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Inulinase production by *Geotrichum candidum* using Jerusalem artichoke as sole carbon source

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Abstract

Inulinase is a versatile enzyme used in many fields, especially in food industr, to produce high fructose syrups. Jerusalem artichoke (JA) (Helianthus tuberosus L.) has a high inulin storage capacity. In the present study, inulinase production by Geotrichum candidum was achieved using JA as a sole and cheaper carbon source, when compared with traditionally used substrates like pure inulin and sucrose. The current study was carried out in two parts. Firstly, ten strains of G. candidum were isolated from different samples and screened for high inulinase production. In the second part of the study, fermentation conditions such as JA concentration, initial pH, cultivation temperature, agitation speed and incubation time were tested in order to optimise inulinase activity. The results showed that maximum inulinase activity was obtained as 45.62 U/ml in optimised fermentation medium using 3.0% (w/v) of JA. Consequently, JA has a potential as an effective and economical substrate for inulinase production.

Key words: *Geotrichum candidum*, Jerusalem artichoke, Inulinase, Optimisation

Introduction

Inulin is the major carbohydrate reserve material in the roots and tubers of onion, garlic, leek, Jerusalem artichoke (JA) (*Helianthus tuberosus* L.), chicory, dandelion, burdock and dahlia, and it has thus been part of human diet for many centuries. Inulin consists of linear chains of β-2,1-linked D-fructofuranose residues terminated by a glucose residue through a sucrose-type linkage at the reducing end. Many inulin sources are being used as a renewable raw material in the production of inulinase, i.e. ethanol, acetone, butanol, pullulan, gluconic acid, sorbitol, inulooligosaccharides and ultra-high-fructose syrup in pharmaceutical industries [1-4]. Inulinase (2,1-β-D-fructan fructanohydrolase, EC 3.2.1.7) is an important commercially used enzyme which is usually extracellular, inducible and versatile. Fructose production can also be achieved by processing starch with α-amylase, amyloglucosidase and glucose isomerase, but with a yield of fructose of only 45% while inulinase produce fructose with 95% [3, 5-9]. Microbial inulinases (endo- and exo-inulinases) catalyse the conversion of inulin into fructose and fructooligosaccharides (FOS) [2, 3, 10-12].

The selection of suitable substrates and a survey of the available materials are important factors in designing a technical enzymatic process. Among various substrates employed for inulinase production, inulin-containing plant materials are preferred due to their cheapness feature when compared with the pure substrates [13, 14]. Various plant materials such as garlic, onion, JA, sugarcane molasses (SCM), corn steep liquor (CSL), wheat bran, rice bran, coconut oil cake, and corn flour have been used as substrates in inulinase production [9, 11, 15-17]. Cost and availability are significant considerations; therefore an appropriate substrate selection is an important step in the development of an enzyme production process. Recently, JA has drawn much attention as a potential energy crop [1], because of its resistance to frost and plant diseases and its ability to grow in poor land and desert conditions. Besides, it has been used in the production of ethanol and inulinase [3, 16,

18], due to its high inulin content (16-20% of fresh weight) [6] and its economical and ready availability. Thus it was selected as sole carbon source in the current study.

Earlier studies focused on inulinase production using various microorganisms such as yeasts (*Kluyveromyces, Pichia*), filamentous fungi (*Aspergillus, Penicillium*) and bacteria (*Xanthomonas, Staphylococcus, Streptococcus)* [2, 4, 9, 11, 15, 19-23]. The yeast *Geotrichum candidum* has been rarely used for this purpose [3]. To our knowledge there is not any report about inulinase production by *G. candidum* using JA. It is well known that this yeast is preferable due to its GRAS (Generally Regarded As Safe) status, high sucrose fermenting ability when compared to many other yeast cultures. Furthermore, it was shown to secrete a large amount of inulinase into the medium without additional nutrient supplementation in submerged fermentation [3]. Therefore, the aim of this study was to investigate inulinase production by *G. candidum* using JA tubers as sole carbon source and to optimize the fermentation conditions to obtain high enzyme activity.

