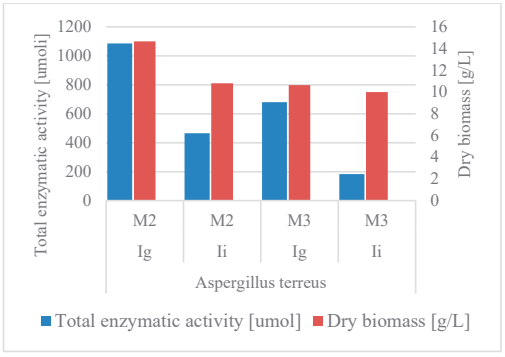

Figure 1. Bioprocess optimization

The optical density, dry biomass, final pH value, and enzymatic activity of extracellular inulinase were used to track inulinase production by the evolution of cell growth.

The *Aspergillus terreus* strain had a relatively uniform distribution of biomass development on the chosen medium variants, and enzymatic activity was higher on the Ig-M2 variant, with a correlation between biomass development and enzymatic activity on the same culture medium.

For the study of biomass development, the growth curves of the *A. terreus* strain were performed in static and agitated fermentation for 7 days in optimal conditions for the development of cultures on liquid media.

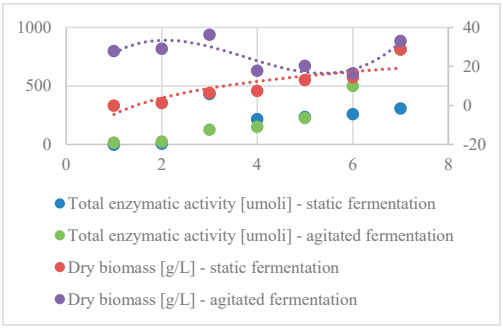

Figure 2: Growth curve of *Aspergillus terreus* strain and enzymatic activity

Along with the biomass study development over time, the effect of aeration on biomass development and enzymatic activity were also analyzed (Figure 2). In agitated fermentation, the strain developed significantly faster; more biomass accumulated in the static fermentation medium only on the seventh day. Stirred fermentation increased enzymatic activity as well, with a significant difference on the fifth day. The highest enzymatic activity was determined after 6 days of bioprocessing.

2. The effects of different Carbon sources on cell growth and enzyme production

On the variant of medium containing inulin and orange peel as a carbon source, the strain produced the highest biomass and had a higher enzymatic activity than when inulin or orange peels was used as a carbon source (Figure 3).

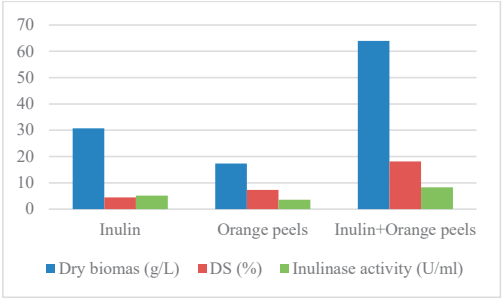

Figure 3. The effect of C source on *Aspergillus terreus* inulinase production

On the environmental version with inulin, the strain progressed well in terms of biomass, but considerably improved enzymatic activity on the environmental variant with orange peel and inulin as carbon sources.
It's worth noting that using agri-food waste as a substrate boosts the strain's bioproductivity.

3. Inulinase purification

The supernatant obtained by centrifugation of *A. terreus* culture, with inulinase activity, was purified by two successive steps: ion exchange chromatography on DEAE-Cellulose after fractionated precipitation with ammonium sulphate (Table 2).
Following the purification stage, a precipitate with higher inulinase activity is formed (approximately 76% of the initial activity).
The eluted inulinase was purified over 37.16 times with a yield of 52.3% in the second phase, which consisted of purification on a DEAE-Cellulose column (Table 2 and Figure 4).
Following the chromatographic purification study, increasing the degree of purification results in an improvement of the specific activity.

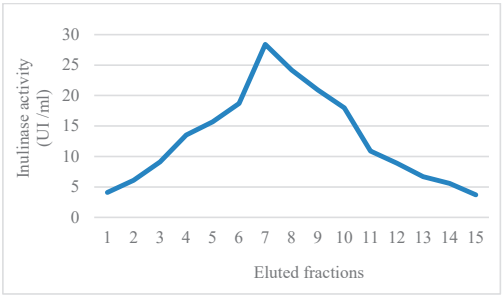

Figure 4: Ion exchange chromatography for inulinase purification using a DEAE-Cellulose column and a gradient of 0.1-1 M NaCl in pH7 acetate buffer in 3 ml fractions

The *Aspergillus terreus* strain was identified as the most important in terms of inulinase bioproductivity on diverse nutritional substrates. These results are equivalent to those reported in the literature using the *Aspergillus niger* strain, as a model (Rawat et al., 2015; Cruz et al., 1998; Singh & Chauhan, 2016).

Table 2. Purification of inulinase from *Aspergillus terreus*

Stages	Volume (ml)	Activity (U/ml)	Total activity (U)	Protein (mg/ml)	Specific activity (U/mg protein)	Purification factor
Fermentation supernatant	170	11.67	1984	1.09	10.62	1
Dialyzed precipitate	33	45.7	1508	0.64	71.4	6.73
Eluted from DEAE-Cellulose Purification	27	29.2	788.4	0.074	394.6	37.16

CONCLUSIONS

The *Aspergillus terreus* strain was identified as the most important in terms of bioproductivity of inulinases on diverse nutritional substrates among the *Aspergillus* sp. strains previously tested according to the stage of biomass and inulinase production.
It was performed with several carbon sources, namely inulin and agri-food by-products (orange peels), to study the optimization of the bioprocess for producing inulinase with *Aspergillus terreus* ICCF 262 strain performed on enzymatic and specific activities. These tests revealed that inulinase is more active when a mixture of approximatively 2% inulin and orange peel powder is used as a carbon and energy source rather than just inulin.
After post-biosynthesis processing of the fermentation medium, a solution with a 29.2 U/ml inulinase content and a yield of 39.7% was obtained.
It should be highlighted that using agri-food waste (in our case, orange peel) as a substrate enhances strain bioproductivity by supplementing the fermentation environment, with an increase in inulinase production yields, also having a favorable impact on their production.

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