Materials and Methods

Materials

All chemicals were purchased from Sigma Chemical Co. (St. Louis, USA) and Fluka (Buchs, Switzerland). JA tubers were purchased from local markets in Erzurum, Turkey, were washed with cold water, sliced with a blender and then dried in a Pasteur oven at 80 °C. The dried slices were milled to a fine powder with mill [24] and termed JA powder (JAP).

Isolation of *G. candidum* strains

Isolation of the microorganisms was carried out according to the technique that was determined by Mughal *et al.* [3]. Ten strains of *G. candidum* were isolated from different samples (e.g. soil and JA tubers) by serial dilutions on yeast glucose agar (YGA, Fluka, Buchs, Switzerland) with additional inulin (1.2%, w/v) as carbon source which helped in the selection of colonies having higher enzyme activity. The petri dishes were incubated at 30 °C for 3 days. Isolates were identified and their morphological characteristics were studied according to Onion *et al.*, [25]. The isolates were maintained on YGA plates without inulin addition and subcultured every three weeks. The strains were then screened for exo-inulinase production. The strain designated as *G. candidum* OC-7 achieved the highest enzyme activity, thus it was selected for further studies and stored at 4 °C.

Fermentation medium

The fermentation medium was composed according to Baysal *et al.*, [24] as follows: 1.0% JAP, 0.23% NH_4NO_3, 0.37% $(NH_4)_2HPO_4$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.15% yeast extract. The pH of the medium was adjusted to 6 with 1 M NaOH or 1 M HCl. Medium was autoclaved at 121°C for 15 min. After sterilization, the medium was cooled to room temperature and one loopfull of yeast cells was transferred as inoculum material into each flask containing 100 ml of medium. The flasks were incubated in a rotary shaker incubator at 150 rpm.

Analytical methods and inulinase assay

Extracellular invertase activity in the culture filtrate was measured as described by Mughal *et al.*, [3]. Inulinase activity was assayed by measuring the reducing sugar released from inulin. The culture medium was centrifuged at 5000 rpm for 15 min and the supernatant was used as the inulinase source. The reaction mixture consisting of 0.1 ml of supernatant and 0.9 ml of sodium acetate buffer (0.1 M, pH 5.5) containing 2% inulin (w/v), was placed in glass test tubes and incubated at 50 °C for 15 min. The reaction mixture was then assayed for reducing sugar using the 3,5-dinitrosalicylic acid (DNS) method [26]. For this, 1.0 ml of DNS reagent was added to each tube and the tubes were placed in boiling water for 5 min to stop the enzyme activity. After cooling to room temperature, the volume of each tube was filled up to 8 ml with distilled water. Absorbance was determined in a spectrophotometer at 592 nm. A

blank with distilled water instead of the enzyme solution was run in parallel. One unit of inulinase activity is defined as the amount of enzyme catalyzing the liberation of 1 μmol of glucose from inulin per min under the assay conditions. The standard curve was prepared for DNS using glucose.

Statistical analysis

Each experiment was repeated at least three times with two replicates. Analysis of variance was conducted using one-way ANOVA test using SPSS 13.0 for Microsoft Windows. $P \leq 0.05$ was considered as significant. All values are expressed as mean ±SD (n=6).

Results and Discussion

In developing a fermentation process, the optimization of cultivation conditions and selection of appropriate substrates in the most favourable concentrations have primary importance due to their impact on the economy and feasibility of the process. Many studies have been intended to obtain high-yielded production using optimization processes with low-cost. For this purpose different substrates (such as carbon or nitrogen sources), inducers (such as sodium or calcium salts) or different applications (such as treating producer-microorganism with chemical reagents that cause mutations or expose the microorganism to magnetic fields) were used to occur changes in the metabolism of the microorganisms [27, 28]. The improvement strategies used in microbial enzyme productions, were generally interested in classical optimization methods. There are many reports about classical optimization methods based on varying one-factor-at-a time [29, 30].

Isolation and screening of *G. candidum* strains

It is well known that there has been a growing popularity in isolation of microorganisms from various sources for the production of industrially substantial microbial products. Therefore, the exploration of new *G. candidum* strains to produce an enzyme such as inulinase may represent a significant contribution. The current study was performed in two parts. Firstly, it was aimed to select the most inulinase-productive strain to use in further studies. A total of ten *G. candidum* strains were isolated from different samples and screened for their inulinase activities. The obtained findings showed that different strains of the same microorganism may exhibit fundamental metabolic differences even if the same metabolite is produced. When designing industrial production processes these different behaviours must be defined in the laboratory in order to achieve optimal production conditions. Although it was found at the end of the screening process that inulinase enzyme was produced by all isolates and the differences between the results of some strains were significant (data not shown). The isolate which was designated as *G. candidum* OC-7 achieved the maximum enzyme activity among other isolates, so this isolate was chosen for further studies.

Optimization of fermentation conditions

To determine optimal fermentation conditions for inulinase production, the JAP concentration varied between 1-4% (w/v). As seen from Fig. 1 the highest enzyme activity (21.1 U/ml) was achieved by the usage of 3% of JAP. Baysal *et al.*, [24] determined the suitable concentration of 1.0% JAP for *Aspergillus niger;* however the usage of this concentration resulted in the lowest enzyme activity (16.66 U/ml) for *G. candidum* in the current study. The differences between the enzyme activities are significant and the concentration above 3% of JAP caused decreasing in the inulinase activity. This would be resulted from the higher minerals in the JA; in fact Paturau [31] reported that the inhibition of the enzyme synthesis would be effected by the higher minerals content.

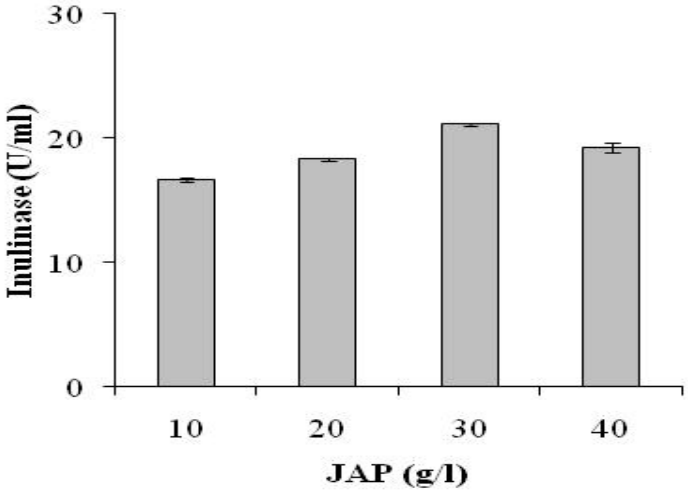

Fig. 1 Effects of different concentrations of JAP on inulinase production*
*Fermentation conditions: pH 6,0; 25°C, 150 rpm, 48 h

It is well known that the initial pH of the fermentation medium and incubation temperature are very important factors for production in high yield of a microbial substance. In recent studies, the optimum pH values for inulinase production by various microorganisms were reported to vary between 4.8-7.5 [3, 8, 12, 13]. The present experiments showed that optimal pH for a higher inulinase activity (23.88 U/ml) was 5.0 (Table 1). Chen *et al.*, [12] cultivated *Aspergillus ficuum* at 30 °C for 5 days and performed the enzyme reactions at various temperatures (from 20 to 80 °C) in order to obtain optimum temperature for inulinase activity; whereas, in the current study, the *G. candidum* was cultivated in different temperatures and maximum inulinase activity (36.95 U/ml) was obtained at 30 °C (Table 1). The agitation speed, another effective fermentation condition, was also investigated. It was tested at 100, 150, 200 and 250 rpm. As seen from data presented in Table 1, the highest inulinase activity was obtained at 200 rpm. However, Mughal *et al.* [3] indicated the optimal agitation rate at 350 rpm, in the current study inulinase activity above or below 200 rpm started decreasing.

Table 1. Effects of initial pH, incubation temperature and agitation speed on inulinase activity

Culture parameters	**Inulinase activity**
Initial pH[1]	**(U/mL)**
4.0	18.95±1.41
5.0	23.88±1.82
6.0	21.17±1.18
7.0	20.43±1.08
Temperature[2] (°C)	
25	27.88±0.82
30	36.95±1.74
35	34.94±2.11
40	31.48±1.78
Shaking speed[3] (rpm)	
100	29.26±2.67
150	36.95±1.74
200	41.85±2.07
250	38.08±1.47

[1]**Fermentation conditions**: Temperature 25 °C, agitation speed 150 rpm, time 48 h
[2]**Fermentation conditions**: pH 5.0, agitation speed 150 rpm, time 48 h
[3]**Fermentation conditions**: pH 5.0, temperature 30 °C, time 48 h
All values are the mean of two replicates from three independent experiments (±SD).

G. candidum is a preferable yeast due to its lower fermentation time [3]. Many investigations were made to obtain higher enzyme productivity in shorter time due to a substantial gain. For this purpose, the optimum cultivation time was investigated. Mughal *et al.*, [3] observed maximum inulinase activity after 48 h of fermentation, whereas, the highest enzyme activity (45.62 U/ml) in the present study was obtained after 40 h of fermentation (Fig 2). Having more yields in a shorter time has a great impact in commercial productions. This could be resulted from natural structure of JA as a fermentation medium component instead of sucrose or strain differences of the microorganism. The enzyme activity started decreasing gradually after this time. The reduction in enzyme activity probably was caused by depletion of nutrients in the medium (substrate limitation) or accumulation of some autotoxic products of the organism in the medium [32].

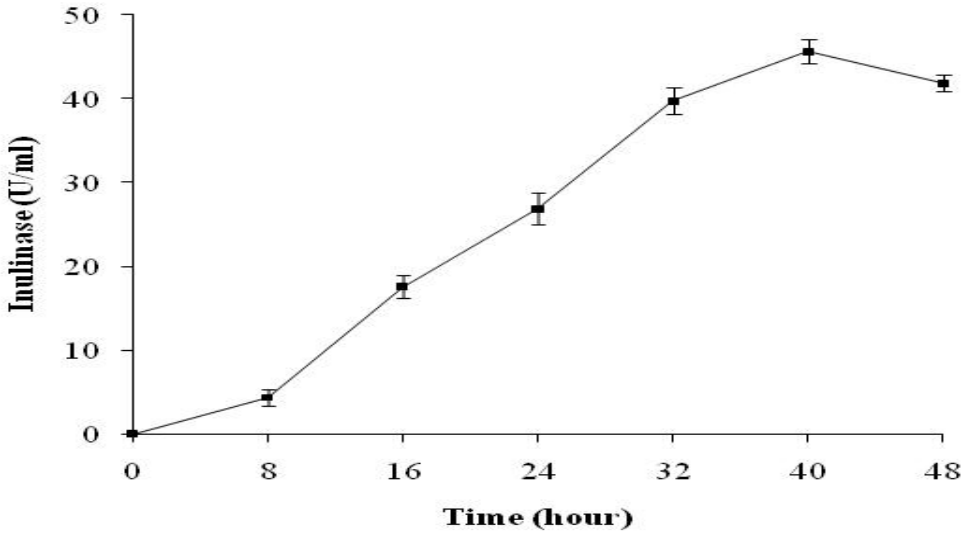

Fig. 2 Effects of cultivation time on inulinase production*
*Fermentation conditions: %3.0 of JAP, pH 5.0, 30°C, 200 rpm

Conclusion

This work presents the production of inulinase, a versatile enzyme used in many fields, by an economical way, with high activity in a shorter time. JA is a rich source of inulin and has many industrial applications due to its easy and cheap availability when compared with ordinary substrates. Therefore, it was used in different studies as a fermentation medium component due to its high inulin content. Furthermore, the obtained data showed that JA has a substantial potential without any additional carbon source in inulinase production by *G. candidum.* According to these results, it can be said that JA could effectively be used as a cheaper nutrient source in the production of inulinase and further studies will run using JA for the production of other commercially valuable enzymes.

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